Human Dental Pulp Stem Cells: Characterisation and \textit{in vitro} 3D Bone Ontogeny

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ABSTRACT

Tissue engineering has emerged as a practical approach to tackle with the prosthetic industry limitations. Its methods merge aspects from developmental biology, engineering, material sciences and medicine, with the aim to produce fully-functional bone tissue ex vivo, to further replacement or regeneration of real bone injuries and/or defects. Traditional bone tissue engineering cell culture technique, includes the seeding of SCs in three-dimensional matrices, cultured in fed-batch rotating bioreactors, working jointly with biological cues to produce biomimics. Nonetheless, fed-batch bioprocessing has found some difficulties. Namely, mass transport limitations in the seeded scaffolds and accumulation of cellular waste, yielding poor nutrition and oxygenation of the cells. Thus producing heterogeneous distributed cell/bone constructs. Perfusion of media has been proven to improve mass transport in the culture system, along with removing cellular waste. To overcome heterogeneity, finding the adequate cells and proper cues to drive osteogenic differentiation, are as important as the bioprocess to host the culture. hDPSCs are a promising source of stem cells for the production of bioactive biomaterial for skeletal tissue reconstruction. They lodge immunosuppressive and regenerative functions, high proliferation rates and ease in access. Their source and the subtle nature of the extraction procedure, harbour less moral concerns and variability than ESCs and most of the MSCs.

In this study, the characterisation of hDPSCs as MSCs under the minimal criteria set by The International Society for Cellular Therapy was performed. Further, the osteogenic differentiation of hDPSCs encapsulated in alginate/gelatin hydrogel subjected to suspended culture in a novel perfusion-RWV bioreactor was studied, and compared with traditional fed-batch and static culture methodologies. Finally, the effect of osteogenic cues as physiological BMP2 and simvastatin were studied to enhance the designed bioprocess.

The characterisation results demonstrated that passage 4 donor hDPSCs were ideal to perform the 3D osteogenic differentiation. These cells, allowing enough production of cells while maintaining the multipotent phenotype of the parental cells under several conditions, including highly dense long-term culture. These cells were able to undergo
osteogenesis in 2D and 3D. The novel high throughput perfusion-RWV bioreactor bioprocessing, demonstrated to be successful in the mitigation of nutrient and oxygen transport limitations, external to three-dimensional cell/alginate constructs, performing above fed-batch RWV bioreactor and static culture, and able to produce more homogeneous, denser and functional bone constructs, rich in mature osteoblasts and mineralising osteocytes. Both BMP2 and simvastatin, demonstrated to enhance the quality of bone constructs produced by the perfusion-RWV unit, yielding more homogeneous constructs, with higher alkaline phosphatase activity, mineralisation and showing a more mature gene pattern. Interestingly. BMP2 produced constructs rich in mature osteoblast, while simvastatin, constructs rich in osteocytes. In particular, this experiment proved effective in producing osteogenic differentiation with minimal use of BMP2, offering a potential mean to avoid dosage dependant safety risks of BMP2.

In conclusion, this thesis reports the development of a novel bioprocess to produce homogeneous bone tissue constructs able to support the transfer of the “on the bench” research, to the clinical facility.
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Overview

The need for bone tissue engineering

In the United Kingdom, the number of bone fractures reaches approximately 2.3 million annually. 70,000/100,000 hip and knee replacement surgeries respectively, are carried out yearly. The use of surgical engraftment implants and bone screws for bone failure is increasing every year. A report from Global Industry Analysts (GIA), “Orthopaedic Prosthetics: A Global Strategic Business Report”, sets the global orthopaedic prosthetic market at £12.7 billion and projects by 2017 the market reaching £15.2 billion. Yet, prosthetic replacement failure (e.g. peri-implant bone loss, infections and allergic reactions) is frequent. This is due to implants not being able to undergo physiological remodelling along with the host bones (Yuan et al., 2011). Around 5% of all prosthetic replacements will fail eventually. This occurs as a result of implant rejection, corrosive attack, wear and mechanical loading, and due to lack of osseointegration or delayed union. These numbers dramatically rise to 20% for high-impact fractures (NHFD, 2012, Brydone et al., 2010, Yuan et al., 2011). Furthermore, infections and rejections of implants could result in additional loss of bone, cartilage, muscle and tendon mass (Brydone et al., 2010); and ultimately, in the requirement for secondary implant. If we consider the size of the prosthetics industry and their limitations, it is clear that there is a necessity for further development into an actual cure.

Tissue engineering (TE) encompasses a toolset capable of tackling with the prosthetic industry limitations using a more biological approach. It studies embryo development and adult healing phenomena, to further pioneer materials to reconstruct and mimic not only the mechanical characteristics of bone, but also the functionality of the damaged tissue. TE uses cell therapy and the development of biomimetic biomaterials to provide solutions for these problems with greater safety and long-term durability (see Figure 7 The Tissue Engineering principles, for a schematic view).

Research in this field has been able to provide a wide variety of stem cell sources, with bone mimicry potential. Including embryonic (ESCs), hematopoietic (HSCs), bone marrow-derived (BM-MSCs), umbilical cord blood-derived (UCB-MSCs), adipose tissue
(ADSCs), muscle (MDSCs), and dental pulp (DPSCs) (Seong et al., 2010, Peng et al., 2009, Polak et al., 2008, Placzek et al., 2009); and by designing and using different bioprocessing systems, has been able to study the pathways associated with development, as well as to produce different kinds of tissue mimics. Tissue such as skin, neurons, pigment cells, cardiac muscle, bone, teeth, cartilage, tubule cells, pancreatic cells, thyroid cells and lung cells, able to promote and foster in vivo tissue regeneration (Placzek et al., 2009, Hwang et al., 2009, Pagkalos et al., 2010b, Itskovitz-Eldor et al., 2000, Peng et al., 2009).

Figure 1 Tissue engineering approach

Human dental pulp stem cells (hDPSCs) are a promising new source of stem cells for the production of bioactive biomaterial for skeletal tissue reconstruction. As MSCs-like cells, they lodge immunosuppressive and regenerative functions. They are multipotent ectomesenchymal stem cell (MSCs from the neural crest), capable of differentiating into
osteoblasts, osteocytes, odontoblasts, adipocytes, chondrocytes and neural cells (Grottkau et al., 2010). With high proliferation rates and ease in access. DPSCs are located in the cell-rich zone of the dental pulp of adults’ teeth (similar to the human exfoliated deciduous stem cells (SHEDs) in babies). Their source and the subtle nature of the extraction procedure, harbour less moral concerns than their predecessors’ embryonic stem cells (ESCs) and most of the MSCs.

Cells from impacted, wisdom and exfoliated teeth were previously discarded as medical waste. Now they have an upcycled clinical use for understanding regenerative cues of the body and implementing regenerative medicine. They can be suitable for bone therapy, as they can differentiate into all the cells in the osteoblastic and osteocytic lineages, as well as synthesise and secrete most of the extracellular matrix secreted by the cells they would replace.

The variability and low potency of adult MSCs (in comparison with immortalised cells and other murine stem cells) hinders their clinical potential and classical static culture processing has not been able to aid. Hence, a better bioprocessing is essential. MSCs culture requires to comply with several specific processing requirements to be of clinical use (quantity, quality, etc.) and the TE approach not only can tackle these requirements, but also optimise them.

This PhD thesis summarises the efforts carried out in the past four years. The aim is to support the use of perfusion rotating wall vessel systems as a platform for bone tissue engineering of hDPSCs, with the use of minimal growth factors. In particular, the characterisation of hDPSCs as MSCs-like cells, was carried out, to further study the use of different bioprocesses, as a tool to bypass classical bioprocessing weaknesses in bone regeneration, as well as the biological cues to produce all the osteoblastic and osteocytic lineage cells.
Novelty

I will be discussing how a novel rotating wall vessel system, coupled with perfusion flow of culture media, with minimal use of growth factors (physiological level) or drugs, can achieve bone mimicry, starting from undifferentiated hDPSCs in a 3D environment. Ultimately, leading to the induction of the whole range of cells in the osteoblastic phenotype, from pre-osteoblasts to the terminally differentiated osteocytic cells to produce cells for clinical uses.

Similar platforms have been used to study osteoblastic differentiation of ESCs and different MSCs with the use of growth factors, but using concentrations of several orders of magnitude higher (Liu et al., 2007, Ghodadra and Singh, 2008, Karbanova et al., 2010). In addition, the ex-vivo differentiation of hDPSCs in three-dimensional constructs has never been followed and characterised till this extent, studies will normally pre-differentiate cells to then move to an in vivo platform.

The importance on reducing the concentration of growth factors proceeds from the risk of cancer associated to their use (Singh and Morris, 2010, Carragee et al., 2013a) and their high price. The production of all cell types associated with bone, ensures that the constructs will be able to generate functional bone, once it is used as implants.

In addition, a fully differentiated human bone-like construct represents a great step in regenerative medicine, as it would allow researchers to perform preclinical studies for mechano-biology and drug delivery in a cheaper platform, with the potential to bear closer similarities (bioequivalence) to the human body. Rather than performing studies in vivo. Testing of new drugs in an ex vivo tissue, to minimise the generation and use of human-animal chimeras, as well as avoiding animal sacrifice in preclinical trials, should be our moral aim. Although in vivo studies are considered to be the “gold standard”; in vitro studies, if improved, could exceed in biological value. Reduced costs, more direct assessing of product performance and less ethical considerations, are some of the advantages. But more importantly, avoiding the low bioequivalence of highly variable drug testing studies in vivo. In vitro mimicry of tissues can in time provide a cheaper, more defined and ethically accepted platform for stem cells research.
1 Literature review
1.1 Bone
Bone tissue refers to a heterogeneous family, constituted by hierarchically structured composite biomaterials of organic and mineral nature. These share a basic building block, the hydroxyapatite crystals and the mineralised collagen fibrils. The most important members of the family are all the tissues normally called bones; but cartilage, enamel, dentin, cementum and mineralised tendons, also form part of the family. In general, bones will differ in the proportions of the common components (collagen fibrils, hydroxyapatite crystals and water) and in their structural motifs/ architecture at all levels of complexity. The diversity of structures they present, reflects their specific functionality, as mechanical support and as reservoirs of calcium and phosphate for metabolic functions (Weiner and Wagner, 1998, Jager and Fratzl, 2000).

Bones are composed in 20-30% in weight of an organic phase, 60-70% of mineral substances and the remaining is water. The organic phase consists of mineralised collagen fibrils, non collagenous proteins (around 200 different types, less than 10% of the total protein content), functional bone cells (osteocytes), bone forming cells (osteoblasts), bone resorbing cells (osteoclasts) and osteoinductive growth factors and molecules. Assemblies of parallel and staggered molecules of type I collagen protein, form channels, where the reminder compounds will be allocated. The mineral phase consists only of hydroxyapatite crystals ($Ca_5(PO_4, CO_3)_3(OH))$).

The skeleton has mainly two arranged macro-architectural forms, cancellous/ spongy bone (around 20% of total bone) and cortical/ compact bone (the remaining 80%), different proportions of these two structures will be present in the different bone family members.

To understand the complexity of the tissue in discussion, the next section describes the micro and macro bone structure in a more detailed manner.

1.1.1 Structure of bone
The main components in bone are hydroxyapatite crystals, type I collagen fibrils, non-collagenous proteins and water. Factors that determine the properties and function of different bones are: a) the proportion and organisation of crystals and water, b) the size of the crystals, c) the structures these form with the collagen fibrils, d) the orientation
and porosity of the construct and e) how these constructs organise into higher order structures (Weiner and Wagner, 1998, Jager and Fratzl, 2000). Bone relative proportions vary systematically between bone types. Although the collagen concentration is almost constant, the increase of minerals proportion occurs at expense of water content. Furthermore, the mineral phase will increase with ageing as the crystals continue growing over time (by thermodynamic factors). How they build in higher and more complex structures, establish the different characteristics of the overall biomaterial.

Mineralised Collagen fibril form arrays in parallel alignment (fibres): collagen fibrils can fuse with a contiguous fibril, forming fibres. However, there will be coexistence between mineralised and un-mineralised fibrils, producing local alignment of crystal layers in adjacent fibrils (Weiner and Wagner, 1998). The fibres organise in a variety of pattern depending on their direction, the formation of clusters and how packed they are, namely: arrays of parallel fibrils (lamellae), Woven fibre structure, plywood-like structure (e.g. sementum) and radial fibril arrays (e.g. dentin).

Cylindrical motifs, osteons and canaliculi: remodelling and regeneration of bones results in the formation of cylindrical motifs. Osteoclast resorption of bone tissue generates a matrix of tunnels. These are then refilled by lamellar bone produced by osteoblasts, leaving minuscule channels that work as blood vessels called osteons and a group of even smaller channels called canaliculi, in them we find osteocytes with secretory function that will exchange nutrients through the bones.

Cortical and cancellous bone: macro-structurally, there are two types of bone, the cortical (or compact) and the cancellous (or spongy, trabecular). Normally, bones have a dense cortical outer layer, and a spongy cancellous structure within its interior. These are recognized by the degree of porosity, or how fenestrated they are.
Figure 2 Structure of compact bones.

Cancellous bone has a microstructure rich in irregular, unorganized and unpacked bundles of lamellae, meanwhile trabecular bone has an organized, cylindrically shaped and packed lamellae (Rho et al., 1998).

A whole bone is a construct of all the described levels structured to perform a specific function. As a result, each bone type will have a characteristic porosity; crystals, osteons and canaliculi distribution; and mechanical resistance.

1.1.2 Bone cells

There are three different types of cells in bone tissue: Osteoblasts, or bone forming cells; Osteocytes, or bone functional cells; and Osteoclasts, or bone resorbing cells.

Osteoblasts secrete the organic bone matrix. They can grow and will be active in all areas where bone growth, regeneration or remodelling is proceeding. They are allocated on the surface of bone. When these get surrounded and isolated by bone matrix, they develop into osteocytes.

Osteocytes are mature osteoblasts. They are found within the bone matrix, confined inside lacunae. These communicate to each other through processes running within
canaliculi. They control the formation and resorption of new and old bone respectively and work in signalling events between the rest of the organism. Thus, they deal with metabolic requirements and mineral homeostasis of bone.

Osteoclasts perform extracellular bone matrix resorption. The joint labour of these and osteoblasts ensures the equilibrium in bone structure, constituents and density. This fine equilibrium is managed by Osteocytes.

1.1.3 Osteogenesis
The skeleton develops from three different mesenchymal cell lineages. The somites (or primitive segment), precursor of the axial skeleton; the lateral plate mesoderm, precursor of limb skeleton; and the cranial neural crest, precursor of the branchial arch, craniofacial bone and cartilage. Osteogenesis can occur following two different mechanisms: intramembranous ossification (IO), where mesenchyme differentiate directly into vascularised osteoblasts at ossification centres; or endochondral ossification (EO), in which the formation of mineralised bone is intermediated by the development of a cartilaginous scaffold (Gilbert et al., 2000). Both mechanisms are accompanied by the secretion of a complex extracellular matrix (collagen, bone sialoprotein, proteoglycans, other proteins and crystalline salts), that initiates tissue mineralisation to form the calcified bone (Heng et al., 2004b).

In general, bone development involves four phases: 1) the migration of mesenchyme with osteogenic potential to the skeletogenesis spot, 2) mesenchymal–epithelial interactions, condensation (or the aggregation) and commitment of mesenchymal cells, and the successive differentiation into chondrogenic and/ or osteogenic lineage.

In the next section, the two mechanisms of osteogenesis will be described in a more detailed manner.

1.1.3.1 Intramembranous ossification
IO starts in the second month of embryogenesis, and is characteristic of the development of flat bones of the skull (e.g. frontal, parietal, occipital, and temporal bones) and the clavicles. Mesenchyme from the neural crest proliferates and differentiates forming dense clusters of cells called “Ossification Centres”. The cells will have two possible paths, develop into capillaries, or change their shape and become
osteoid secreting osteoblasts (pre-bone), a collagen-proteoglycan matrix. This matrix is able to bind with calcium salts, letting the pre-bone get calcified. The osteoblasts left trapped between osteoid will become osteocytes and develop secretory properties. From these nodules of osteoblasts, calcified spicules will start to spread. Furthermore the area will be enclosed by new layers of compact mesenchyme (periosteum) that will differentiate into osteoblasts and secrete a new layer of calcified matrix in parallel to the spicules, and in due process, propagating the bone formation (Gilbert et al., 2000), and thus, the growth of the bone. Bones developed at this stage, are woven-like bones. With further development, the woven bone will be replaced by mature lamellar bone. In the most inner part of the new bone, spongy bone will persist. Blood vessels are going to infiltrate the spongy bone and trabeculae will be formed around them. The vascularised tissue will become red marrow (Marieb and Hoehn, 2013).

In Figure 3 Structure of flat bones, the layout produced by IO can be seen with compact bone in the exterior, spongy bone in the inner part, and blood vessels and red marrow in the interstice between the trabeculae.

Figure 3 Structure of flat bones.

1.1.3.2 Endochondral ossification

EO starts in the second month of embryonic development and will result in the formation of all the bones of a human being, but the flat bones of the skull and clavicles (formed by IO). Bones are developed through mesenchyme formation of a cartilage model, and its further breakdown and gradual replacement with bone during foetal development and postnatal growth (Mackie et al., 2008). The process happens in five distinguishable stages. In the first stage, mesenchyme will proliferate, and get
committed to differentiate into hyaline cartilage (articular). In the second stage, the committed cells will flatten and aggregate into clusters and differentiate into chondrocytes (cartilage forming cells). In the third stage, the chondrocytes will proliferate and arrange in the form of the specific bone they will build. The cells start secreting cartilage specific extracellular matrix. In stage four, the chondrocytes stop the proliferation and start to grow. Increasing their volume dramatically, becoming hypertrophic chondrocytes. The matrix they secrete, contain collagen X and more fibronectin. This matrix will be mineralised with carbonate calcium. In the fifth stage, the cartilage scaffold will be infiltrated by cells from the periosteum (layer of tissue surrounding the bone): blood vessels, osteoclasts (bone-resorbing cells), and cells with osteogenic (osteoblasts) and hematopoietic (bone marrow) properties. This “Ossification Front” forms the periosteal bud. The hypertrophic chondrocytes start dying by autophagic cell dead and are resorbed by the osteoclasts. Later, this area will become yellow bone marrow. Mesenchymal cells from the periosteum and the periosteal bud, start proliferating and secreting extracellular bone matrix (osteoid) attached to the remnants of the hyaline cartilage scaffold left by the osteoclasts. The process starts from the centre (primary ossification centres), and propagates to the longitudinal ends (secondary ossification centres), allowing ossification centres to form in the space where once cartilage was. Eventually all the cartilage is replaced by trabecular bones, except at the articular surfaces. Where hyaline cartilage remains and the inner part where the marrow cavity is formed (Gilbert et al., 2000, Mackie et al., 2008). Further development makes the external layers to become cortical bone. The remaining cartilage in the articular surfaces, will allow for the bone to grow longitudinally during post-natal development (Mackie et al., 2008).

See Figure 4 Endochondral Ossification (Marieb and Hoehn, 2013), for a schematic representation of the five stages of EO.
1.1.3.3 In vitro bone ontogenesis

The *in vitro* mechanism followed by MSCs to commit to osteogenic lineage and to produce the histo-architecture of neo-tissular bone recapitulates and is closely related to its analogue *in vivo* mechanism. It is governed by the intrinsic capabilities of the cells and the expression of several signalling, growth factors and cytokines in an orchestrated cross-talking to regulate its key stages (Veillette and McKee, 2007, Caplan, 1988). It will require the commitment of the undifferentiated multipotent cells to the osteogenic lineage, and the differentiation into pre-osteoblasts (osteoinduction) (Urist, 1965, Albrektsson and Johansson, 2001). Signalling pathways normally associated with osteoinduction are: WNT Signalling, TGF-β Signalling, FGF Signalling and Bone Morphogenetic proteins signalling, being the last one, one of the most studied. In Figure 5, a scheme of this cross-talk can be seen.

1.1.3.3.1 Regulation of osteogenesis by Bone morphogenetic proteins signalling

The bone morphogenetic proteins (BMP) family, is a secreted cytokine/ growth factor subfamily of the transforming growth factor-β (TGF-β) superfamily of signals. They are multifunctional cytokines regulators of development, proliferation, differentiation, adhesion and apoptosis in different cell types at embryonic and adult ages (Kirkbride et al., 2008, Miyazono, 2000). Abundant in lung, spleen and colon, and in low but significant levels in heart, brain, placenta, liver, skeletal muscle, kidney, pancreas, prostate, ovary and small intestine.
The BMPs signalling pathway has widely been recognized as the main player in a series of developmental processes in the body, and the disruption of them has been connected to a number of bone diseases (Chen et al., 2012). They induce and are essential for cartilage, tooth and bone formation, crystallisation and maturation, having major relevance in the differentiation of stem cells in vitro and in vivo into osteogenic lineage, through the induction of the expression of gene Runx2 in early stage osteogenesis (preosteoblasts), and subsequently with the expression of Osteocalcin (BGLAP) in late state osteogenesis (osteoblasts) (Wozney, 2002, Chen et al., 2004, Bei and Maas, 1998).

Both BMP2 and BMP7 have been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the use in certain types of bone fracture, making these two growth factors really important in the regenerative medicine field (Ghodadra and Singh, 2008, Starman et al., 2012).
Figure 5 The BMP and TGFβ canonical signalling pathways: BMPs contact surface receptor BMPR-II, forming an heterodimeric complex with surface receptor BMPR-I, BMPR-I gets phosphorylated and interacts with the transcription regulators R-SMADS (SMAD1/5/8) that react propagating the signal through C-SMAD (SMAD4) and translocate to the nucleus, then they interact with DNA binding proteins, to regulate gene expression, in a cascade of genes (Runx2, dlx5, Msx2 will trigger Osterix) that will end up in osteoblasts differentiation. The TGFβ contact TGFβR-II, forming an heterodimeric complex with TGFβR-I, TGFβR-I gets phosphorylated and interacts with
R-SMADS (SMAD2/3) that react propagating the signal through SMAD4 and translocate to the nucleus, then they interact with DNA binding proteins, to regulate gene expression, in a cascade of genes (Runx2) that will lead to osteoblasts differentiation (Cai et al., 2012, Song et al., 2009, Yavropoulou and Yovos, 2007). Dexamethasone through interacting with the glucocorticoid receptor (GCR) protein suppressing the inhibitory action of NFkB (by suppressing TNF-α signalling inhibitory effect and its NFkB activation) on BMPs induced osteoblastic differentiation.

BMPs and TGF-β signalling pathways are two highly interactive pathways, and their cross-talk is fundamental in the osteoblastogenesis and osteoclastogenesis (Chen et al., 2012). Both pathways share inhibitors and activators. The central signal messengers for BMP and TGF-β are the Smad proteins. There are three subclasses of Smads: the pathway restricted Smads (R-Smads, i.e. Smad1/2/3/5/8), the common mediator Smads (C-Smads, i.e. Smad4) and the inhibitory Smads (I-Smads, i.e. Smad6/7). BMPs and TGF-β are mediated by Smad4 and inhibited by Smad7. A diagram of the canonical pathway can be seen in Figure 5.

The BMP signal transduction requires the interaction between two distinct types of transmembrane receptors forming a complex (BMPR-II, a high affinity receptor for ligand binding and BMPR-I, an activator of the signal transduction), that will spread the signal through the cytoplasm by the interaction of pathway restricted Smads (Smad1/5/8 or r-Smads) and common mediator Smads (Smad4 or c-Smads) to finally translocate the signal to the nucleus and regulate/generate a cascade of gene expression that will end up in osteoblastic differentiation. The binding of BMPs to BMPR-II results in transphosphorylation and activation of the BMPR-I by BMPR-II, activating the BMPR-I and the subsequent signalling by the heterodimeric receptor complex. BMPR-I will bind, phosphorylate and then activate the Smad1/5/8 transcriptional regulator, which as well will bind and form a complex with Smad4 to subsequently translocate into the nucleus. Inside the nucleus, the r-Smad/c-Smad complex will directly interact with DNA as well as with DNA-binding proteins directing the transcription of the targeted genes. The gene cascade will start with Runt-related transcription factor 2 (Runx2 or CBFA1), followed by expression of collagen I (Col1) and osteopontin (OPN or Spp1), osterix (OSX) in the preosteoblast stage (Komori, 2010,
Matsubara et al., 2008). Mature osteoblasts keep expressing Col1 and start expressing osteocalcin (Bglap), but Runx2 is kept in a constant level. The non-canonical, smad independent signalling pathway (not described here), through a different mechanism converges to the same gene, Runx2.

Runx2, Sp7 transcriptional factor (SP7) and canonical Wnt signalling will be determinant for osteoblastic differentiation. After BMPs signalling triggers the induction of Runx2, the initial transcriptional factor to form a preosteoblast, Runx2 and Wnt signalling finally direct the preosteoblast to a mature stage (Komori, 2010). After commitment to osteoblasts lineage, cells will express different bone matrix protein genes, depending on the stage of the cell development, immature preosteoblasts will weakly express collagen I (Col1) and in a higher level osteopontin (OPN or Spp1) and osterix (OSX), forming unpacked woven bone (Komori, 2010, Matsubara et al., 2008). Mature osteoblasts will strongly express Col1 and osteocalcin (Bglap), forming a densely packed lamellar bone. Finally, when osteoblasts become osteocytes, they will express dentin matrix acidic phosphoprotein (Dmp1), sclerostin (SOST) and podoplanin (E11).

A scheme showing the transformations from MSC to osteocyte can be seen in Figure 6.
1.1.4 Summary and Conclusions of this section
Bone is a heterogeneous tissue with high structural complexity. The composition of the major components will ulteriorly define its function. This complexity is accompanied by a convoluted net of developmental pathways, that will allow all the different structural patterns in bone to be generated. In order to bio-mimic this tissue in vitro, these developmental pathways need to be studied, understood and controlled. A proper system has to be used to create the adequate microenvironment, that will enable this to happen. Tissue engineering has proven to be a discipline with all the tools required to face the challenges involved, and is slowly becoming a leading actor in the development of bone related clinical appliances and implants.

In the next section, tissue engineering and the multidisciplinary approach it can provide to tackle bone regeneration, will be described.
1.2 Bone tissue engineering
Regeneration has been of great interest to investigators since long ago. Research has been done with the focus in understanding the biological mechanisms beneath the development and regeneration of biological tissues, and the tools this knowledge could engineer/ pioneer to help medicine and the prosthetic industry. That’s how in the last twenty years, the advances on medicine and biological sciences guided engineering in the footpath of Tissue Engineering (TE).

Langer and Vacanti defined TE as “an integrated research field that applies the principia of engineering and life sciences for the development of biological replacements that restore damaged tissue function” (Langer and Vacanti, 1993). Furthermore the nature of TE research, positions improve functionality of tissues at a glance (Salgado et al., 2004). From a clinical perspective this encompasses the production of in vitro engineered tissues for in vivo transplantation.

Bone tissue is a major focus of interests for TE. Its complexity has drawn the attention of many researchers with the believe that bone could be recovered through enhancing regenerability or by biomimicry, rather than using prosthetics.

The orthopaedic industry, studying complex materials and designing devices, has produced spare bone substitutes able to partially recover damaged bone. Nevertheless, its focus has not been to find cures, but design putative spare devices. Commonly, bone cements, metals and screws are used to fill or pin gaps and replace portions of bone; autologous and allogeneic engraftments are used to help healing of fractures with partial success. These methodologies work for the most common and simple cases, but as mentioned in the previous section, there is an important number of cases in which they fail. For these cases, graft surface enhancement with bioactive osteoconductive growth factors, has been tried, but with limited success. The lack of functional cells, hinders the inclusion of the grafts to the metabolic normal mechanisms of the bone environment, and in due process its maturation towards functional tissue. Thus, it is required to find a better answer for those questions (Navarro et al., 2008, Hench and Polak, 2002).
Figure 7 The Tissue Engineering principles

Bone TE envisions new horizons: Going the logical one-step above the prosthetic industry, trying to produce in vitro engineered bone tissue for in vivo transplantation, studying the bone healing process. And in due process, providing the next generation of tailor-made biomaterials that mimic bone tissue. Also, offering alternatively to repair or regenerate failing bone as a much viable method of accomplishing its goals.

The use of living cells to produce biological substitutes to replace, repair and/ or regenerate failing bone with functional living bone-alike constructs requires the growing/ fabrication/ implantation of bone ex vivo, and/ or the growth in vivo (Polak et al., 2008). From a cellular perspective, the actors involved in bone regeneration are: cells, extra cellular matrix (ECM), signalling, cellular dynamics and the interactions between them.

The constant research in TE, has lead to the finding of a variety of cell sources for its purposes, including: embryonic stem cells (ESCs), hematopoietic stem cells (HSCs), adult stem cells (ASCs), bone marrow-derived mesenchymal stem cells (BM-MSCs), umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), adipose tissue-derived stem cells (ADSCs), muscle-derived stem cells (MDSCs), and dental pulp stem cells (DPSCs) (Seong et al., 2010, Peng et al., 2009, Polak et al., 2008, Placzek et al., 2009); and
manipulating these cells in vitro and in 2D static systems it has been possible to mimic different types of tissues: bone cells, teeth cells, cartilage cells, skin cells, neurons, pigment cells, cardiac muscle, , tubule cells, pancreatic cells, thyroid cells and lung cells (Placzek et al., 2009, Hwang et al., 2009, Pagkalos et al., 2010b, Itskovitz-Eldor et al., 2000, Peng et al., 2009).

However, researching cells on its own cannot provide a comprehensive and suitable solution for bone regeneration. Bone cells are enclosed in a 3D complex microarchitecture coordinated by intricate signalling dynamics. This 3D environment is subjected to mechanical stresses and in constant interaction with adjunct tissues.

This reasoning has led bone TE bioprocessing methodology to be involved in four areas of research: cells, scaffolding, nutrition/ signalling and bioprocessing. These four components are key to emulate the conditions in which the body normally regenerates/ develops bone. The proper healthy cells (type and source) must be chosen, seeded and grown in order to produce sufficient amount of bone cells; the scaffolding to provide the microenvironment required to support the cell growth, to develop and mature into bone and to interact and integrate with the surrounding tissues after implantation; signals to stimulate the correct response from the cells (growth factors or ECM); and finally, the system to seed the cells/ scaffolds and provide the fluid dynamics/ mass transfer/ mechanical stress to deliver the signalling properly (Polak et al., 2008).
Figure 8 Scheme for in vitro development of bone

In Figure 8 Scheme for in vitro development of bone, in A) the static and B) the dynamic path for developing bone tissue in vitro can be seen, where cells are expanded, then encapsulated to finally be scaled up and differentiated in the selected platform.

In the next sections each of the four areas of study will be reviewed in a more thorough manner. What type of cells is suitable for bone, which scaffold will provide the milieu for the bone cells and ECM deposition, what cues will foster bone differentiation and finally what platform should be used to deliver the cues and to sustain a healthy cellular growth.

1.2.1 Cell sources for bone Tissue Engineering

Cells with potential for bone TE can be supplied exogenously by donors or by biopsies from the patients (autologous), then expanded and differentiated in vitro, to further be transplanted. To accomplish this, it is necessary to find a reliable and renewable source of healthy cells, that could be easily isolated and expanded into required quantities (quality is age dependant), that is compatible with humans, and that with the appropriate cues, is capable of enhancing the body’s regeneration, to finally become part of it (Boheler and Fiszman, 1999).
The natural choice of cells with bone TE potential are Osteoblasts. These wouldn’t present an immunogenic reaction from the body when acquired autologously and further expanded *in vitro*, they wouldn’t require differentiation and they would be expected to be fully functional bone cells. However, these cells come with a series of limitations: the invasive extraction is time consuming and damaging for the patient or donor, low number cell extraction yield and with poor expansion. The scale-up of these cells, comes followed by senescence and the loss of their phenotypes (Luyten et al., 2001). Additionally, cells could have compromised their quality if the donors present an impaired functionality of osteoblasts product of bone diseases.

Xenogeneic cell (non-human) could cope with the low number of cells. However, they come with immunogenic reactions and phenotypic mismatch, ultimately leading to rejection of the tissue and further health complications.

The early development of embryos and the self-repair mechanisms of the body, together harbour a source of cells that complies the requirements of TE, the stem cells. These have the remarkable potential to expand and give rise to all the tissues in the body. Being in charge of the development of the body at early stage, and the growth and regenerative processes after birth. Since their discovery, they have become an obvious place to research for alternative treatments to tackle the disadvantages and weaknesses of the prosthetic industry. If properly handled and guided, they posses the key to empowering the regenerability of the body, and ultimately of curing many different diseases and injuries.

To choose the proper source of cells, it has to be considered: 1) The immune response of the body towards them, 2) Its ability to proliferate, 3) Its location and ease of extraction, and 3) the feasibility to differentiate into specific functional cell types. This is a huge advantage for stem cells (SCs), especially the embryonic stem cells (ESCs), over any kind of somatic cells. As SCs are normally involved in repairing damage tissue, they do not need to be local, they have certain level of ubiquity in their use, although some of them are subjected to specific cell lines or a closely related family. It will depend on the potency of the cells:
• Totipotent stem cells: cells with the ability to give rise to all the cell lineages of an organism (embryonic, somatic, and germ cells), being able to produce a whole organism; in mammals only the zygote and the first cleavage blastomeres are totipotent.

• Pluripotent stem cells: cells with the ability to give rise to the three germ layers (endoderm, mesoderm, ectoderm). In this group of cells we find the ESCs and the induced pluripotent stem cells (iPSCs).

• Multipotent stem cells: adult cells with the ability to give rise to multiple cell types of one lineage. Good examples are the bone marrow-derived mesenchymal stem cells (BMSCs).

• Unipotent stem cells: cells able to give rise only to one cell type. Good example is spermatogonial stem cells (can only generate sperm).

• Somatic cells: cells that form every tissue from the body of an organism, different from a gamete or any kind of stem cells.

Figure 9: Cell sources (Seong et al., 2010)

1.2.2 Pluripotent stem cells
SCs are clonal precursors able to self-renew or to differentiate into other specialised cells. There are different types of stem cells, presenting unique challenges and benefits for the manufacture of therapeutics for the clinic (Placzek et al., 2009).

1.2.2.1 Embryonic stem cells
SCs of embryonic origin comprise: embryonic stem cells (ESCs) that are derived from the inner cell mass of blastocyst-stage embryos; as well as embryonic germ (EG) cells, that are cells derived from the developing foetal gonadal ridge. Additionally, embryonal carcinoma (EC) cells that develop from testicular tumour can be considered as ESCs,
although this is controversial (Heng et al., 2004a, Vallier et al., 2009). These cells are capable of indefinite expansion and can give rise to any cell lineages (pluripotent). An early study made by Smith et al. showed that murine ESCs in absence of heterogeneous feeder cells can sustain undifferentiated growth, when in presence of cytokines with differentiation inhibitory activity (DIA), as Leukemia Inhibitory Factor (LIF) (Smith et al., 1988). In the absence of these conditions, ESCs lose its self-renewal capacity and pluripotency and do not persist more than a small number of days. Similar observations were made by Watanabe et al. when studying human ESCs and the use of Rho kinase (ROCK) inhibitor, when cultured with or without a growth inactivated feeder layer (Watanabe et al., 2007, Claassen et al., 2009). Therefore, under the proper conditions, ESCs can be maintained in an unlimited self-renewal undifferentiated state, or be differentiated into any cell lineage in vivo and in vitro.

**Table 1: The different signalling pathways involved in self-renewal and pluripotency of hESCs**

<table>
<thead>
<tr>
<th>Genetic factor</th>
<th>Name</th>
<th>Family</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oct 4 (Oct-3, Oct-3/4, Pou5f1)</td>
<td>Octamer binding factor</td>
<td>POU</td>
<td>initial development and maintainance of pluripotency</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducers and activators of transcription</td>
<td>DNA-binding</td>
<td>activation via LIF and glucoprotein 130 is esencial for maintaining pluripotency</td>
</tr>
<tr>
<td>Nanog</td>
<td>Tex determining region Y box 2</td>
<td>Homebox</td>
<td>maintain ESC self renewal in absence of LIF/gp130/STAT3</td>
</tr>
<tr>
<td>Sox 2</td>
<td>HMG</td>
<td>self renewal growth</td>
<td></td>
</tr>
</tbody>
</table>

In Table 1, some of the signalling pathways associated with growth and pluripotency can be seen, a thorough description can be seen in Figure 10 Stem cell pathways (Walia et al., 2012) where all the known pathways associated with pluripotency and differentiation are shown.

None of these pathways suffice to sustain the self-renewal and pluripotency of hESCs, and hence prevent spontaneous differentiation. Factors and signalling remain to be elucidated in further studies (Humphrey et al., 2004, Daheron et al., 2004).
These characteristics of ESCs allow them to be one of the most useful cell sources for TE and regenerative medicine. Lineage specific differentiation of ESCs can be directed under specific culture conditions and by manipulating the microenvironment. Recently, substantial attention has been given to directing ESCs differentiation into osteogenic lineage and other lineages of the human body (Seong et al., 2010).

Currently, efforts are being made to produce patient-tailored ESC lines that overcome immunological rejections. However, ESCs have negative aspects to consider: they are capable of teratoma formation, the differentiated fate they follow is very difficult to control, and they elicit ethical considerations due to the destruction of the embryo (Placzek et al., 2009). All these factors hinder the application of ESCs in cell therapy. And in the process to cope with them, the induced pluripotent stem cells were developed (Takahashi et al., 2007).

1.2.2.2 Induced Pluripotent Stem cells

Induced pluripotent stem cells (iPSCs) are adult somatic cells reprogrammed in vitro to an embryonic-like pluripotent state by the introduction and forced expression of the main genes and factors associated with pluripotent cells (Walia et al., 2012). Mouse and human fibroblasts can be reprogrammed by transduction with a complex combination of transcriptional factors (Oct 3/4, Sox2, Klf4, and c-Myc or alternatively Oct3/4, Sox2, Nanog, and Lin28). Yamanaka et al. reprogrammed fibroblasts by defined factors, without the use of vectors (Takahashi et al., 2007, Yamanaka, 2007). The cells resulting from the reprogramming, attain an ESC-like state able of indefinite proliferation, with the sustained capacity to differentiate into cell types characteristic of all the three germ layers in vitro and in vivo. These express most of the markers associated with pluripotency, having epigenetic and morphological characteristics similar to those of ESCs. Although the potential of iPSCs compared with that of ESC fluctuates between similar to less effective depending on the cell lines tested and the differentiation analyses performed (Bilousova et al., 2011). However, it remains to be demonstrated whether both cell types rely on similar mechanisms to maintain pluripotency and to drive their differentiation (Vallier et al., 2009).
The known molecular pathways acting in maintenance of pluripotency and differentiation can be seen in the Figure 10 Stem cell pathways (Walia et al., 2012).

Although iPSCs do not elicit ethical considerations as their close relatives, the ESCs; they have lower expansion and differentiation rates. But most importantly, they still lack in safety standards. Not only they carry the possibility of producing teratogenesis in the hosts as ESCs, but also they introduce a new negative factor to consider, the use of dangerous vectors (lentiviruses, retroviruses, adenoviruses, plasmids, transposons and recombinant proteins) required to achieve cell reprogramming (Das and Pal, 2010).
1.2.3 Multipotent stem cells

Multipotent stem cells or adult mesenchymal stem cells (MDCs) are anchorage-dependent, fibroblastic spindle-shaped, undifferentiated cells found throughout the adult body. They belong to specific niches in different organs, playing a critical role in postnatal tissue development and healing (Yu et al., 2007). These cells heal damaged tissue and replace dying cells. They present an immunomodulatory effect, *in vitro* and *in vivo* studies have demonstrated that these cells can suppress T cells proliferation and decrease the production of various inflammatory cytokines (Fibbe et al., 2007). Adult stem cells have been used clinically since 1950s, when stem cell transplantation was introduced for the first time, using bone marrow-derived stem cells. Since then, many types of MSCs have been identified in the body, offering alternative possibilities, which originate from easier to harvest and less invasive cell sources, such as fat, skin, olfactory cells and peripheral blood (PB) (Placzek et al., 2009).

MSCs have been isolated from the connective tissue of almost every organ, suggesting a role as a storage and regenerative pool for the various mesenchymal tissues. Several studies have been successful in evaluating the capability of MSCs for their feasibility and efficacy in healing cartilaginous, osseous, tendon defects or even in treating genetic disorders such as osteogenesis imperfecta or Duchenne’s muscular dystrophy (Schultz et al., 2000).

MSCs sources, bone marrow-derived mesenchymal stem cells (BM-MSCs), umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs), adipose tissue-derived stem cells (ADSCs), muscle-derived stem cells (MDSCs), and dental pulp stem cells (DPSCs) (Seong et al., 2010, Peng et al., 2009, Polak et al., 2008, Placzek et al., 2009); and manipulating this cells they have been able to produce different kinds of cell tissues. When stem cells are supplemented with hydrocortisone, insulin, T3, EGF, VD₃ and ascorbic acid, they differentiate into skin cells when grown in collagen/ poly(l-lactic acid)–co-poly(3-caprolactone) nanofibrous scaffolds (Jin et al., 2011). Moue and human SCs *in vitro* can be differentiated to neural cells, when cultured in growth-factor-minimized medium, in what is called intrinsic transition (Kamiya et al., 2011). pigment cells, cardiac muscle, bone cells, teeth cells, cartilage cells, tubule cells, pancreatic cells, thyroid cells and lung cells are other examples of cells that have been produced
bioprocessing stem cells (Placzek et al., 2009, Hwang et al., 2009, Pagkalos et al., 2010b, Itskovitz-Eldor et al., 2000, Peng et al., 2009).

In the postnatal state, cells with osteogenic potential persist in the bone marrow, in dental pulp and in other cell niches. They play an integral part in bone growth and remodelling, as well as in bone repair during postnatal life (Heng et al., 2004b). These cells continuously replicate, while some become committed to cell lineages such as bone, cartilage, tendon, ligament, and muscle. The differentiation of these cells is a complex multistep pathway involving distinct cellular transitions. Progression depends on the presence of specific bioactive factors, nutrients, and other microenvironmental cues whose controlled action orchestrate the differentiation phenomenon (Bruder et al., 1994).

The International Society for Cell Therapy (ISCT) proposes as sufficient criteria to define a human MSCs the following: MSC must be plastic-adherent when maintained in standard culture conditions, must express the surface markers CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR when measured in flow cytometry. They must differentiate to osteoblasts, adipocytes and chondroblasts in vitro. These panel of criteria represents the commonly used and allows a standardised study of MSCs (Dominici et al., 2006).

1.2.3.1 Dental pulp stem cells.

Dental pulp stem cells (DPSCs) are an interesting and particular source for adult stem cells. They have ease in access and extraction, a high proliferation ability and multipotency and they do not elicit ethical considerations as their extraction is one of the less invasive between SCs.

DPSCs are particular, they come from a family of MSCs called the ectomesenchyme, as it can be seen in Figure 11 Stem cell fate. In embryonary development, they arise from the neural crest, which is committed to ectomesenchyme by order of FGFs signalling upon entering the pharyngeal arches. Hence it is directed by epithelial cues within the arches, making the cells STRO-1 positive, as many mesenchymal stem cells. These cells comply with the minimal criteria for MSCs set by the ISCT as well as being positive for pluripotent markers like SSEA4, OCT3/4, NANOG, SOX2, LIN28 (Blentic et al., 2008).
They can be used as a source of cells for bone tissue, dental/periodontal or non-bone tissue regeneration therapies, DPSCs are self-renewing and able to express markers of bone, cartilage, teeth, vascular and neural tissues, suggesting their multipotential capacity (Karbanova et al., 2011, Shi and Gronthos, 2003, Kawashima, 2012, Wei et al., 2007).

**Figure 11 Stem cell fate**

Previous studies have shown the capability of DPSCs to regenerate their microenvironments with outstanding fidelity, including the surrounding mineralised structures of bone and dentin (Shi and Gronthos, 2003), being able to differentiate into bone-like constructs and dentin-pulp-like tissues when cultured with media supplemented with dexamethasone, ascorbic acid 2-phosphate and inorganic phosphate (Gronthos et al., 2000, Yu et al., 2007, Duailibi et al., 2008, Lee et al., 2011, Ito et al., 2011, Liu et al., 2011, Mangano et al., 2010, Khanna-Jain et al., 2010), and into cartilage and into muscular tissue (Zainal Ariffin et al., 2012, Lizier et al., 2012).

Research has been done in search of the proper techniques for directing the differentiation of DPSCs taking in consideration the effect of different growth/transcriptional factors, cytokines and other biological compounds in monolayer culture.
Results have shown that transforming growth factor (TGF-β) and 5-Aza-20-deoxycytidine (DNA demethylation agent) have myogenic action (Maeda et al., 2004, Nakatsuka et al., 2010, Song et al., 2012, d’Aquino et al., 2007), fibroblast growth factor β (bFGF) and epithelial growth factor (EGF), neural differentiability (d’Aquino et al., 2007, Bei and Maas, 1998, Bilousova et al., 2011); and bone morphogenetic factor (BMPs), dexamethasone and natural polysacaride biomaterials as chitosan and glucosamine have osteogenic and odontogenic capability (Aberg et al., 1997, Bei and Maas, 1998, d’Aquino et al., 2007, Huang et al., 2010).

Figure 12 DPSCs multipotency (stemsaveblog.com, 2012)

Collections of DPSCs have been evaluated as potential subjects for iPSCs banks by retroviral transduction of the conventional reprogramming transcriptional factors: Oct3/4, Sox2, Klf4 and c-Myc, with positive results, and with the effective generation of iPSCs even without the use of c-Myc that is an oncogen with tumorgenicity (Tamaoki et al., 2010, Takahashi et al., 2007).

As hDPSCs harbour the potential to overcome many of the difficulties that other SCs present, and as their quality as SCs is not hindered by difficult access or ethical considerations, they should be exhaustively researched for further engineer cell
therapeutics to be applied in bone regenerative medicine and stem cell healing products in general.

1.2.4 Human dental pulp stem cell 3D bioprocessing
Traditionally, cells have been grown in 2D static monolayered culture systems, where cells are grown attached to a supportive plane in their basal surface and are exposed to culture media in their upper face. Consequently, cells in the basal area receive limited contact with nutrients and signals, developing heterogeneously. Cells allocated into planar cell culture flasks become flatter, divide abnormally and lose their differentiated phenotype (Baker and Chen, 2012). Thus, many findings in 2D static culture are not reproducible in vivo or in tissue explants. The efforts to solve the gap between the culture techniques and the in vivo tissue have derived into the development of 3D static and dynamic culture systems. These try to mimic the host tissue microenvironment more accurately. Adding a scaffolding machinery, mechanical stimulus and perfusion of culture media through the cells. Enhancing the exchange of nutrients and removal of waste between the cells and the media required for proper cell expansion and differentiation. It has already been seen by several researchers that the 3D environment allows the cells to maintain most of their characteristics and markers under prolonged culture periods (Godara et al., 2008, Baker and Chen, 2012, Sailon et al., 2009).

In this section scaffolds, cell culture systems, nutrient requirements and environmental conditions will be reviewed.

1.2.5 Scaffolds
In vivo, bone cells are contained in a tissue specific 3D environment that hosts the cells and their interactions. Herein, water, nutrients, cytokines and growth factors are delivered to the cells. To regain functionality of damaged bone, this microenvironment has to be temporarily reproduced in the form of a scaffold. These matrices supply a supporting microarchitecture for the cells to grow and adhere. Providing a template for normal cell-cell interactions and the mass transfer for nutrients and signalling delivery. In other words, the scaffold plays the role of an artificial 3D ECM (Hutmacher, 2000, Sheridan et al., 2000).
SCs can be seeded into 3D biocompatible and bioresorbable scaffold, allowing the slow resorption of the scaffolding while the growth of cells and development of the tissue happens either ex or in vivo.

A scaffold has to be designed considering the restrictive semi-permeable properties of the materials in use, as cells get physically isolated from the exterior. It has to allow the essential molecules required by the cells to diffuse through its pores, and leave outside the undesired particles (Uludag et al., 2000). The permeability and size of pores won’t allow access to bigger molecules. Accordingly, we have to consider its mass transfer properties and the particular needs of the cell type.

After transplanting the new grown tissue, osseointegration, osteoinduction, osteoconduction, and fast/ abundant vascularisation is on demand; otherwise there could be a negative immune response, or the lack of oxygenation could hinder, or even kill the cells (Abbah et al., 2006, Sheridan et al., 2000).

In general, the fabrication of a scaffold for bone tissue requires a highly biocompatible material, hence not having any potential immune response (osseointegration). It has to allow the migration of cells from the surroundings to inhabit the scaffold (osteoconduction) and aid their osteogenic commitment (osteoinduction). Moreover, it has to be bioresorbable in a controlled rate, allowing the cells to replenish the area with bone ECM and in the process, to generate a new organically generated neotissue. The rate of biodegradability should be tightly similar with the time required for the injury to be regenerated.

Metals, ceramics and polymers from natural and synthetic origin have been studied as possible bone scaffolds. Nevertheless, metals and most of the ceramics cannot be used for this purposes, as they are not bioresorbable.

Bioceramics and bioglasses commonly used are: the natural coralline hydroxylapatite (HA) or synthetic as calcium phosphate(CaP), synthetically produced HA or β-tricalcium phosphate (β-TCP), SiO₂, CaO, Na₂O and 45S5 bioglass®. All of them being biodegradable, osteoconductive and osteoinductive. However, these materials present several limitations. They are brittle and have poor mechanical features and in vivo they
can be degraded unexpectedly by osteoclastic activity, hence compromising their quality (Rezwan et al., 2006).

There is a wide range of regulatory approved biodegradable and bioresorbable polymers used in manufacturing scaffolds with osteoconductive and osteoinductive features. These include natural-based materials, with a polysaccharide/protein nature, such as: alginate, collagen, chitosan, starch; and the synthetics-based materials, such as: poly(α-hydroxy acids) (PHA), polylactic acid (PLA), poliglycolide (PGA), poly(lactic co-glycolic acid) (PLGA), poly(ε-caprolactone) (PCL). These polymers undergo degradation and allow the vascularization of the engineered tissue in transplants. Bioactive compounds (as growth factors) can be embedded in these polymers and slowly secreted along with the degradation of the polymers (Hutmacher, 2000, Augst et al., 2006). However, the biopolimers have poor mechanical properties and variable physical properties, depending on the sources of the protein matrices. A feasible method to cope with these drawbacks has been the use of hydrogel microcapsules.

Hydrogel microcapsules with a diameter of 0.3-1.5 mm are suitable for encapsulation of cells and in vivo transplantation. Hydrogels are highly hydrated polymer materials, able to overcome the drawbacks of their solid biopolymers counterparts (Drury and Mooney, 2003). They provide a large surface area to volume ratio, hence high mass transport. The interstitial space between microcapsules allows angiogenesis, and the appearance of blood vessels in the neo-tissue is fundamental to properly heal bone. They are more resistible to mechanical stress than ceramic scaffolds, a desirable feature for 3D bioprosessing. Encapsulation of cells can be achieved by reacting polymers with different charges and forming an aqueous biocompatible matrix (Uludag et al., 2000). However, the use of highly toxic solvents in the polymerisation methods is disadvantageous for the encapsulated cells. As well, the difficulties in the technique make difficult to incorporate bioactive compounds in most of the polymeric scaffolds.

Alginates are biopolymers widely utilised in the industry and in clinical studies. Alginates are found in natural sources as an heterogeneous population of polymers, they are purified from different species of seaweed, brown algae and bacteria (Augst et al., 2006). They have a number of useful features as biodegradability, osteoinductivity,
osteocompatibility and osseointegration. They can be bead shaped with microcapsule size, as well as being easily produced hydrogels without the use of solvents. They are frequently used clinically as a delivery carrier for proteins and cytokines, to stimulate healing of tissue, as a vehicle for cell transplantation and as an immunological wall.

1.2.5.1 Alginate scaffolds

Alginites are natural hetero-polysaccharides isolated from brown algae such as Laminaria hyperborea and lessonia. Alginic acid is constituted by an heterogeneous mixture of guluronic acid (G), D-manuronic acid (M), with different concentration ratio depending on the source (Augst et al., 2006). They are linear and unbranched copolymers, ionically and covalently linked in varying sequences (i.e. GGGG, MMMM or GMGM). These can be stabilised with Na⁺ forming sodium alginate, and then gelified by cross-linkage with multivalent cations (e.g. Ca²⁺, Ba²⁺ or Sr²⁺). Using different alginites (arrangement and ratio of the G/M-blocks) as well as the type and concentration of cations will produce gels with different properties.

The mechanical properties of the hydrogel beads, stiffness and swelling, can be controlled by physical factors as alginate solution concentration, G/M ratio, and the cross-linking density (time, temperature and stirring dependant). Normally the higher the concentration of polymer, the stiffer the gel. However, the viscosity of the pre-gelled solution will be higher as well and the mechanical stress produced by handling the solution could damage the cell content. Managing the elasticity of the beads and avoiding high levels of viscosity product of the concentration, can be achieved by manipulating the G/M ratio and by addition chelating agents. Gelling at lower temperatures allows a more organized structure and hence enhancing mechanical properties. The composition of the pre-gelling solution affects the swelling properties and the pore size (ranging from 5-18 nm in beads of 80-120 µm, with a density of 1.06-1.12 g/cm³) (Klein et al., 1983, Hutmacher, 2000). Remarkably, the gelling process and the resulting beads produced, is highly reproducible when same conditions and regents are used.

Alginate microcapsules have been used for cell culture and bone tissue engineering, on its own and as composites, with other materials as gelatin, chitosan, collagen. Many
researchers have reported bone regeneration using this scaffold loaded with SCs with success. When using alginate as a drug delivery system with BMPs, they have been able to produce mature osteoblasts (Saito et al., 2005, Li et al., 2005). Adding Gelatin to the scaffolds has been considered to strengthen the hydrogel to be used in long term culture. As alginate alone, when ionically cross-linked, loses Ca$^{2+}$ ions and could collapse (Basmanav et al., 2008). Furthermore, cell adherence and proliferation has been seen to significantly improve. (Venkatesan et al., 2015).

Alginate microcapsules can fill irregularly shaped areas and be implanted in vivo with a minimally invasive procedure. These can host different types of MSCs and be cryopreserved maintaining cell viability for banking for future use (Venkatesan et al., 2015). In vivo studies have demonstrated alginate osseointegration, osteoinductivity and osteoconductivity (Alsberg et al., 2001).

![Figure 13: Guluronic acid (G) and Manuronic acid (M) acids](image)

When cells are grown in alginate hydrogels (or most of the 3D scaffolds) on standard static culture flasks, the morphology of the cells and the environment for cells to grow, in comparison to the traditional monolayered static culture, is closer to the one found in vivo. Nevertheless, the transport of oxygen, nutrients, metabolites, wastes and other important molecules through the scaffold occurs by diffusion. This has negative repercussions in the culture, as the high metabolic demand by the cells is not achieved, ultimately forcing the starving cells to migrate from the inner scaffold, to the surface, or dying. If the culture is prolonged, cells from the surface of the scaffold travel to the flask surface. This phenomenon hinders the osteogenicity and osteoinductivity of the scaffold, and renders poor osteoconduction when transplanted in vivo. The cells leaving the scaffold would become flattened with all the implications previously discussed. The
dynamics of the *in vivo* environment cannot be achieved by this setting alone. Mechanical stimulation caused by hydrostatic pressure and shear stress have a pivotal action in osteocyte functionality, osteoblast growth and mineralisation.

The requirement of a bioprocessing dealing with the local and global transport and the dynamic characteristics of bone, becomes unavoidable and fundamental. In the next section, this will be described in detail.

### 1.2.6 Stem Cells Bioprocessing

Bone tissue is a complex structure subjected to active interactions, between multiple cell types under mechanical stresses, and a dynamic 3D micro and macro architecture/local and global environment. The development, maintenance and regulation of stem cell populations for bone-related cell therapeutic products requires the very difficult task of replicating this complex and specific microenvironment and the dynamicity of its transport characteristics. Thus, the design of adequate tailor-made bioprocessing systems able to achieve such high standards is on demand (Salgado et al., 2004). The desirable bioprocessing should consider control, reproducibility and the possibility to scale up, as well as the routine good manufacturing practices (GMP) required for clinical applications.

Different bioprocessing systems using shear stress have been under study. The mechanisms in which shear stress affects cell culture are not yet established. Nonetheless, it is clear that it is an important biophysical signal, with cell growth and bone mechano-transduction effect. These mechanisms comprise calcium signalling, mechanical force transmission along fibrous cytoskeletal networks, cell-cell communication, and disruption of ligand-receptor binding, between others (Akins et al., 1997, Morrison et al., 1992, Talbot et al., 2010).

In Figure 14, pictures of different bioprocessing systems can be seen, they will be discussed in detail.
**Figure 14 Different bioprocessing systems**

The spinner flask bioreactor (SF) is a simple and inexpensive dynamic culture system in which, cell-seeded scaffolds are attached to needles secured to the lid of the vessel or seeded into microcarriers and the culture media is subjected to turbulence by a magnetic stirrer. The amount of shear stress depends on the stirrer speed. This bioprocessing has successfully been used to generate bone constructs. Sikavitsas and colleagues used a system consisting of murine MSCs (mMSCs), porous PLGA disks (D=12.7 mm and L=6 mm) and SF, they found that proliferation, mineralisation, ALP activity and osteocalcin expression are enhanced compared to static culture (Sikavitsas et al., 2002). Stiehler et al. found similar proliferation and mineralisation results when bioprocessing hMSC in cubic PLGA scaffolds (8x8x5 mm$^3$) with a SF (Stiehler et al., 2009), they assessed a bigger panel of genes, finding COL1A1, BMP2, OPN, RUNX2 and OSX to
be upregulated when compared to static culture. However, they found OC, ALP and BSP expression to be downregulated (Stiehler et al., 2009). Jin and co-workers bioprocessed murine pre-osteoblasts in porous PCL-PLPC microcarriers (D=0.35 mm) in SF, to further successfully assess a mice in vivo model (Jin et al., 2014). Nonetheless, the constant stirring of culture media causes turbulent flow around the scaffolds and the cells can form a dense layer in its surface. Oxygenation and nutrition of the cells in the inner scaffold will be disrupted, hindering the healthy development and differentiation of cells and the tissue constructs ultimately produced. In addition, the shear stress supplied by the magnetic stirrer is not homogenous, causing heterogeneous production of calcified scaffolds (Salgado et al., 2004, Sikavitsas et al., 2002, Martin et al., 2004). Another bioprocessing, the Rotating Wall Vessel Bioreactors (RWV) such as the NASA’s developed High Aspect Ratio Vessel Bioreactor (HARV) or the Slow Turning Lateral Vessel (STLV) are fed-batch culture systems able to create a low shear environment along the horizontal axis, which allows cells to be cultured in static suspension. Qiu and co-workers did an early study with murine MSC/osteosarcoma cells in bioceramic hollow microspheres (D=0.2-0.3) bioprocessed by RWV, demonstrating the production of calcium phosphate crystals, with characteristics comparable with HA (Qiu et al., 1999). Botchwey et al. developed a hollow, lighter-than-water microcarriers of bioerodible PLGA (D=0.8 mm), they seeded the scaffold with human osteosarcoma cells and processed the cell constructs in RWV. They demonstrated that the system was able to significantly enhance cell number, ALPase activity, hystochemical phenotypic expression and early mineralised matrix synthesis (Botchwey et al., 2001). Sikavitsas et al. used a system consisting of murine MSCs (mMSCs), porous PLGA disks (D=12.7 mm and L=6 mm) and RWV, they found that mineralisation, ALP activity and osteocalcin expression are enhanced compared to static culture. However, proliferation was decreased (Sikavitsas et al., 2002). Hwang et al. studied mMSCs encapsulated in alginate hydrogels (D=3-3.5 mm) and differentiated in suspended RWV culture, showing evidence of differentiation by gene expression and assessing the material with histology, ATR-FTIR, X-ray microanalysis and mechanical properties (Hwang et al., 2009). These systems are efficient to reduce diffusion limitations of nutrients and when the scaffolds’ density is similar or lower than the density of media they have proved to be useful in producing ex vivo bone, achieving ECM deposition and mineralisation/ALP activity/ Osteocalcin
expression similar or better than the parameters achieved in static culture. RWV-based bioreactors, when working in the right setting, have shown to achieve better expansion and bone differentiation than the SF or static culture (Rauh et al., 2011). Only the work from Sikavitsas et al. with RWV bioreactors shows decreased osteogenic effect. Their findings suggest that when the density of the scaffold is higher than the density of the media, the scaffolds crash against the vessel’s walls with the rotation, thus expansion and differentiation are hindered, achieving poor mineralisation and ALP activity (Venkatesan et al., 2015, Sikavitsas et al., 2002). However, none of these bioprocessing has been able to recapitulate the conditions of the microenvironment found in bone tissue. There is still much to do to understand the complex interaction between the bioprocessing and the microarchitecture, i.e. the local and global cues to produce bone tissue, thus further development is critical to achieve bone mimicry in vitro and to be able to use this tissue in vivo.

To attempt to close the gap between the bioprocessing and the in vivo requirements, a third family of bioreactors was pioneered. Fed-batch feeding strategies from spinner flasks and RWV bioreactors is left behind, to adopt a perfusion feeding strategy.

Perfusion culture systems are diverse in design (containers, cartridges, chambers) but they all have in common a continuous exchange of medium. By generating a laminar fluid flow of culture media through the scaffold, mass transport of nutrients and oxygen is enhanced as well as providing the system with mechanical stimuli. Perfusion ensures supplementing essential nutrients to all the cells in the scaffold, while removing the metabolic waste throughout the cultivation process.

These systems are supplied with a peristaltic pump to drive culture media through the scaffold. Media can flow either in an open or a closed loop system, through gas permeable silicone tubes. The system could be supplied of an oxygenator device and/or gas permeable membranes to ensure adequate oxygenation. Media flow mode can be controlled to be steady, pulsatile or oscillated, hence a dynamic shear stress can be studied. Jacobs and colleagues performed an early study on the differential effect of flow type and bone mineralisation. Their findings suggest that oscillating flow is less potent stimulator of bone cells than either steady or pulsing flow. Furthermore, lower
frequencies were observed to produce higher intracellular calcium concentration for the dynamic flows. These results coincide with the net fluid transport of the fluid profile (Jacobs et al., 1998). Another report performed by Sinlapabodin and colleagues studied the effect of perfusion flow rate (1-3-5 ml/min) in cell density and osteogenic differentiation. They showed that high flow rates can cause the detachment of cells and limit ALP activity and intracellular calcium content. As well, they indicated that the lower flow rate gave better expansion, but less ALP and internal calcium content than the intermediate flow (Sinlapabodin et al., 2016). Accordingly, each bioreactor-scaffold-cell type will have to be assessed for the adequate flow rate. Many studies have been done with steady flow. Wang and colleagues cultured murine osteoblasts seeded-β-TCP scaffold (5x5x5 mm3) inside a perfusion chamber, achieving enhanced proliferation, calcification and ALP/OC gene expression, with a successful in vivo validation (Wang et al., 2003). Gomes et al. and Hosseinkhani et al. utilised a perfusion cassette-like system to bioprocess murine cells in different scaffolds, a starch fibre mesh (D=8 mm, L=1.5-2 mm) and a collagen/PGA (D=8 mm, L=1.5-2 mm) respectively. Both groups were able to produce bone constructs with enhanced proliferation and gene expression compared to static culture. Additionally, Gomes group did in vivo validation with positive results supported by histochemistry (Gomes et al., 2006, Hosseinkhani et al., 2006). Different cells/scaffold pairs with osteogenic potential have been successfully bioprocessed under perfusion flow (Bancroft et al., 2003, Janssen et al., 2006, Qian et al., 2013, Gardel et al., 2014, Kleinhans et al., 2015). Expansion and gene expression have been shown improved compared to static culture and to SF. Further, these in vitro results, have been frequently subjected to in vivo models validation, with positive results in terms of osteoinduction and osseointegration to the host animals.

Several of these reactors are already available in the market with differing ease of use, cost/efficiency, and capability of monitoring and controlling biophysical and chemical parameters. However, further optimization, modification, or the design of new bioreactors, as well as the full understanding of signalling and the culture medium, are required to produce the proper osteoblast-seeded biomaterial able to translate accurately from the in vitro concept, to the in vivo neo-tissue completion.
In Table 2: Current studies about stem cell differentiation towards bone tissue using various bioreactors, a summary of the mentioned publications, plus a selection of other studies can be found.

1.2.7 Nutritional requirements

hDPSCs and MSCs in general are found in low numbers. Their quality and quantity is age and health dependent, and traditional long-term culture technique hinders their self-renewal and multipotent features. A scale-up strategy to produce high quality cells is on demand.

It is necessary to understand the nutritional necessities and essential bioactive factors required in culture medium, the global system to provide the adequate cues at local level; and the environmental conditions, as oxygen and pH, which directly effect important pathways. These parameters are required to be controlled, for the cells to grow healthy and undifferentiated, and are priority for a successful culture strategy (Ng et al., 2014).

Growth and differentiation are mediated by a number of growth factors, transcription factors and extracellular matrix proteins. These act as molecular switches to activate or repress specific gene expression programmes (Shi and Gronthos, 2003, Niwa et al., 2000). Hence, these switches Influence the architecture, intercellular interactions, and functions of the cells.

Culture medium must support cell attachment and expansion in primary and passaged cultures as well as in 3D environments (Jung et al., 2012b). Micro and macro nutrients are the substrates for metabolism or biosynthesis. If regulatory metabolic pathways are compromised, homeostasis will be hindered. Hence, minerals, amino acids, carbohydrates, lipids and vitamins normal to the in-vivo cellular environment must be provided in the culture medium. Most micronutrients for cell stability are provided by adding foetal bovine serum (FBS) to basal medium. If these are not present in either FBS or basal medium, they have to be supplemented to guarantee production of healthy cells and genomic and phenotypic stability. Other important component are antibiotics to prevent microbial and fungal contamination of the culture.
Table 2: Current studies about stem cell differentiation towards bone tissue using various bioreactors

<table>
<thead>
<tr>
<th>Bioreactor type</th>
<th>Scaffold</th>
<th>Cells</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirred Flask</td>
<td>PLGA discs</td>
<td>murine MSC</td>
<td>Growth↑, Ca↑, ALPase↑, OC↑</td>
<td>(Sikavitsas et al., 2002)</td>
</tr>
<tr>
<td>Stirred Flask</td>
<td>PLGA cubes</td>
<td>human MSC</td>
<td>Growth↑, Ca↑, ALPase↑, OC/ALP/BSP↓, COL1A1/BMP2/OPN/RUNX2/OSX↑</td>
<td>(Stiehler et al., 2009)</td>
</tr>
<tr>
<td>Stirred Flask</td>
<td>PCL/PLPC microcarriers</td>
<td>murine pre osteoblasts</td>
<td>Growth↑, Ca↑, ALPase↑, OC↑, in vivo</td>
<td>(Jin et al., 2014)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>SiO₂ bioceramic</td>
<td>murine MSC/osteosarcoma</td>
<td>Ca↑, HA↑</td>
<td>(Qiu et al., 1999)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>PLAGA microcarriers</td>
<td>human osteosarcoma</td>
<td>Growth↑↑, Ca↑, ALPase↑, histochem+</td>
<td>(Botchwey et al., 2001)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>PLGA discs</td>
<td>murine MSC</td>
<td>Growth↓, Ca↑, ALPase↑, OC↑</td>
<td>(Sikavitsas et al., 2002)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>Alginate</td>
<td>murine ESC</td>
<td>Ca↑, HA↑, RUNX2/OSX↑</td>
<td>(Hwang et al., 2009)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>Demineralised human bone</td>
<td>murine osteoblasts</td>
<td>Growth↑, Ca↑↑, ALPase↓, ALP/OC↑↑, in vivo</td>
<td>(Song et al., 2008)</td>
</tr>
<tr>
<td>Rotating Wall Vessel</td>
<td>Alginate hydrogels/bioglass</td>
<td>murine MSC</td>
<td>Growth↑, ALPase=, RUNX2↑</td>
<td>(Bancroft et al., 2003)</td>
</tr>
<tr>
<td>Perfusion chamber</td>
<td>Flat surface</td>
<td>Human fetal osteoblasts</td>
<td>Ca↑↑</td>
<td>(Jacobs et al., 1998)</td>
</tr>
<tr>
<td>Perfusion container</td>
<td>β-TCP</td>
<td>murine osteoblasts</td>
<td>Growth↑, Ca↑↑, ALPase↓, histology+, ALP/OC↑↑, in vivo</td>
<td>(Wang et al., 2003)</td>
</tr>
<tr>
<td>Perfusion cassettes</td>
<td>collagen sponge discs</td>
<td>murine osteosarcoma</td>
<td>Growth↑, Ca↑↑, ALPase↑</td>
<td>(Hosseinkhani et al., 2006)</td>
</tr>
<tr>
<td>Perfusion bioreactor</td>
<td>starch fibre mesh</td>
<td>murine MSC</td>
<td>Histocem+, BMP2/TGFb1,FGF2/VEGF↑, PDGF-A=, in vivo</td>
<td>(Gomes et al., 2006)</td>
</tr>
<tr>
<td>Perfusion cassette</td>
<td>Collagen/PGA</td>
<td>murine MSC</td>
<td>Growth↑, Ca↑, ALPase↑, OC↑, histology+</td>
<td>(Janssen et al., 2006)</td>
</tr>
<tr>
<td>Perfusion bioreactor</td>
<td>HA/polyamide</td>
<td>murine MSC</td>
<td>Growth↑, Ca↑↑, ALPase↑, OC↑</td>
<td>(Qian et al., 2013)</td>
</tr>
<tr>
<td>Perfusion cylinder</td>
<td>starch/PCL</td>
<td>goat MSC</td>
<td>Growth↑, Ca↑, HA↑, histology↑, in vivo</td>
<td>(Gardel et al., 2014)</td>
</tr>
<tr>
<td>Perfusion bioreactor</td>
<td>β-TCP</td>
<td>sheep MSC</td>
<td>Histology↑, histomorphology↑</td>
<td>(Li et al., 2014)</td>
</tr>
<tr>
<td>Perfusion cartridge</td>
<td>P(LLA-co-CL)</td>
<td>human MSC</td>
<td>ALPL/COL1A1↑, RUNX2/SPP1↑↑, BGLAP↓, histology+</td>
<td>(Kleinhans et al., 2015)</td>
</tr>
<tr>
<td>Perfusion cartridge</td>
<td>Silk/gelatin/HA</td>
<td>MC3T3-E1/murine MSC</td>
<td>Growth↑, Ca↑, HA↑, ALPase↑</td>
<td>(Siniapabodin et al., 2016, Li et al., 2014)</td>
</tr>
</tbody>
</table>
In vivo, carbohydrates supply the cellular energy requirement. Through the glycolysis and the Citric acid cycle (also known as TCA cycle, Krebs cycle) the cells metabolise the carbohydrates and produces ATP to function. Hence, their uptake will be controlled by the energy requirement characteristic of every different cell type. In addition, they are used for the synthesis of certain amino acids, fat and nucleic acids and for intracellular carbohydrate intermediates. But these represents a small proportion of the total glucose consumption. In vitro, carbohydrates will be provided in basal medium as glucose. It is known that high glucose concentrations could be harmful for certain cell types (Jung et al., 2012b). Thus a low glucose concentration is normally used in MSC culture (5.5mM). Lactate, a by-product of glucose metabolism, can reduce growth and multipotency when its concentration reach levels higher than 16 mM, so its concentration has to be followed carefully.

Amino acids are the raw material for protein synthesis, they are of extreme importance for the majority of the cellular processes. All cells need 12 essential amino acids and they come as part of basal media, arginine, cysteine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tyrosine, tryptophan and valine. Particular amino acids as glutamine have to be added in specific concentrations as they play a specific role, either in growth or differentiation. Glutamine plays an important role as it provides the nitrogen of purine and pyrimidine of nucleic acids and is essential for the synthesis of the mono-, bi- and tri-phosphate acid glycosides.

Lipids that stimulates growth, for instance, cholesterol, linoleic acid, oleic acid and phospholipids are found in serum. Hence media with serum won’t require supplementation of lipids. In contrast, serum free and differentiation media will. Hormones of lipidic nature have roles in growth and differentiation and require to be added to the media. For instance, hydrocortisone is a hormone with growth promoting properties its action mainly happens when cells are in log growth phase but has negative osteo-regulatory characteristics (Rowe et al., 1977). Simvastatin is a known lipoprotein with osteogenic properties, acting through the bone morphogenetic proteins pathway (Pagkalos et al., 2010a, Chen et al., 2010).
Growth factors and cytokines are normally from proteic or lipidic (steroid hormones) nature, they are key for most of the regulatory processes to maintain cell function and their undifferentiated/differentiated state and have to be added to media. Serum will have a complex pool of them with importance in growth and attachment of cells.

Vitamins primarily act as coenzymes or prosthetic groups supporting enzymatic functions in metabolic processes. Essential vitamins required in cell culture comprise fat-soluble vitamins such as A, D, E, K, thiamine, riboflavin, niacin pyridoxine, pantothenic acid, folate, vitamin B12, biotin. Another essential, Ascorbic acid (vitamin C), has antioxidant properties, and works as a growth promoter. Vitamins C and D are reported to have osteoinductive properties, and play a key role in calcium homeostasis (Rowe et al., 1977, van Leeuwen et al., 2001).

Inorganic salts play a role in retaining the osmotic balance of the cells and in specific functions. Most mammalians have a plasma osmotic pressure of 260-320 mOsm/kg, humans in particular, have a plasma osmotic pressure of 290 mOsm/kg and cells grow healthy when maintain between 10% of this value. Sodium, potassium, calcium, magnesium, nitrogen, chloride, sulfate, bicarbonate and phosphate are included in basal media. Other functions associated with inorganic salts are cell attachment aid, signalling transduction intermedation, membrane potential regulation and intracellular charge. Inorganic trace elements as molybdenum, vanadium, iron, zinc and selenium, copper, manganese come as part of basal media. Although their function remains unclear, they are known to play a role as enzyme cofactors.

Serum provides most of the essential requirements for cell viability and clonogenicity that are not found in basal medium and is an extremely complex fluid. With an undefined nature, it is known to be constituted by a comprehensive range of biological factors, with a balanced action in growth promotion, inhibition and differentiation. Growth factors found in serum as PDGF, EGF, IGF-1, IGF-2 are known to be related with cell proliferation and differentiation. Adhesion factors as fibronectin support cell attachment and expansion. The main functions of serum comprise providing: essential nutrients, adherence and expansion factors, hormones, various growth factors and binding proteins.
A table with the approximate components and concentrations of serum published by Jung and colleagues can be seen in Figure 15.

The composition of serum is unknown and jointly with other culturing reagents comes from animal origin. They pose numerous safety issues in clinical therapy, including possible infections and severe immune reactions. Additionally, serum composition varies from batch to batch. Hence, constant attempts have been made to develop defined Serum-free and xeno-free culture media. For a successful serum/xeno-free media to be developed, the same essential requirements provided by serum must be attained. A well-defined culture media will provision the production of homogeneous culture enriched with a desired cell type, enhancing cell bioprocessing protocols, which is essential for increasing clinical efficiency of cell therapy devices. As serum is still ill-defined, most formulations developed on the knowledge gathered from serum, present limited performance, with less viability and slow rates being some of their problems. Until the defined media does not reach the culture conditions required to produce a quality cell product, serum will still be the golden standard in culture media.
<table>
<thead>
<tr>
<th>Constituent</th>
<th>Range of concentration</th>
<th>Constituent</th>
<th>Range of concentration^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins and polypeptides</td>
<td>40–80 mg/mL</td>
<td>Polymines:</td>
<td>0.1–1.3 µM</td>
</tr>
<tr>
<td>Albumin</td>
<td>20–50 mg/mL</td>
<td>Putrescine, Spermidine</td>
<td></td>
</tr>
<tr>
<td>Fetuin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10–20 mg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>1.0–10 µg/mL</td>
<td>Urea</td>
<td>170–300 µg/mL</td>
</tr>
<tr>
<td>Globulins</td>
<td>1.0–15 mg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease inhibitors:</td>
<td>0.5–2.5 mg/mL</td>
<td>Inorganics</td>
<td>0.14–0.16 M</td>
</tr>
<tr>
<td>α1-antitrypsin</td>
<td></td>
<td>Calcium</td>
<td>4.0–7.0 mM</td>
</tr>
<tr>
<td>α2-macroglobulin</td>
<td></td>
<td>Chlorides</td>
<td>100 µM</td>
</tr>
<tr>
<td>Transferrin</td>
<td>2.0–4.0 mg/mL</td>
<td>Iron</td>
<td>10–50 µM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Potassium</td>
<td>5.0–15 mM</td>
</tr>
<tr>
<td>Growth factors:</td>
<td></td>
<td>Phosphate</td>
<td>2.0–5.0 mM</td>
</tr>
<tr>
<td>EGF, PDGF, IGF1 and 2</td>
<td>1.0–100 ng/mL</td>
<td>Selenium</td>
<td>0.01 µM</td>
</tr>
<tr>
<td>FGF, IL-1, IL-6</td>
<td></td>
<td>Sodium</td>
<td>135–155 mM</td>
</tr>
<tr>
<td>Amino acids</td>
<td>0.01–1.0 µM</td>
<td>Zinc</td>
<td>0.1–1.3 µM</td>
</tr>
<tr>
<td>Lipids</td>
<td>2.0–10 mg/mL</td>
<td>Hormones</td>
<td>0.1–200 nM</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>10 µM</td>
<td>Hydrocortisone</td>
<td>10–200 nM</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>0.1–1.0 µM</td>
<td>Insulin</td>
<td>1.0–100 ng/mL</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>0.01–0.1 µM</td>
<td>Triiodothyronine</td>
<td>20 µM</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.7–3.0 mg/mL</td>
<td>Thyroxine</td>
<td>100 nM</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>1.0–2.0 mg/mL</td>
<td>Vitamins</td>
<td>0.01–10 µg/mL</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.6–1.2 mg/mL</td>
<td>Vitamin A</td>
<td>10–100 ng/mL</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.6–1.2 mg/mL</td>
<td>Folate</td>
<td>5.0–20 ng/mL</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0.5–2.0 mg/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>2.0–10 µg/mL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The range of concentrations is approximate and is intended to convey only the order of magnitude.

<sup>b</sup>In fetal serum only.
1.2.8 Remarks and conclusions of this section

Cells are the machinery, growth factors the cues, scaffold the local microarchitecture, while the bioprocessing the equivalent of the global interactions. All this factors require to be fully understood and orchestrated for a succeeding osteogenic bioprocessing and have to be standardised in order to be able to bring bone tissue engineering from the laboratory bench to the clinical use in regenerative medicine.
2 Aims and objectives
The use of hDPSCs in bone tissue engineering and regenerative medicine requires the development of an unambiguous and efficient protocol for selectively directing MSCs differentiation towards the osteogenic lineages in vitro. Additionally, clinical applications require an automated and scalable bioprocessing able to achieve the required production, minimising sample manipulation. Most of the traditional methods for MSCs culture involve laborious procedures and handling of samples, the expansion of healthy undifferentiated cells to the desired quantity and their further differentiation to produce homogenous osteogenic cells in the adequate three-dimensional environment is not easily achieved. Furthermore, two-dimensional and three-dimensional static culture systems are not suitable for large scale production and differentiation of MSCs and require the use of high concentrations of expensive growth factors, cytokines and biological cues to achieve differentiation.

The Aims of this project are to develop the effective methodology to produce bone-like tissue constructs for clinical use. through the lineage specific differentiation of hDPSCs, especially into osteoblasts, and the development of three dimensional cost-effective bone constructs within a bioreactor culture system for clinical applications. Specifically, this can be achieved through the characterisation of hDPSCs as MSC-like cells in a 2D environment as this represents the starting material in a three-dimensional culture. Subsequently, the focus would be on the development of an integrated, single step culture process for direct osteogenic differentiation of hDPSCs by encapsulation in alginate/ gelatin hydrogels and the use of perfusion of culture medium through a rotating wall vessel bioreactor, resulting in an efficient and reproducible culture system for bone tissue engineering applications.

The specific objectives are:

a. The characterisation of hDPSCs as MSCs-like stem cells in two-dimensional static culture, by following the minimal criteria defined by The International Society for Cellular Therapy (ISCT), with the examination of the undifferentiated and proliferative quality of the cells. And ultimately, developing of an in vitro protocol for the generation of a specific cell population with high osteogenic potential from hDPSCs.
b. Determination of the effect of perfusion in dynamic rotatory wall bioreactor culture in the expansion and osteogenic differentiation of hDPSCs in three-dimensional culture, using encapsulation in alginate/ gelatin hydrogels with the examination of the quality of the bone neo-tissue constructs and comparison with Horizontal Aspect Ratio Vessel (HARV) bioreactor and three-dimensional static culture. Ultimately, generating an *in vitro* protocol for the production of cell populations from osteoblastic lineage with high therapeutic potential.

c. To investigate the effect of Bone morphogenetic protein 2 at physiological level and simvastatin as enhancers of osteogenesis in perfusion-RWV bioreactor system and in alginate/ gelatin scaffolds with the examination of the quality of the bone neo-tissue constructs. Ultimately, developing an *in vitro* protocol for the generation of high quality, homogeneous, functional and mature cell population from osteoblastic lineage with high therapeutic potential.
3 Materials and methods
3.1 Overview of the experiments
This study addressed the in vitro bone mimicry capacity of hDPSCs from donors, encapsulated in 3D alginate beads and the effect of Rotating Wall Vessel bioreactor coupled with perfusion flow of culture media; following cell fate through the whole process. The study was structured to follow three different aspects of the cells bioprocessing;

a) The characterisation of hDPSCs as MSCs-like cells following the minimal criteria defined by The International Society for Cellular Therapy (ISCT).

b) Comparison between the in vitro osteogenic differentiation capability of hDPSCs encapsulated in alginate beads in different bioprocessing systems: 3D static culture, High Aspect Ratio Vessel bioreactor (HARV) and In-house designed Perfusion Rotating Vessel bioreactor.

c) Enhancement of the differentiation capabilities of Perfusion Rotating Vessel bioreactor by addition of Simvastatin and physiological levels of bone morphogenetic protein 2 (BMP-2).

3.1.1 Culture media

- Maintenance medium: Minimum essential medium Eagle - alpha modification (αMEM, Gibco, UK) with 10% Fetal Bovine Serum (FBS, Gibco), 2 mM L-glutamine (L-GLu, Gibco), 0.1 mM L-Ascorbic Acid 2-Phosphate (L-ASAP, Sigma), 100 U/ml Penicillin/Streptomycin antibiotics (Pen/Strep, Gibco) and 0.25 ug/ml fungizone antimycotic (amphotericin B, Gibco)*.

- Osteogenic differentiation medium: maintenance medium plus 5 mM β-glycerophosphate (β-GP, Sigma), 0.1 mM dexamethasone (Dex, Sigma), 0.5 mM ASAP (Sigma), 1.8 mM KH₂PO₄ (Sigma).

- Maintenance BMP-2 medium: maintenance medium, plus 300 pg/ml Bone BMP-2 (Peprotech).

- Maintenance Simvastatin medium: maintenance medium, plus 10 uM Simvastatin (Sigma).

- Osteogenic differentiation BMP-2 medium: osteogenic medium, plus 300 pg/ml BMP-2 (Peprotech).
• Osteogenic differentiation Simvastatin medium: osteogenic medium, plus 10 uM Simvastatin (Sigma).

• Chondrogenic differentiation medium: growth medium plus 50 uM ASAP (Sigma), 100 nM Dex (sigma), 5 ug/ml human insulin (Sigma I-9278), 1 ng/ml TGFb1, 400 uM proline, 1x non-essential amino acids (Sigma)

• Adipogenic differentiation medium: growth medium plus 0.5 mM 3-isobutyl-1-methylxantine (IBMX, Sigma), 1 uM Dex (Sigma), 10 ug/ml human insulin, 200 uM indomethacin (Sigma)

* fungizone anti mycotic was used only in 3D experiments. For 2D static culture, it was omitted.

3.1.2 hDPSCs extraction
Normal human impacted third molars were collected from adults (19–29 years of age) under approved guidelines set by The UK Stem Cell Bank and Department of Health in the Code of Practice for the use of human stem cell lines. Tooth surfaces were cleaned and cut around the cementum-enamel junction by using sterilized dental fissure burs to expose the pulp chamber. The pulp tissue was gently separated from the Crown and root and then digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 h at 37°C. Single-cell suspensions are obtained by passing the cells through a 70-μm strainer. The extraction and isolation of the hDPSCs was done in Leeds Dental Institute, in collaboration with the University of Leeds.

3.1.3 hDPSCs maintenance
hDPSCs were routinely cultured on tissue culture flasks (T-flasks) in a humidified incubator set at 37°C and 5% CO₂. Single-cell suspensions of naïve hDPSCs (< passage 5) were seeded into T-flasks with a density of 5x10³ cells/cm² and then incubated for 3-4 days in maintenance medium to get cell expansion. When achieving 80% confluence of the T-flask surface, cells were detached with TrypLE express (Thermo Fisher scientific) and passaged by re-plating at low density (5x10³ cells/cm²). Cells were fed every 3 days with fresh hDPSC maintenance medium.
3.1.4 hDPSCs characterisation
To characterise the undifferentiated stage of the cells prior to the induction of 3D bone differentiation, The International Society for Cellular Therapy (ISCT) defined, minimal criteria for cells to be considered multipotent stromal stem cells were followed. With this purpose, cells were analysed through passaging for multipotency markers in flow cytometry and they were driven to trilineage mesenchymal differentiation. As well, their clonogenic potential was measured by assessing fibroblast colony forming units (CFU-F).

3.1.5 hDPSCs osteogenic differentiation in 2D static culture
Single-cell suspensions of naïve hDPSCs (< passage 5) were seeded into T-flasks in a humidified incubator set at 37°C and 5% CO₂ with a density of 5x10³ cells/cm², maintenance medium was used and then incubated for 3-4 days until achieving 80% confluence of the T-flask surface. After, osteogenic differentiation was triggered using osteogenic medium for the next 21 days. During this period, cells were fed every 3 days with fresh hDPSC osteogenic medium.

3.1.6 hDPSCs adipogenic differentiation in 2D static culture
Single-cell suspensions of naïve hDPSCs (< passage 5) were seeded into T-flasks in a humidified incubator set at 37°C and 5% CO₂ with a density of 5x10³ cells/cm², maintenance medium was used and then incubated for 3-4 days until achieving 80% confluence of the T-flask surface. After, adipogenic differentiation was triggered using adipogenic medium for the next 21 days. During this period, cells were fed every 3 days with fresh hDPSC adipogenic medium.

3.1.7 hDPSCs chondrogenic differentiation in 2D static culture
Single-cell suspensions of naïve hDPSCs (< passage 5) were seeded into T-flasks in a humidified incubator set at 37°C and 5% CO₂ with a density of 5x10³ cells/cm², maintenance medium was used and then incubated for 3-4 days until achieving 80% confluence of the T-flask surface. After, chondrogenic differentiation was triggered using chondrogenic medium for the next 21 days. During this period, cells were fed every 3 days with fresh hDPSC chondrogenic medium.
3.1.8 Alginate encapsulation of hDPSCs

80% confluent undifferentiated hDPSCs were detached from T-flasks with TrypLE express (Thermo Fisher scientific), dissociated into single cell suspension, washed two times with phosphate buffered saline (PBS) and centrifuged to get a cell pellet. The cells were counted and resuspended at a density of $2.5 \times 10^6$ cells/ml (20,000 cells/bead) in a sterile-filtered solution composed of 1.1% (w/v) alginic acid (Sigma) and 0.1% (v/v) gelatin (Sigma) in PBS (pH 7.4) as described in our previous study (Randle et al., 2007b). The cell suspension was driven through a peristaltic pump (Model P-1, Amesham Biosciences, U.K.) and dropped from at least 30 mm height into gently stirred cross-linking solution (100 mM calcium chloride ($\text{CaCl}_2$) and 10 mM N-$(\text{s-hydroxyethyl})$ piperazine-N-$(2$-ethane sulfonic acid) at pH 7.4). The flow rate of the pump was adjusted to give single droplets using a 25-gauge needle (Becton Dickinson, Oxfordshire, UK). Upon contact with the solution, gelling of the alginate droplets started and after 5 minutes in the solution, bead-shaped gels with an approximate diameter of 1.3 mm were obtained. The droplets remained in the solution being stirred for 5 more minutes at room temperature to allow the beads to set. Finally, the beads were washed three times in PBS and placed into normal hDPSC growth or differentiation culture media (Randle et al., 2007a) as required for the experiment. To avoid contamination, tubes were autoclaved and washed three times with filtered-sterile PBS before the encapsulation process.

3.1.9 Three-dimensional culture of hDPSCs for osteogenic differentiation

Encapsulated cells were transferred to a 175 T-flasks, a 55 ml Perfusion Rotating Wall bioreactor (BSEL in-house design), or a 55 ml HARV bioreactor (Synthecon RWV-HARV, Cellon, Bereldange, Luxembourg). Each vessel was loaded with 250 alginate beads. The rotation rate of the vessels was optimized in previous works in our lab, according to the cell growth kinetics to avoid increasing shear stress in the vessel. Beads were collected at each sample point to further be stored, fixed or depolymerized for biological analysis of the resulting cells and mineralised scaffolds.

For the first 7 days of experiments, beads were seeded in 175 T-flasks and expanded undifferentiated with maintenance medium for the next 7 days. After, the beads were transferred to either a 55 ml Perfusion Rotating Wall bioreactor (BSEL in-house design),
a 55 ml HARV bioreactor (Synthecon RWV-HARV, Cellon, Bereldange, Luxembourg), or left in a 175 T-flask. To perform osteogenic differentiation, the seeded vessels were loaded with any of the differentiation media for another 21 days of culture, according to the culturing plan. The time plans for 3D bone differentiation are described in Table 3 and Table 4. Growing cells in beads were photographed using a Leica DM IL microscope (Leica, Wetzlar, Germany) and CellSens software (Olympus, Munich).

Table 3: Experimental plan, comparison of different bioprocessing for bone tissue engineering.

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium</th>
<th>Maintenance</th>
<th>Osteogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control - flask</td>
<td></td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>HARV</td>
<td></td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>Perfusion</td>
<td></td>
<td>7</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 4: Experimental plan, enhancing 3D bone ontogenesis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Medium</th>
<th>Maintenance BMP2</th>
<th>Maintenance Simvastatin</th>
<th>Osteogenic BMP2</th>
<th>Osteogenic Simvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>28</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BMP2 only</td>
<td></td>
<td>7</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Symvastatin only</td>
<td></td>
<td>7</td>
<td>0</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>Osteo BMP2 Osteo Symvastatin</td>
<td></td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>21</td>
</tr>
</tbody>
</table>

3.1.10 Fabrication of a perfusion RWV bioreactor vessel

A novel in-house designed computer-controlled RWV bioreactor was used in the 3D experiments. Which is able to deliver continuous oxygenation, supply of nutrients, and removal of metabolic wastes. The gas exchange was achieved via using a gas-permeable membrane and the nutrients supply/waste removal, via perfusion of medium with a peristaltic pump. The design of the bioreactor was performed by Jae Min Cha, a previous PhD student in BSEL. The design and fabrication of the bioreactor system are described in chapter V. The materials specification for the gas-permeable membrane and all parts of bioreactor can be found in Table 5 and Table 6.
### Table 5: Specifications of gas permeable membranes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>dimensions</td>
<td>16x16 cm²</td>
</tr>
<tr>
<td>membrane thickness</td>
<td>0.004 inch polydimethylsiloxane</td>
</tr>
<tr>
<td></td>
<td>0.001 inch polytetrafluoroethylene</td>
</tr>
<tr>
<td></td>
<td>0.005 inch total thickness</td>
</tr>
<tr>
<td>surface area of gas permeable</td>
<td>6030 cm²</td>
</tr>
<tr>
<td>membrane at atm pressure</td>
<td>1.1e-2 cm/s</td>
</tr>
<tr>
<td>supplier information</td>
<td>Specialty silicone products inc.</td>
</tr>
<tr>
<td></td>
<td>3 McCrea hill road.</td>
</tr>
<tr>
<td></td>
<td>Ballston spa, NY 12020, USA</td>
</tr>
</tbody>
</table>

### Table 6: Information of materials

<table>
<thead>
<tr>
<th>Parts</th>
<th>Materials</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base plate</td>
<td>White Delrin/Acetyl Plastic</td>
</tr>
<tr>
<td>Support walls</td>
<td>White Delrin/Acetyl Plastic</td>
</tr>
<tr>
<td>Oxygenator</td>
<td>White Delrin/Acetyl Plastic</td>
</tr>
<tr>
<td>Oxygenator tubing</td>
<td>Platinum Cured Silicone Rubber Tube (3mm ID x 4mm OD)</td>
</tr>
<tr>
<td>Tubing</td>
<td>Silicon tubing, Tygon (1.6mm ID x 4.8mm OD)</td>
</tr>
<tr>
<td>Vessel</td>
<td>Virgin PTFE (Teflon)</td>
</tr>
<tr>
<td>Steel rods</td>
<td>1/8&quot; OD x 0.028&quot; Wall. 316 Stainless Steel Tubing</td>
</tr>
<tr>
<td>supplier information</td>
<td>Metal &amp; Plastic Ltd (Delrin, Teflon)</td>
</tr>
<tr>
<td></td>
<td>- PO Box 432 Watford, Hertfordshire WD1 8YP, UK</td>
</tr>
<tr>
<td></td>
<td><strong>London Fluid System Technologies Swagelok London</strong> (St steel tubing)</td>
</tr>
<tr>
<td></td>
<td>- Kingley Park, Station Road, Kings Langley, Herts WD4 8GW UK</td>
</tr>
<tr>
<td></td>
<td><strong>Silex Ltd (silicon tubing)</strong></td>
</tr>
<tr>
<td></td>
<td>- Unit 5 Broxhead Trading Estate, Lindford, Bordon, Hampshire GU35 0NY, United Kingdom</td>
</tr>
</tbody>
</table>
3.1.11 Alginate decapsulation of hDPSCs
The beads were dissolved at each time point for sampling using a depolymerisation buffer consisting of 50 mM tri-sodium citrate dihydrate, 77 mM sodium chloride and 10 mM HEPES (Magyar et al., 2001).

3.1.12 Biological evaluation techniques
The general approach was to show the results in a genetic, then in a protein and finally in a structural level. The viability of cells in the hydrogel beads was measured by using LIVE/DEAD assay, Celltiter-Glo 3D and Cell counting kit 8. The gene and protein expression were measured by means of quantitative real time polymerase chain reaction (qPCR) and flow cytometry or immunostaining. The structural analysis was done by histology, scanning electron microscopy (SEM) with X-ray microanalysis (SEM/EDS) and attenuated total reflectance-Fourier transform infra-red spectroscopy (ATR-FTIR). The human antibodies to be used as markers of the cell state and to characterise them by flow cytometry or immunostaining were: anti-CD44, anti-CD73, anti-CD90, anti-CD105, anti-CD14, anti-CD11b, anti-CD34, anti-CD45, anti-CD79α, anti-CD19 and anti-HLA class II, Runx-related transcription factor 2 (Runx2 – early osteoblasts marker), anti-Osteopontin (OPN/SPP1 – mature osteoblasts marker), anti-Osteocalcin (BGLAP – mature osteoblasts marker), anti-Podoplanin (PDPN/E11/gp38 – early osteoclast), anti-Sclerostin (SOST – mature osteocyte) (Yu et al., 2010, Sabokbar et al., 1994).

3.1.13 Live/Dead assay
Intracellular esterase activity and an intact plasma membrane are distinguishing characteristics of live cells. This assay discriminates the live and dead cells within the alginate hydrogels, by staining with cell membrane-permeant dye, 3',6'-Di(O-acetyl-2',7'-bis[N,N-bis(carboxymethyl) aminomethyl] fluorescein tetraacetoxymethyl ester (calcein AM, Invitrogen)), to produce cytoplasmic green fluorescence to indicate esterase activity and with membrane-impermeant ethidium homodimer-1 (EthD-1) to label nucleic acids of membrane-compromised cells with red fluorescence to indicate loss of plasma membrane integrity. Beads are collected and washed with PBS. A 4mM EthD-1/ 2mM calcein AM solution diluted in PBS was added to beads and incubated at 37°C with gentle shaking for 30 minutes in the dark. A negative control is produced by using 100% (v/v) methanol for 5 minutes instead of PBS. After incubation, the solution
is aspirated and a small amount of PBS is added to the beads to prevent dehydration. Samples are then photographed for analysis.

3.1.14 Celltiter-Glo 3D cell viability assay
A quantitative method to determine the number of viable cells in 3D cell culture, by determining the amount of ATP present, which is a marker for metabolically active cells. 3 hydrogel beads were harvested (n=3) and deposited in opaque-walled 96 well plate with 100 uL of media. 100 uL of Celltiter-Glo 3D reagent were added to each well, mixing vigorously for 5 minutes to dissolve the scaffold and induce cell lysis. From this step onwards, everything was done in darkness. The plate was allowed to incubate at room temperature for an additional 25 minutes. The luminiscence was measured with a GLoMax 96 microplate luminometer (Promega).

3.1.15 Cell counting kit 8 viability assay
Cell counting kit 8 (CCK-8; Sigma), is a tetrazolium salt based kit (as MTT or MTS). It uses 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8), a reagent reduced by cellular dehydrogenases producing an orange formazan dye. The amount of formazan produced by this reaction, is proportional to the living cell number. 3 hydrogels beads were harvested (n=3) and deposited in 96 well plate with 100 uL of media. The samples were pre-incubated in the incubator at 37°C and with 5% CO₂. 10 uL of CCK-8 reagent were added to each well and then samples were incubated in the incubator for 4 hours. After, the supernatant absorbance at 450 nm was measured with GLoMax 96 microplate luminometer (Promega).

3.1.16 Real Time Polymerase Chain Reaction (qPCR)
Total RNAs from the samples is extracted by using RNeasy kit according to the manufacturer’s instructions. If necessary dissolve the beads with depolymerisation buffer for 20 minutes, RNA is reversed transcribed into complementary DNA (cDNA) using the Reverse Transcription System. Briefly, cocktail for cDNA synthesis consisting of 1 µg of RNA, 4 µl of MgCl₂ (25 mM), 2 µl of dNTPmix (10 mM), 2 µM of RT 10X Buffer, 0.5 µl recombinant RNasin ribonuclease inhibitor, 0.6 µl AMV reverse transcriptase (15U) and 1 µl random primers (0.5 mg) in a total volume of 10,1 µl per reaction. The RNA samples are heated at 70°C for 10 minutes and then immediately cooled on ice for
5 minutes. After the cocktail is added to RNA samples, they are incubated at 25°C for 10 min, and then reverse transcription is performed by setting the program for 92°C burst for 20-40 cycles depending on the sample. 50 μl cDNA samples containing the mastermix, 5 μl 10X PCR Buffer (-Mg), 1 μl dNTP mixture (10 mM), 1.5μl MgCl₂ (50mM), 38.1 μl DEPC-treated water. Add 1 μl of each primer (sense and anti-sense), 0.4 μl Platinum Taq DNA Polymerase (5 U/ μl) and 2 μl of cDNA sample. Run the real time PCR machine. The list of genes and specific PCR conditions is indicated in Table 7.

Table 7: Primers for osteogenic differentiation characterisation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Size</th>
<th>AT(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GGAGCGGAGATCCCTCAAAAT</td>
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<td>62</td>
</tr>
<tr>
<td></td>
<td>GGCTGTTGTCATCTCTCATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Runx2</td>
<td>TGGTTACTGTCAATGGCGGGTA</td>
<td>101</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>TTCAGATCGTTGAACCTTGCTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Col1a1</td>
<td>GAGGGCCAAGACGAAGACATC</td>
<td>140</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>CAGATCAGTCATCGCACCAAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALPL</td>
<td>ACCACCAGAGATGAACCCA</td>
<td>79</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>CGTTGTCTGAGTACCAGCCC</td>
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<td></td>
</tr>
<tr>
<td>SOST</td>
<td>ACACAGCCTCGTGTAGTG</td>
<td>123</td>
<td>61</td>
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<tr>
<td></td>
<td>GGTTCATGCTTGTGTTCTGCC</td>
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<td></td>
</tr>
<tr>
<td>BGLAP</td>
<td>CACTCCTGCCCCTATTGGC</td>
<td>112</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>CCCTCCTGCTTGGACACAAAG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.1.17 Flow cytometry analysis

Cells were trypsinised, counted and washed. Pellets with 100,000 cells were formed by centrifugation. The cell pellets were fixed for 40 minutes at room temperature in 4% (w/v) paraformaldehyde (PFA: BDH Laboratory supplies), washed twice with cell staining buffer, consisting of 1% bovine serum albumin (BSA, Sigma) and 0.02% sodium azide in PBS. and placed into a tube. The samples were then permeabilised by incubating for 45 minutes in 0.1% triton x-100 (Sigma), 1% BSA in PBS, washed with staining buffer and then blocked by incubating for 10 minutes in 10% serum in staining buffer. A good blocking solution consists of serum of the same animal species than the host of the
secondary antibody. The blocking solution was removed and samples were washed 2 times in staining buffer. Cells were incubated overnight in a humid chamber with the primary antibodies diluted in staining buffer, for the control sample, only the staining buffer was added. The solution was removed and washed twice with staining buffer. Samples then were incubated for 1 hour in the dark and at room temperature, with the corresponding secondary antibodies diluted in PBS (including the control). From this step onwards, everything was done in darkness. The secondary antibody solution was removed and samples were washed in rinse buffer 1X. The pellet was resuspended in 300 ml of staining buffer and the samples were ready to be measured in flow cytometer (BD Fortessa flow cytometer; BD biosciences/ Guava easyCyte 8HT benchtop flow cytometer; Merck Millipore).

3.1.18 Immunocytochemical stainning
Hydrogels were collected and fixed for 90 minutes at room temperature in 4% (w/v) paraformaldehyde (PFA: BDH Laboratory supplies), washed twice with rinse buffer 1X, consisting of 20 mM Tris-HCl buffer (Sigma), 0,15% NaCl and 0,05% tween-20 (Sigma) in PBS and placed into tubes. The samples were then permeabilised-blocked by incubating for 45 in permeabilisation-blocking solution. A good blocking solution consists of serum of the same animal species than the host of the secondary antibody, in this study, rabbit and goat serum were used. The permeabilisation-blocking solution was removed and samples were washed 2 times in rinse buffer 1X. samples were incubated overnight in a humid chamber with the primary antibodies diluted in 0.1% triton, 1% BSA in PBS, for the control sample, only the diluent was added. The solution was removed and washed twice with rinse buffer 1X. Samples then were incubated for 1 hour in the dark and at room temperature, with the corresponding secondary antibodies diluted in PBS (including the control). From this step onwards, everything was done in darkness. The secondary antibody solution was removed and samples were washed in rinse buffer 1X. The rinse buffer was removed and the samples were ready to be observed under the microscope.

3.1.19 Alkaline Phosphatase (ALPase) activity
Although the mechanism is yet not fully understood, ALPase has a fundamental roll in the deposition of the mineral salts and calcification of the bone matrix, thus making it a
valued marker for the bone mineralisation. Cellular ALPase was assessed colorimetrically by measuring ρ-nitrophenol phosphate (ρNPP) content. Three beads (n=3) were collected from each group and dissolved in depolimerisation buffer for 20 minutes, centrifuged at 400g for 10 minutes and the supernatant was removed and washed with PBS. 200 µL of ALPase buffer and 200 µL of ρNPP were added to each sample, pipetted vigorously and then incubated at 37°C for 30 minutes in the dark. To stop the reaction 400 µL of 0.5N sodium hydroxide were added to the samples. Finally, 100 µL of the solution were analyse at 410 nm wavelength with ELISA reader (MRX II plate reader; Dynex technologies, West Sussex, UK).

3.1.20 Media analysis
The metabolic activity of the cells is directly related to the metabolic stress they are living and will impact the cells proliferation and differentiation. The concentration profile of nutrients (glucose, glutamine, lactate, ammonia, glutamate) and pH was measured using Bioprofile 400 Analyzer (Nova Biomedical, Flintshire, UK) taking 1.0 ml samples of culture media supernatant to be harvested at several time points during the culture. Fresh media was used as control.

3.1.21 Paraffin embedding and sectioning for histological analysis
The paraffin wax embedding of the hydrogels for further sectioning and histological analysis was done following the same procedure for all experiments as described: beads were fixed in the appropriate fixative depending on the sample point (see Table 8), dehydrated through a series of graded ethanol baths (70% for 3 minutes; 95% for 3 minutes, 2 times; 100% for 3 minutes, 2 times; 1:1 Paraclear:Ethanol for 10 minutes; Paraclear (Polysciences, Inc) for 10 minutes, 2 times) to displace water, then infiltrated with paraplast plus (Sigma) for 1 hour to finally be embedded with new paraplast in an histology cassette for up to 1 hour to form a block. It is important not to keep the beads in hot paraffin too long or samples could be damaged. Processed tissues can be stored in the cassettes at room temperature indefinitely, or serially sectioned with a thickness of 4 um with a microtome into glass slides.
Table 8: Different fixatives for histology

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Time of Experiment</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol 70%</td>
<td>First 2 weeks of experiment (first week, day 0 and 7 in growth media, and first week, day 0 and 7 of differentiation)</td>
<td>* Without differentiation, beads get dissolved in common fixatives.</td>
</tr>
<tr>
<td>Neutral buffered formalin (Formaldehyde 4% in PBS)</td>
<td>Last 2 weeks of experiment (day 14 and 21 of differentiation)</td>
<td>**most commonly used fixative. **</td>
</tr>
</tbody>
</table>

**3.1.22 Alizarin red S (ARS) Staining for calcium deposition**
Paraffin-embedded slides were deparaffinised and rehydrated, by placing them in paraclear and decreasing concentrations of ethanol. After, the slides were immersed in a 40 mM alizarin red S (Sigma-Aldrich) solution (pH 4.2) for up to 5 minutes at room temperature, checking for the red colour granules forming in the slides under the microscope. When the colour was formed, the solution was removed and the slides were washed with running water. The slides then were dried in a series of increasing concentrations of ethanol, to finally be mounted with 10% (V/V) glycerol (Sigma) and observed in the microscope.

**3.1.23 Von Kossa staining for calcium deposition**
Paraffin-embedded slides were deparaffinised and rehydrated, by placing them in paraclear and decreasing concentrations of ethanol. Slides were immersed and incubated for 20 minutes in 1% silver nitrate solution under ultraviolet light, then rinsed in several changes of distilled water. The unreacted silver was removed immersing the slides in 5% sodium thiosulfate for 5 minutes. Nuclear fast red was used as counterstain. The slides were immersed in fast red for 5 minutes, rinsed with distilled water and then dried in a series of increasing concentrations of ethanol, to finally be mounted with 10% (V/V) glycerol and observed in the microscope.

**3.1.24 Haematoxylin & Eosin staining**
Paraffin-embedded slides were deparaffinised and rehydrated, by placing them in paraclear and decreasing concentrations of ethanol. To stain with Mayer’s haematoxylin (Sigma), the slides were immersed in haematoxylin solution for 3 minutes, washed in tap water for 5 minutes, dipped 12 times in acid ethanol (1 ml of concentrated HCl in
400 ml of 70% EtOH) to destain, rinsed 2 times for 1 minute in tap water and one more time in deionised water for 2 minutes. If desired, slides can be left in water over night. To stain with Eosin Y, the remaining remaining water was blotted and the slides were immersed in eosin y solution (Sigma) for 30 seconds. The slides then were dried in a series of increasing concentrations of ethanol, to finally be mounted with 10% (V/V) glycerol and observed in the microscope.

3.1.25 Scanning electron microscopy with X-ray microanalysis
Hydrogels were rinsed in PBS to remove any medium, fixed by submerging in warm 3% glutaraldehyde solution (Sigma) in Sorenson’s buffer (80.4 ml of Na₂HPO₄, 19.6 ml of KH₂PO₄ and 100 ml of dH₂O) for 3 hours, and washed two times with Sorenson’s buffer for 5 minutes. For post-fixation, beads were submerged in osmium tetroxide (Sigma), under the hood and in a cold bath for 15 minutes. Beads were rinsed carefully in Sorenson’s buffer, to avoid structural damage of the samples. Samples were dried in a series of increasing concentrations of ethanol and mounted on stubs. Mounted samples were coated with gold in a sputter coater () and then viewed. Digital image capture was performed in Jeol JSM5610LV SEM and x-ray microanalysis was performed in Jeol JSM6400 SEM fitted with Oxford Instruments INCA energy dispersive analytical system (EDS) for elemental x-ray.

3.1.26 Attenuated Total Reflectance-Fourier Transform Infra-red spectroscopy
Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectral analysis was conducted using an ATR-FTIR spectrometer (Varian 7000 FT-IR, Varian Inc. CA, USA) with Isys software (Spectral Dimension, Malvern, Aus). Sample beads were placed onto the measuring surface of the ATR crystal (oil analyser, Specac Ltd., Kent, UK). Excess water was removed and spectra was acquired. Three beads were measured per treatment, and the relative amount of hydroxyapatite in each of the bead was compared by calculating the ratio between wavelength values at 1415 and 1035 [cm⁻¹], both characteristic for the organic (mostly collagen I) and inorganic composition of bone (mostly hydroxyapatite) respectively.

Attenuated total reflection Fourier transform infrared (ATR-FTIR) imaging was performed using a continuous scan FTIR spectrometer (Varian 7000 FT-IR, Varian Inc. CA, USA) jointly to a large sample compartment extension (Varian LS) and a 64 x 64 focal
plane array (FPA) detector. Sample beads were placed onto the measuring surface of the ATR crystal (oil analyser, Specac Ltd., Kent, UK) with the surface in contact with the crystal. Excessive water was carefully removed and the sample was sheltered to prevent further evaporation, thus ensuring that the bead remains hydrated during measurements. Images were acquired with 8 cm\(^{-1}\) spectral resolution with 32 co-adding scans. A sample measurement would normally require less than 1 minute to be processed. Three beads (n=2) were measured from each sample. Spectral data was analysed using the Isys software (Spectral Dimension, Malvern, Aus). Factor analysis was applied to the spectral region of 1110-990 [cm\(^{-1}\)]. Results were analysed simultaneously by concatenating all images together before the factor analysis. Allowing a direct comparison between images. Image showing the distribution of hydroxyapatite developed in the hydrogels was extracted using factor analysis. Further, a binary image was generated with a threshold of 10% to outline the pixels that were considered to detect hydroxyapatite.

3.1.27 Statistical analysis
Samples for quantitative analyses were measured in replicates (two or three). Error bars on all graphs represent the standard deviation of their average values. Comparable values from each group were subjected to statistical analysed with student t-test or analysis of variance (ANOVA) with a significance of p<0.05 or p<0.001 (* or ** respectively).
4 Characterisation of human dental pulp stem cells
4.1 Introduction

Adult MSCs have been found in most of the tissues of the human body, such as: dental pulp, bone marrow, peripheral blood, adipose tissue. In addition, MSCs have been found in various neonatal tissues, such as: placenta, amnion, umbilical cord and cord blood. These differently sourced MSCs, have been seen to carry differences in their genotype and phenotype. Several important surface SC markers in addition to proliferation rate, life span and differentiation capacity, present differences ranging from subtle to considerable (Hass et al., 2011). Moreover, diverse methodologies of extraction and expansion can further increase these differences. The scale-up of production of stem cells requires Safeguarding the resulting cells are sufficiently akin and differentiable to be consistently used in cell therapy procedures. Hence characterising MSCs represent the first mile stone of any stem cell strategy development.

hDPSCs differ from mesenchyme as they have an ectodermal origin and arise from the neural crest. Nevertheless, these cells share several characteristics with MSCs, clonogenic, rapidly proliferating and with the potential to differentiate into several tissues. To define them as ectomesenchyme with cell therapy potential, it’s required to follow exactly the same criteria used to define regular MSCs and in the process assess their MSCs equivalence.

4.1.1 Minimal criteria for MSCs

Based on currently available data, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) issued a position statement in 2006, proposing a set of standards defining the minimal criteria for cells to be considered MSCs for laboratory-based research and for pre-clinical studies (Dominici et al., 2006). They propose three criteria: 1) cells must be plastic adherent when grown under standard in vitro culture conditions, 2) 95% or more of the expanded cells, must express the surface markers CD73 (ecto-5’nucleotidase; cell-cell interactions, migration and modulation), CD90 (Thy-1 antigen; cell-cell interactions) and CD105 (Endoglin; TGF-β receptor III) when measured by flow cytometry, Other cell surface marker characteristic of MSCs is CD44, it’s thought to help cells to maintain stemness and the ISCT recommends reporting its expression as well; hematopoietic cells are the most common contaminant in MSCs samples, cells must be negative (2% or less positive) for the markers: CD14/
CD11b (monocytes and macrophages), CD34 (primitive hematopoietic and endothelial), CD45 (leukocyte), CD79α/CD19 (B cells) and HLA class II (leukocyte). 3) the most distinctive property of MSCs is their tri-lineage differentiability under standard in vitro culture conditions. Differentiation to osteoblasts, adipocytes and chondrocytes must be demonstrated and it can be shown by the following in vitro stainings Alizarin red S, Oil red O and Safranin O respectively.

4.1.2 Clonogenicity of MSCs and lost of multipotency
MSCs are highly proliferative cells able to form colonies of functional clones under normal culture conditions. These colonies when grown in incubators can be driven to differentiate into specific cell types, or spontaneously differentiate into osteoblasts, adipocytes and chondrocytes. However, long term culturing has been demonstrated to hinder cell proliferation and/or their differentiation capacity, thus, primitive MSC-like properties may be lost through successive passaging.

4.1.3 Tri-lineage differentiation of hDPSCs
Directed differentiation is a critical aspect for MSCs, strategies and requirements for tri-lineage differentiation of hDPSCs have to be studied before designing bioprocessing. The differentiation in vitro of MSCs will depend largely on the culture conditions, the specific inducers used, and the nutrients for biosynthesis.

4.1.3.1 Osteogenic differentiation of hDPSCs
Undifferentiated hDPSCs can be induced to Osteogenic differentiation by treatment with BMPs, a family of growth factors which act as direct cues for differentiation by driving a chain of events that triggers the expression of runx2 and causing the initiation and propagation of genes in charge of the development and maturation of osteoblasts (e.g. collagen I, osteopontin, alkaline phosphatase and osteocalcin). Dexamethasone (Dex) and simvastatin are two drugs that act as indirect differentiation cues, by interacting with the BMP pathway, they down-regulate the effect of tumor necrosis factor (TNF-α), a inflammatory mediator with inhibitory effect in osteogenesis. Dex has been reported to protect cells from apoptosis in confluent culture. β-glycerol phosphate (β-GP) is required as a local source of inorganic phosphate ions for the mineralising tissue (forming part of hydroxyapatite) and can cause differentiation alone by acting as an intracellular signal, or enhance the effect of other cues. Ascorbic acid (ASAP) which
stimulates ECM secretion (collagen type I) is a vital cofactor for enzymes that hydroxylate proline and lysine in pro-collagen, in its absence, collagen is not properly formed (Pittenger et al., 1999, Langenbach and Handschel, 2013). The most common medium for osteogenic differentiation was first reported in 1985 by Tenenbaum and Heersche, it uses Dex, β-GP and ASAP in serum medium and it has become the golden standard for osteogenic differentiation (Tenenbaum and Heersche, 1985).

For characterization purposes, osteogenic differentiation can be confirmed histologically by the appearance of calcium nodules and by the expression of ALP.

4.1.3.2 Chondrogenic differentiation of hDPSCs
Undifferentiated hDPSCs can be induced to Chondrogenic differentiation by treatment with TGF-β, a family of growth factors which act as direct cues for differentiation by driving a chain of events that triggers the expression of SOX9 and causing the initiation and propagation of genes in charge of the development and maturation of chondrocytes (e.g. Col2, Ihh, Runx2, Colx, ALP, VEGF). TGF-β1, TGF-β3, Dex, Insulin, insulin-like growth factor 1 (IGF-I), or BMP4 which acts as chondrogenic inducer proceeding through TGF-β signaling are typically used in chondrogenic differentiation mediums. Although the mechanism in which insulin acts is still unidentified, but it’s known to be essential. If not included, the differentiation is not possible (Mueller et al., 2013).

For characterization purposes, chondrogenic differentiation can be confirmed histologically by the appearance of proteoglycan-rich ECM (Pittenger et al., 1999).

4.1.3.3 Adipogenic differentiation of hDPSCs
Undifferentiated hDPSCs can be induced to Adipogenic differentiation by treatment with transcription factors cyclic adenosine monophosphate messenger (cAMP) which triggers cAMP response element-binding protein (CREB), these drive the expression of CCAAT/enhancer-binding proteins (C/EBPs) and of transcription factor peroxisome proliferator activated receptor gamma (pPAR-δ) which regulates adipose tissue development and maturation (Rosen and MacDougald, 2006). The most common media for Adipogenic differentiation uses 1-methyl-3-isobutylxanthine (IBMX), Dex, insulin, and indomethacin, this cocktail enhances intracellular cAMP and triggers adipogenic gene expression. Insulin through the Wnt pathway induces β-catenin in due process it
promotes proliferation and differentiation of preadipocytes. Dex alone is known to be important for the tri-lineage differentiation, and when combined with IBMX they regulate pPAR-δ and C/EBPs and promote adipogenesis.

For characterization purposes, adipogenic differentiation can be confirmed histologically by the appearance of lipid-rich vacuoles within the cells (Pittenger et al., 1999).

4.2 Objectives
An efficient strategy for scaling up the production and differentiation of hDPSCs for cell therapy, requires understanding the behaviour of cells under normal MSC culture conditions. This chapter focuses on the characterisation of the hDPSCs as MSC-like cells by studying the undifferentiated expansion, the passaging and the further tri-lineage differentiation, as well as the nutritional and environmental requirements of hDPSCs. Characterising hDPSCs by the ISCT defined criteria, represents the foundations for designing a bioprocess for bone tissue engineering.

4.3 Experimental Process
Isolated donor samples of hDPSCs were cultured and characterised by their ability to proliferate in culture with an attached well-spread morphology, by the presence of a consistent set of marker proteins on their surface, and by their extensive differentiation to multiple mesenchymal lineages under controlled in vitro conditions.

Prior to the tri-lineage differentiation, hDPSCs were passaged 12 times for morphologic, kinetic and flow cytometry characterisation, to assess which passage would allow to expand sufficient cells for the further differentiation, meanwhile maintaining the established minimal criteria.

Clonogenicity, metabolic activity and tri-lineage differentiation were assessed for the selected passage and were measured by CFU-F, bioprofiling, histological analysis and in particular for bone differentiation, runx2 expression and ALP activity were measured.
4.4 Results

4.4.1 hDPSCs expansion and maintenance

The expansion and maintenance of undifferentiated hDPSCs, while retaining their multipotency, is an important step in designing a bone tissue bioprocessing, since it provides the starting material for the further differentiation. Establishing the appropriate passage that will retain the multipotent characteristics as well as the proliferation rate of the parental cells, meanwhile allowing to suffice the amount of cells required, was the first step in this characterisation.

To establish the optimal condition for the hDPSCs expansion, Passage 4 cells from 2 donors (n=6) were seeded with different densities (Low=2000 cells/cm\(^2\), Medium=5000 cells/cm\(^2\), High=10000 cells/cm\(^2\) and then allowed to expand for 5 days. Every day cells were counted to assess their growth. As shown in Figure 16, the medium initial density of approximate 5000 cells/cm\(^2\) showed faster growth achieving the same cell number than the achieved by the high cell density culture. The doubling time for low density was of 1.51 days, for medium density of 1.475 days and for the high density of 2.21 days. Accordingly, the medium cell seeding density was used in all further studies.

Figure 16 hDPSCs expansion during 120 hours of culture in function of cell seeding, cell number was measured. For each group, n=3 (*: significantly different compared to the sample point, P<0.05)
As previously stated, when hDPSCs are undifferentiated, the surface antigen CD73, CD90 and CD105 are highly expressed. When differentiation starts, the expression is down-regulated until totally disappearing at the end of the process. As well, CD14/CD11b, CD34, CD45, CD79α/CD19 and HLA class II are always negative.

Passage 1 cells from 2 donors were plated at density 5x10^3 cells/cm² and individual attached cells were identified on the culture dish. When 80% confluence was achieved, cells were sub-cultured into new T75 flasks with the same cell density. With every new passage, cells were counted for proliferation analysis and compared with the parental cells (passage 1). This process was repeated for 12 successive passages. Population doubling time, is an important index indicating how proliferative the given cells are in culture. Cells were found to have an average doubling time of 1.568 ± 0.29 days with a minimum of 28 hours and a maximum of 48, with an average live/dead ratio of 21 ± 4.64. The proliferation rates of the first five passages of the cells did not show significant differences compared with the parent cells. These results can be seen in Figure 17.

![Figure 17: Doubling times and live/dead ratios for 12 passages of undifferentiated hDPSCs. For each group n=3 (*: significantly different compared to the parent cells, P<0.05)](image-url)

A flow cytometry characterisation of clonal cells was performed. Cells from selected passages were analysed with flow cytometry and their multipotency was compared to the parent cells (Passage 1). Cells were stained with surface markers for CD44, CD73, CD90, CD105, CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II and were found
to be homogeneous and indistinguishable from the parent culture until passage 4 with expression above 98% for CD73, CD90, CD105 as well as for the additional marker CD44. After passage 4, the markers presented fluctuation, but with a tendency to decrease. The histograms and percentage of expression of CD73, CD90 and CD105 for every passage can be seen in Figure 18 and Figure 19 respectively. It is important to comment that histograms for passage 8 and 10, appear different, as the measurements were done by different flow cytometer than the other samples (Guava easyCyte 8HT flow cytometer, Millipore).

![Figure 18 Histograms for the markers CD73, CD90 and CD105 measured by flow cytometry, where the red line indicates the negative control and the blue line, the positive samples. 10,000 events were acquired.](image1)

![Figure 19 Percentage of cells with positive expression for the markers CD73, CD90 and CD105 measured by flow cytometry, 10,000 events per sample were acquired.](image2)
The histograms and percentage of expression of CD44 can be seen in Figure 20 and Figure 21 respectively.

**Figure 20** Histograms for the markers CD44 measured by flow cytometry, where the red line represents the negative control and the blue line, the positive samples, the positive samples. 10,000 events were measured.

**Figure 21** Percentage of cells with positive expression for the marker CD44 measured by flow cytometry.

All the negative hematopoietic markers were grouped into one single channel and analysed. If samples were negative, it meant all the markers were negative. The results for all the selected analysed passages were negative can be seen in Figure 22.
Figure 22 Histograms for the grouped hematopoietic markers CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II in one channel, in each histogram the samples (orange), the unstained cells (red) and the isotypes can be seen (light blue).

These results confirm a well-defined proliferative ability through all the passages, especially from passage 1 to 5, and show how these cells are suitable for long term 3D culture.

4.4.2 Effect of culture duration on surface markers
A flow cytometry characterisation of clonal cells enduring long-term culture was performed. Cells from passage 4 were seeded with a density of ≈5000 cells/cm², cultured for 10 days without passaging and analysed with flow cytometry. Their multipotency was compared to cells from day 1 of culture. The density of cells at the last day achieved 151330 ± 14443 cells/cm² (4.93 doublings). Cells were stained with surface markers for CD44, CD73, CD90, CD105, CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II and were found to be homogeneous and indistinguishable from day 1 culture, with expression above 98% for CD44, CD73, CD90, CD105. The light scatter, histograms and percentage of expression for markers CD44, CD73, CD90 and CD105 can be seen in Figure 23 and Figure 24 respectively.
Figure 23 light scatter plots and histograms for long term culture measured by flow cytometry. A) forward vs side scatter, CD44/CD73 and CD90/CD105 plots for day 1 of culture. B) forward vs side scatter, CD44/CD73 and CD90/CD105 plots for day 10 of culture. C) CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II markers grouped in one channel for day 10 of culture, red colour for the samples, green for the isotypes and blue for the unstained. D) unstained and isotype controls

Figure 24 Percentage of cells with positive expression for the markers CD44, CD73, CD90 and CD105 for days 1 and 10 and cell density, measured by flow cytometry.
4.4.3 **Morphology and clonogenicity of hDPSCs, CFU-F**

Following the determination of the surface antigen expression, the morphology of undifferentiated hDPSCs was followed for 7 days of culture. The cells attached after 3-4 hours to the flask. The cells grew attached to the plastic surface of the T-flasks and started single-cell spreading and then forming colonies. The cells within each colony were characterised by the typical fibroblast-like morphology analogous to the shape and size of BM-MSCs. The shape and size was heterogeneous. In the first day, cells attached to the plastic and the fibroblastic shape was still not clear. By day 2, cells started elongating and the spindle shape was more clear, cells started growing and spreading through the plate. Cells achieved 80% confluence by day 3, confluence by day 4 and then they started growing multi-layered.

![Morphology of hDPSCs under normal growth conditions in light microscopy. A), D) are pictures of cells by day 1 of culture. B) and E) are pictures of cells by days 2. C) and F) are pictures of cells by days 3. G) Displays a confluent plate and H) a multi-layered plate. Scale bar=200 µm.](image-url)
Colonogenic assay was performed to establish the growth potential of hDPSCs \textit{in vitro}. This assay was employed to determine the quality of the isolated stem cells (the level of SCs present in samples). Only true multipotente SCs in samples would adhere and form colonies when cultured with very-low density. The Colony forming efficiency was established by seeding cells from 3 donors, with a density of 100 cells/well in 6 well plates ($\approx$ 10 cells/cm$^2$) and counting the colonies scored after 14 and 21 days of culture. The number of colonies scored between 14 and 21 days has no significant differences with a colony efficiency of 16 ± 2.16 percent and 13.3 ± 2.62 percent respectively. Each colony originates from a single parental cell and displays a wide variation in cell morphology and growth potential. No new colonies were formed in this period, but the size of the colonies was very diverse. In Figure 26 pictures of the resulting colonies can be seen as well as close ups to the colonies showing the maintenance of the spindle shaped morphology and how the colonies grew in size.

4.4.4 Metabolic activity of hDPSCs

Following the multipotency and growth kinetics assessment, in order to evaluate indirectly the proliferative capability of hDPSCs in 2D static culture, the metabolic activity of hDPSCs cultured under normal conditions of culture was evaluated by measuring for 3 days the time-course concentration of key substrates and metabolites in culture medium. Consumption of glutamine and glucose, production of ammonia and lactate, concentration of oxygen and detection of pH were acquired every 4 hours with a bio-profiler. During the whole culture, media was not exchanged, allowing to assess the metabolic stress that cells were suffering during an entire medium exchange cycle (normally of 3 days). The same quantity of media sampled (1 ml) was restocked with fresh media to maintain a constant volume in the culture.
Figure 26 CFU-F of hDPSCs under normal growth conditions. A) hDPSCs colonies after 14 days of culture (n=3). B) scored hDPSCs colonies after 14 days of culture (n=3). C) scored hDPSCs colonies after 21 days of culture D) a close up to a colony of 14 days of culture. E) a close up of a colony after 21 days of culture. F) higher magnification of a colony after 21 days of culture
Figure 27 Change with culture time of key substrates and metabolites in culture medium. A) Consumption of glutamine and production of ammonia. B) Consumption of glucose and production of lactate. C) Oxygen. D) pH.

The glutaminolysis is a key metabolic pathway to produce energy for proliferative cells and as glutamine is lysed into glutamate to produce ATP, it releases 1 molecule of ammonia. Glutamine was consumed from 2.83 [mmol/L] to 1.88 [mmol/L] and the same number of molecules of ammonia was produced, going from the initial concentration of 1.49 [mmol/L] to 2.43 [mmol/L]. Glutamine and ammonia kinetics can be seen in Figure 27A. The glycolysis is a key metabolic pathway to produce energy for proliferative cells and as glucose is digested to produce ATP, it releases 2 molecules of Lactate. Glucose was consumed from 5.2 [mmol/L] to ≈0 [mmol/L] and twice the number of molecules of lactate was produced, going from the initial concentration of ≈0 [mmol/L] to 11.67 [mmol/L]. Glucose and lactate kinetics can be seen in Figure 27B. The oxygen tension in the medium slowly decreased, fluctuating between 180 [mmHg] and 150 [mmHg]. The oxygen kinetics can be seen in Figure 27C. Cell culture was established with an unadjusted pH, results could improve if pH was adjusted to a starting value of 7.4. The initial pH was 7.7 and gradually decreasing to 7.5 as culture time passed. The pH kinetics can be seen in Figure 27D.
4.4.5 In vitro tri-lineage differentiation of hDPSCs

Following the multipotency and kinetic studies, a tri-lineage directed differentiation assay was performed. To characterise the differentiability of hDPSCs and to assess if they comply with the minimal criteria, cells were differentiated into osteoblasts, chondrocytes and adipocytes. Passage 4 multipotent hDPSCs were seeded in T-flasks and allowed to grow until 80% confluence, then cells were long-term cultured for 21 days, in the presence of ASAP, Dex and β-GP for osteogenic differentiation; ASAP, Dex, human insulin and TGFβ1 for chondrogenic differentiation; or IBMX, Dex, human insulin and indomethacin for adipogenic differentiation media. The differentiation was assessed by histological analysis, following the time-course change in the tainted tissues.

Osteogenic differentiation was assessed by measuring the capacity to form alizarin red-positive condensed nodules, by staining collagen with picrosirius red, by measuring ALPase activity and by flow cytometry analysis of Runt-related transcriptional factor 2 (Runx-2). Positive staining of nodules started as early as day 7, nodules kept growing in number and size through the whole experiment to finally conquer most of the growth surface of the plate. The start of osteogenic differentiation is characterised by an up-regulation of Runx-2, followed by its subsequent down-regulation. In Figure 28 and Figure 29, the change in Runx2 positive cells can be seen, and as expected, it starts high and goes down with the time-course of the differentiation.

Early osteogenic differentiation is characterised by an up-regulation of the enzyme alkaline phosphatase, followed by a consequent down-regulation. Flask coating with collagen and gelatine has been reported to help enhance attachment of cells and differentiation. Flasks were coated with collagen and gelatine and the resulting ALPase activity after 14 days of culture was compared with uncoated flask. The enzymatic activity of ALPase was quantified using a colorimetric assay and demonstrated that both coatings produced higher enzymatic activity than the uncoated flasks. From the two coats, collagen gave better performance than gelatin. Albeit, the differences were not statistically significant and coating was considered unnecessary. The results can be seen in Figure 31.
Chondrogenic differentiation was assessed by measuring the capacity to form safranin O-positive extracellular matrix containing proteoglycans. Positive nodules started appearing from day 14 and then they grew in number and and size.

Adipogenic differentiation was assessed by measuring the capacity to deposit Oil red O-positive triglycerides and lipids. In the first week of differentiation the presence of lipidic expression was null. It was after day 14 when evidence of differentiation was found. In unstained pictures, the lipidic nodules can be seen with less effort as well as the adipogenic morphlogy. Although scarce staining, the presence of nodules by day 14 and 21 is sufficient to support and sustain a positive differentiation.

![Figure 28 Hystograms for Runx-2 expression measured by flow cytometry for A) day 7, B) day 14, C) day 21 and D) unstained and isotype controls. 10,000 events were acquired.](image)

![Figure 29 Runx-2 expression during 21 days of osteogenic differentiation measured by flow cytometry. n=3, 10,000 events were acquired.](image)
Figure 30 Differentiation time-course followed by hDPSCs during 21 days of differentiation into adipocytes, chondrocytes and osteoblasts. A), B), C) and D) are images of the adipogenic differentiation stained with Oil red O by day 1, 7, 14 and 21 respectively. E), F), G) and H) are images of the chondrogenic differentiation stained with Safranin O by day 1, 7, 14 and 21 respectively. I), J) K) and L) are images of the
osteogenic differentiation stained with Alizarin red S by day 1, 7, 14 and 21 respectively. The arrows indicate in A,B,C cartilage nodules, in C,D and F fat deposition and in G, H and I, calcium deposition.

In Figure 30, the differentiation time-course followed by the cells during the tri-lineage differentiation can be seen.

![Alkaline Phosphatase Activity](image)

Figure 31 Measurement Alkaline Phosphatase activity of hDPSCs differentiated for 14 days in different coatings, collagen, gelatin and uncoated. For each group n=3. No significant differences were found.

To assess the quality of the osteoblasts produced by the hDPSCs differentiated into osteoblasts, cells were frozen for 1 year and then thawed and expanded in growth medium for 2 weeks, the resulting cells were then stained with alizarin red s and picrosirius red to assess the differentiation state. Cells grew to populate the flasks, they formed a multi-layered surface and produced bone extracellular matrix. In Figure 32 the resulting pictures can be seen, were A) shows the calcium nodule deposition and B) the collagen deposition.
4.5 Discussion

MSCs characterisation is a standard procedure to assess the quality of the biological material for further differentiation and developmental studies and plenty of information can be found in literature. However, as hDPSCs come from the ectoderm, their MSC-like state has to be assessed.

hDPSCs are clonogenic multipotent stromal cells that can be obtained autologously from adult teeth with the capability to differentiate into many different specialised animal tissues. Hence these cells have been under study to understand many developmental processes with the aim of developing therapeutics and applications for regenerative medicine and tissue engineering. However, they possess limited stemness and through passaging, their multipotency is slowly lost until cells became quiescent. Most of progenitor cells have a Hayflick’s limit between 50 to 70 doublings. Additionally, the use of donor cells comes associated with an intrinsic heterogeneity of the biological material in comparison with immortalised cell lines. As consequence, the characterisation of the cells in function of their functionality and suitability is of utmost importance as a starting point to develop a 3D bioprocessing with clinical use.

For a successful bioprocessing of hDPSCs with medical purposes to be design, it is essential to be able isolate and enrich the best cell populations that would ensure
optimal functionality, cell viability and clinical effectiveness, capable to construct a particular tissue from the cellular and extracellular perspective. One of the main characteristics of MSCs is their multipotent self-renewal and hDPSCs are similar to other MSCs in aspects including shape and adherence, multipotence and differentiability, however the maintenance of the multipotent capabilities and the differentiability are unique for each MSC type.

A standardised culture method is critical to understand and preserve the undifferentiated state of the cells and support their clonogenicity during cell renewal and expansion. As critical as a preserved expansion methodology, are the defined culture conditions for directing homogenic cell differentiation into specific cell types.

The cell seeding density plays a key roll in the proliferative capabilities of the cells, cell-cell interactions are managed by the Notch pathway, which controls cell proliferation and differentiation. Albeit the mechanism of action is still uncertain, it is known that low density of cells will hinder their proliferative capabilities as they need the contact to develop and form colonies. A high seeding density however, will hinder proliferation as well. In this conditions, a retarded cell growth is produced by contact inhibition, the Notch pathway prepares the cells for differentiation rather than for proliferation, cells go to the non proliferative phase G0/G1 of the cell cycle and markers of early differentiation start being expressed, such as Runx-2 (Galindo et al., 2005). Our results are in correspondence with results of other researchers (Suchán et al., 2007, Bressan et al., 2012) and the medium density of 5000 cells/cm² gave the better proliferation rates with a DT of ≈ 34 hours. The medium density was used for all the further 2D experiments.

The CFU-F efficiency reported by us, with such a low density of starting cells is higher than the efficiency reported for other MSCs and is in conformity with results found in literature (Gronthos et al., 2000). These results support the high proliferative quality of hDPSCs.

Evaluation of cell viability and proliferation rates for long term culture is of major relevance in quality control for cell therapeutics. Previous research has assessed these parameters with dissimilar results (Martin-Piedra et al., 2013, Kerkis et al., 2006, Martin-
Piedra et al., 2014), implying that further analysis should be realised. Viability and proliferation rates (measured as doubling time (DT) were assessed and followed through 12 passages, revealing a viability always over 90% with its peak at passage 4, after viability started slowly but steadily decreasing. The DT steadily increased with each passage, ranging from 28 to 48 hours. Our findings correspond to approximately 25 population doublings per sample and are in accordance with a wide study realised by Suchánek et al. on donors aged 16-66, they informed doubling times of 12-50 hours for the first 40 population doublings. After, DT increases to 60-90 hours (Suchánek et al., 2007, Bressan et al., 2012). Statistical analysis suggests that there’s no significant difference between viability and doubling time between parental cells and cells amid passage 1-6. After, the decrease in quality becomes significant. These results suggest that cells between passage 1 and 6 are at their optimal and should be used preferentially in therapeutics. Albeit, cells from further passage are still good quality and their multipotency should be studied to assure the quality of the cells.

Along with the proliferative capabilities of hDPSCs, their long-term multipotency hasn’t been fully studied. The assessment of the conserved undifferentiated self-renewal state associated with expression of cell surface molecules such as CD44, CD73, CD90 and CD105. As well as the lack of expression of CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II, has to be evaluated to ensure the quality of the cells. These markers were studied for 12 consecutive passages of the cells to assess the number of passages it takes for the cells to get significantly different from the parental cells. Flow cytometry data analysis showed that there is no difference between the parental cells and cells from passage 1-4. After, with the positive markers over 98% and the negative markers under 2%, after the positive markers started to slowly diminish fluctuating and reaching the lowest quality by passage 12. Interestingly, CD105 was the first in decrease, this marker has been previously reported as the most fluctuating MSC marker, Soncini and colleagues demonstrated that amnion mesenchymal stem cells (AMCs) with only 40% of the cell population positive to CD105 were able of tri-lineage differentiation (Soncini et al., 2007). These results suggest that cells between passage 1 - 4 are at their optimal and should be used preferentially in therapeutics. Cells from further passage will
differentiate and will have potential for neo-tissue formation, albeit the results will probably be heterogenic and with poor reproducibility.

Following the viability and immunophenotypic analysis of the 12 first passages, the same scheme was followed for a 10 days long culture without passaging. With the aim to assess the effect of contact inhibition in proliferation and stemness, the parental cells were compared with the resulting cells. Our results gave a DT of 2.033 days, significantly higher than the same passage 4 cells sub-cultured which could yield approximately double the amount of cells. Interestingly, the stemness markers showed no significant difference with the parental cells, retaining all the positive clusters of differentiation above 99% and the negatives under 2%. These results suggest that long term culture without passaging can produce healthy multipotent cells, but has a big impact in the proliferative quality of the cells.

The two main characteristics of MSCs are self-renewal and capability for multipotency differentiation. We already studied the self-renewal of the hDPSCs considering their proliferation rate, their viability and immunophenotype. However, their differentiation capability has yet to be discussed. Osteogenic, chondrogenic and adipogenic neo-tissular formation are considered to be trademark of all MSCs. hDPSCs demonstrated potential for the tri-lineage differentiation with results comparable with other MSCs. Osteogenic differentiation was demonstrated from the first week with ALZ staining and the calcium deposition was well established by days 14 and 21. On day 21 unspecific collagen deposition was seen. Runx-2 is a marker of early osteogenic differentiation and a typical in vitro osteogenic profile, starts with the up-regulation of its expression, until its peak is reached by day 14 of culture and its subsequent down-regulation, when osteoblast maturation is achieved (Compton and Lee, 2014). Our experiments are in concordance with the expected profile and this implies that osteoblasts were grown and started to mature. ALPase activity found its peak by day 14 and no significant differences were found when using different coating, these results suggest that the use of coating for the differentiation won’t provide significant benefits, only having a repercussion in reproducibility, as one extra step is added to the culture. Coating has been seen to enhance culture when cells with lower proliferation rates are in use. However, as hDPSCs are highly proliferative (Gronthos et al., 2002). The produced osteoblasts were
functional after a year frozen and were able to grow and produce osteoblast ECM in regular expansion medium. These results suggest the possibility to store frozen neo-tissue for clinical ready-availability.

Although the adipogenic competence of hDPSCs has not been clearly demonstrated, adipogenic differentiation of hDPSCs was confirmed by the formation of fat precipitates in the samples. By day 7 of adipogenic differentiation, adipocytes were indistinguishable, by day 14 and 21 adipocytes were visible by their morphology. Conversely, little accumulation of lipid deposition was seen. It has been reported that adipocytes appear significantly less frequently than the other cell type, Gronthos and colleagues found null adipogenic differentiation and Pierdomenico and colleagues found restricted adipogenic differentiation (Gronthos et al., 2002, Pierdomenico et al., 2005).

Chondrogenic differentiation of hDPSCs was demonstrated from the second week of culture and onward with positive safranin s staining. In the last week a big portion of ECM containing proteoglicans characteristic of chondrocytes was found, denoting the chondrogenic potential of the cells. These results are in accordance with research done by other groups (Gronthos et al., 2002, Pisciotta et al., 2015). Albeit Pierdomenico and colleagues in their studies found hDPSCs to be negative for chondrogenic differentiation in long term exposure to differentiation media (6 weeks).

4.6 Conclusions
Experimental and clinical data indicate that MSCs have potential for differentiating into various tissues in organ transplantation and in gene therapy, owing to their plasticity under appropriate conditions and their immunomodulatory properties. Through our experiments we have demonstrated that hDPSCs harbour a big potential for bone tissue engineering, they have high viability and proliferation rate, meanwhile maintaining their multipotent phenotype through passaging and high density culture, as well as strong bone differentiation that can be sustained after long term storage. From the conditions studied, it was seen that passage 4 cells are able to produce the required number of cells for 3D culture without significant modifications from the paternal cells (passage 1) and are able to produce good quality bone tissue. Hence, the long-term 3D bioprocess in a perfusion-RWV bioreactor studies will be done in passage 4 cells. Albeit hDPSCs are able to differentiate into chondrocytes and adipocytes, their potential is limited and our
studies and literature found mixed results ranging from well established, to low or even null differentiation. Hence, hDPSCs are suitable to develop a long-term bioprocessing for bone tissue engineering and the conditions established in this chapter will be used to produce the biological material for the next chapters. A further study specific for chondrogenic and adipogenic differentiation should be carried out in order to establish a proper protocol able to differentiate cells homogeneously. Until this is not done, they wouldn’t be recommended for the design of any clinical device involving chondro/adiponeo-tissue.
Effect of perfusion in the 3D bioprocessing for osteogenic differentiation: a comparison between a novel perfusion-RWV bioreactor, a fed batch RWV bioreactor and 3D static culture
5.1 Introduction

In vivo, bone cells are contained in a three-dimensional histoarchitectures that hosts the cells and their interactions. Herein, water, nutrients, cytokines and growth factors are delivered to the cells, and normal development/healing phenomena are hosted.

Traditionally, cells have been grown in two-dimensional static monolayered culture systems, where cells are grown attached to a plastic plane in their base and are exposed to culture media in their upper face. Within regular two-dimensional static culture, hDPSCs have been induced to differentiate into cells from the osteoblast lineage and many advances have been done in understanding mammalian developmental processes. The sequence of events followed by the cells, is believed to recapitulate the in vivo developmental phenomena. However, two-dimensional static culture lacks the characteristic three dimensionality found in the normal environment, resulting on limiting the delivery of many structural, mechanical and biochemical cues required for proper neo-tissue development. In two-dimensional environment, cells in the basal area of the culture flasks, receive limited nutrients and signals. Developing heterogeneously. Additionally, cells become flatter, divide abnormally and lose their differentiated phenotype (Baker and Chen, 2012). Consequently, research outcomes in two-dimensional static culture are not reproducible in vivo or in tissue explants.

The efforts to tackle with the adimensionality of regular two-dimensional culture and close the gap between the traditional culture technique and the in vivo tissue have derived into the development of three-dimensional culture systems. The microenvironment of regular bone can be temporarily reproduced within a scaffold. These matrices provide a supporting microarchitecture for the cells to expand and attach. Providing a template for normal cell-cell interactions and the mass transfer for nutrients and signalling delivery. In other words, the scaffold plays the role of an artificial three-dimensional ECM (Hutmacher, 2000, Sheridan et al., 2000). A three-dimensional setting promise to be closer to what really occurs in the in vivo environment.

The development, maintenance and regulation of hDPSC populations for bone-related cell therapeutic products, not only requires such a very difficult task of replicating the complex and specific histoarchitecture of bone. It also requires reproducing the dynamicity of its transport characteristics. Accordingly, improvement of culture
conditions, including the design of adequate tailor-made bioprocessing systems able to achieve such high benchmarks, appears to be crucial (Salgado et al., 2004).

3D scaffolds or cell aggregates cultured in a suspension inside a controlled environment using bioreactors, offer attractive advantages of ready scalability and relative simplicity and may influence cell viability and turnover of specific stages and types of stem cells (Zandstra and Nagy, 2001).

Several studies have reported the use of different scaffolds and bioreactors in the three-dimensional osteogenic differentiation of ESCs and MSCs, a list of them can be found in Table 2: Current studies about stem cell differentiation towards bone tissue using various bioreactors. Their findings delivered mixed results, but all agreeing that bioreactors and scaffolds provide an advantageous environment leading to obtain improved production of cell constructs. Alginate encapsulation has been recurrently studied as a biodegradable matrix which is adaptable to three-dimensional culture systems for tissue engineering. It has been reported that the jointly use of gelatin with alginate, strengthen the hydrogel to be used in long term culture, as alginate alone, when ionically cross-linked, loses Ca$^{2+}$ ions and could collapse (Basmanav et al., 2008). Furthermore, the incorporation of gelatin to the alginate hydrogel, has been seen to significantly improve cell adherence and proliferation, compared with the alginate only scaffold. (Venkatesan et al., 2015).

Different bioprocessing systems using shear stress have been under development. TE adopted this technology to overcome the limitations from using the impeller-type of bioreactor. Shear stress is an important biophysical signal, with cell growth and bone mechano-transduction effect. It is involved in calcium signalling, mechanical force transmission along fibrous cytoskeletal networks, cell-cell communication, and disruption of ligand-receptor binding, between others (Akins et al., 1997, Morrison et al., 1992, Talbot et al., 2010). Example of units utilising the advantages of low shear stress, is the family of bioreactors categorised as Rotating Wall Vessel (RWV) bioreactors. Originally designed by NASA, they are dynamic systems able to generate a low shear environment, where cells grow in static suspension. These systems are efficient to reduce diffusion limitations of nutrients and useful in producing ex vivo bone.
Achieving ECM deposition (Qiu et al., 1999) and mineralisation/ ALP activity/ Osteocalcin expression similar or better than the parameters achieved in static culture (Botchwey et al., 2001, Song et al., 2008, Hwang et al., 2009).

RWV-based bioreactors, when working in the right setting, have shown to achieve enhanced expansion and bone differentiation (Rauh et al., 2011). However, none of these bioprocesses has been able to recapitulate the conditions of the microenvironment found in bone tissue.

To close the gap between the bioprocessing and the in vivo requirements, a third family of bioreactors was pioneered. Semi-batch feeding strategies were left behind to adopt a perfusion feeding strategy.

Perfusion bioreactors, incorporate with a continuous exchange of medium. By generating a laminar fluid flow of culture media through the bioreactor, mass transport of nutrients and oxygen is enhanced as well as providing mechanical stimuli. A peristaltic pump drives pre-oxygenated medium through the bioreactor/ scaffold and ensures providing a well-mixed environment with essential nutrients and oxygen, meanwhile removing the metabolic waste. Additionally, different flow types can be adopted. The performance of flow type and mineralisation have been studied, the results indicate that a steady flow is a more potent stimulator of bone cells, producing higher intracellular calcium than either oscillatory or pulsing flow (Jacobs et al., 1998). Another report studied the effect of perfusion flow rate (1-3-5 ml/min). The findings suggest that a high flow rate can cause the detachment of cells and limit ALP activity and intracellular calcium content. Furthermore, they suggest that low flow rates give better expansion, but less ALP and internal calcium content than an intermediate flow (Sinlapabodin et al., 2016). Accordingly, each bioreactor-scaffold-cell type will have to be assessed for the adequate flow rate.

Bioreactors are available in the market with differing ease of use, cost/efficiency, and capability of monitoring and controlling biophysical and chemical parameters. However, optimization, modification, or the design of new bioprocesses, are required to produce the suitable osteoblast-seeded biomaterial able to translate accurately from the in vitro concept, to the in vivo neo-tissue completion.
Oxygen tension is known to play a role in expansion and differentiation, as well as proper oxygenation which is important key parameter in bioreactors design. The significance of oxygen concentration control is highlighted by the enormous and intricate mechanisms used to maintain oxygen homeostasis in the body (Csete, 2005). Hypoxia (Oxygen tension of \( \approx 0-30 \, \text{mmHg}, \approx 0-5\% \)), has been reported to improve proliferation and yield of undifferentiated ESCs and MSCs and reduce apoptosis, as opposed to normoxia (\( \approx 150 \, \text{mmHg of } pO2, \approx 20\% \)), which has been described to have an impact on differentiation patterns (Sakdee et al., 2009, Csete, 2005) and in particular, it enhances osteogenic differentiation (He et al., 2010). In vivo, stem cells normally reside in relatively low oxygen niches, and local oxygen is increased when healing happens. It has been suggested that controlling only the oxygen tension might not be sufficient to improve the general efficacy in stem cell cultures.

Most bioreactor currently being used in the field of TE are a fed-batch. However, this design aspect requires to be modified. Fed-batch operation lacks control of the parameters in culture medium such as nutrients, metabolites, and oxygen. Additionally, it accumulates metabolic waste, which can be toxic for cells at inhibitory level (Abranches et al., 2007). Moreover, abrupt medium exchange in fed-batch systems can produce high stress to the cells. As cells grow over the culture time, the degree of stress for cells rises due to increasing rates of nutrient and oxygen consumption, and metabolite accumulation in a batch, a schematic diagram of this can be seen in Figure 33. Perfusion culture systems allow control and monitoring of the medium in real time, metabolic waste removal, eliminate medium exchange stress and reduce the loss of cell-secretion, improving the maintenance of the desirable milieu that aids both expansion and differentiation (Bauwens et al., 2005, Baker and Chen, 2012, Godara et al., 2008). Cell-constructs manufactured inside an inadequate environment; lacking in nutrients, oxygen and biosignals, with waste accumulation; tend to produce poor cell-density and development, cell-type heterogeneity, ECM-poor and uneven spatial cell distribution (Ong et al., 2008). Cells closer to the surface of the cell-construct would be preferably expanded and differentiated. Cells in the cell-construct centre, would travel to the surface to fulfil their nutritional requirements. Ultimately, when the surface is excessively cell-dense, cells would leave the construct to adhere into a free surface
(Sikavitsas et al., 2002). Cell constructs under these circumstances, would bring further problems when implanted in vivo as its functionality would be compromised, the cell-dense surface would produce a harder, over-calcified surface, but the centre would remain soft, translating into poor mechanical properties. Additionally, this over-calcified surface would present a barrier for Vascularisation of the healed bone defect, rendering the cell therapy to failure. Hence, adequate scaffold size and medium perfusion in a bioreactor setting are considered not only a requirement, but indispensable features for the future design of any bioprocess with the possibility of success in cell therapy.

Figure 33 Fed-batch vs perfusion feeding strategy. It can be seen how nutrient consumption and metabolites production accumulate in the bioreactor (modified from (Cha, 2010)).
Our group has previously developed a simplified, integrated, and reproducible bioprocess for three-dimensional osteogenesis from ESCs using encapsulation with alginate/gelatin hydrogel and an in-house developed perfusion-RWV bioreactor with promising results for therapeutic uses (Cha, 2010, Yeo, 2012, Randle et al., 2007a). In the next subsection, the principia behind the development of this bioprocessing are summarised.

5.1.1 Principia behind the design of a perfused bioreactor

The in-house designed perfusion bioreactor incorporates an outer gas-permeable membrane that covers the culture vessel, enhancing gas-exchange, as well as medium perfusion and an oxygenator. Inside the bioreactor, cells are encapsulated in alginate hydrogels that are grown in a suspended-like environment. This section summarises the work done by former PhD students in our laboratory to calculate the bioreactor dimensions (Cha, 2010, Yeo, 2012). Additionally, the diameter of the hydrogels for the adequate oxygenation of the cells encapsulated is described. Fick’s second law was used to define the optimal dimensions of the culture vessel and oxygenator. It estimates the profile in which oxygen diffuses and distributes within the vessel and the oxygenator tubing to have cells shortly exposed to the gaseous environment inside an incubator. Fick’s law is briefly explained below.

Fick’s first law, relates the diffusive flux to the concentration field under steady state. The flux travels from high concentration regions to low concentration regions and is proportional to the concentration gradient (spatial derivative). In one dimension:

\[ J = -D \frac{\partial C}{\partial x} \]

Fick’s second law, derived from the first law by combining with a mass balance to the solute, predicts how diffusion causes the concentration field to change with time:

\[ \frac{\partial C}{\partial t} = -\frac{\partial}{\partial x} J = \frac{\partial}{\partial x} \left( D \frac{\partial C}{\partial x} \right) \Rightarrow \frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \]
Where the parameters in both equations are: \( J \) is the diffusion flux, amount of substance that will flow per unit of area per unit of time, \( D \) is the diffusivity, which is proportional to the squared velocity of the diffusing particles, \( C \) is the concentration in ideal mixtures environment and \( x \) is the position (diffusion length).

Fick’s second law represents how far a specific oxygen density (\( C \)) has propagated in a certain direction (\( z \)) by diffusion in time (\( t \)), and was used to determine the optimal vessel dimensions as well as the length of the oxygenator tubing.

The assumptions for the design were as follows: Gas diffusion is from the air to medium in the vessel. Well-mixed medium and constant gas concentrations in air. Initial oxygen concentration in the medium is assumed zero. Concentration at the contact surface is set as the concentration of oxygen in air. The diffusivity of oxygen in medium is assumed the same value as the \( \text{H}_2\text{O}–\text{O}_2 \) system at 37°C.

The partial differential equation with all the assumptions can be derived from the error function. In Figure 34, the solution of the second law considering the assumptions and the conceptual design of the vessel can be seen. In the resulting equation, oxygen concentration (\( C_{O_2} \)), Time (\( t \)) and length of diffusion (\( z \)) vary together (e.g. one day (86400 sec) after oxygen diffusion starts, 67% of the surface concentration of oxygen can be achieved in a depth of 1 cm, according to the calculation.

\[
\frac{\partial C_{O_2}}{\partial t} = D_{O_2-N_2O} \frac{\partial^2 C_{O_2}}{\partial z^2}
\]

Initial condition: \( C_{O_2} = 0 \) at \( t = 0 \)

Boundary condition: \( C_{O_2} = C_{\text{surface}} \) at \( z = 0 \)

\[
C_{O_2} = \text{erfc} \left[ \frac{z}{\sqrt{4 \cdot D_{O_2-N_2O} \cdot t}} \right] \cdot C_{\text{surface}}
\]

\[
\text{erfc}(\eta) = 1 - \frac{2}{\eta^2} \int_0^\eta e^{-u^2} \, du
\]

\[
D_{O_2-N_2O} = 33 \times 10^{-6} \, \text{cm}^2/\text{sec} \text{ at } 37°C
\]

\[
C_{O_2} = \text{erfc} \left[ \frac{87.04 \cdot \frac{Z}{V}}{\sqrt{t}} \right] \cdot C_{\text{surface}} \text{ at } t \text{ sec}
\]
Figure 34 Solution of Fick’s second law and conceptual design of culture vessel (modified from (Cha, 2010)).

To solve the system, the desired concentration inside the vessel is set in order to estimate the relationship between length (z) and time (t). A plot can be drawn by setting the minimal oxygen concentration. In our design, the concentration was fixed at 80% of the surface concentration and from the resulting plot, it was found that for a distance z =1.5 cm to be 80% saturated, approximately 3 days have to pass. Therefore, the depth from the surface to the perfusion rod of the culture vessel (R-r, see Figure 35) can be set as 1.5 cm with allowing 3 days of the oxygen-saturation time. This length also corresponds to the depth of a 55-ml High Aspect Ratio Vessel (HARV), which was designed to have sufficient gas-exchange through the membrane on the back of the vessel and is a system in used as a comparison system. The other dimensions of the vessel were derived on basis of the diffusion length and the volume (approx. 55ml size), and further modified due to technical reasons for fabrication. The design of the inner cartridge of the cell culture vessel is confirmed by the calculations shown in Figure 35.

\[ V_{tot} = \pi(R^2L - r^2l) \]
\[ R = 1.5 + r \]
\[ L = l + 0.2 \]
\[ V_{tot} = \pi((3R - 2.25)L + 0.2(r - 1.5)^2 = 55ml \]
\[ (3R - 2.25)L + 0.2r^2 = 17.51 \]

if \( r = 1 \text{ cm} \) \( \Rightarrow R = 2.5 \text{ cm} \) \( \Rightarrow 5.25L = 17.31 \)
\[ L \approx 3.5 \text{ cm} \]
\[ l \approx 3.3 \text{ cm} \]
Volume \( \approx 60 \text{ ml} \)

Figure 35 Dimensions calculation and schematics of the vessel (modified from (Cha, 2010)).

A gas-permeable membrane is rolled and tightly fixed to the frame of the vessel, allowing gas-exchange through the wide surface area as determined previously. The membrane is made of a silicon material coated in one side with polytetrafluoroethylene (PTFE, Teflon), to prevent fouling by numerous bio-molecules.
present in culture medium or secreted by cells. The detailed specifications of the membrane can be found in Table 5 and Table 6.

Table 5: Specifications of gas permeable membranes, in the materials and methods section.

In addition to the oxygen provided through the membrane, the culture medium perfused to the culture vessel comes oxygen-saturated by the use of an external oxygenator. See figure

Figure 36 Culture vessel's dual supply of oxygen (Cha, 2010). It can be seen how oxygenated media enters the system through the annulus of the central rod, and then radially to the vessel. It exits through the opposite central outlet. Oxygen from the environment enters the vessel through the membrane-wall.

The external oxygenator is composed of a spiral roll of gas-permeable silicon tubing with a wall thickness of 0.5 mm. The length of the tubing of approximately 3.4 m was designed to be long enough to saturate the culture medium with oxygen whilst the medium passes through the oxygenator. The calculations were done following the second Fick law with the same boundary conditions used to design the vessel. The residence time of medium in the oxygenator would be of 600 min for a total length of 3.4 m.

To calculate the adequate diameter of alginate hydrogels, a similar strategy to the vessel design was followed. To describe the dynamic mass transfer of oxygen from bulk solution into the alginate spheres, the transfer of the oxygen along the media and the
transfer from the medium to the solid and the consumption of oxygen by the encapsulated cells have to be considered.

\[ \frac{\partial C_{O_2-Alg}}{\partial t} = D_e \frac{\partial^2 C_{O_2-Alg}}{\partial z^2} + S_r \]

Where \( D_e \) is the effective oxygen diffusion coefficient, \( C_{O_2-Alg} \) is the concentration of oxygen within the hydrogels, \( C \) is the concentration in ideal mixtures environment, \( t \) is time, \( S_r \) is the rate of oxygen consumption per unit volume, \( k_f \) is the Liquid-solid mass transfer coefficient, as is the surface area of the spheres and \( r \) is the position (diffusion radial position).

The assumptions for the design were as follows: consumption by the cells is considered to be zero, microcapsules are assumed to be homogeneous spheres of equal size, well-mixed medium provides a constant oxygen concentration. Initial oxygen concentration in the sphere is assumed zero. Concentration at the contact surface is set as the concentration of oxygen in the media. The diffusivity of oxygen in the hydrogels has a value of 1.4x10-5 cm2/s, this value is obtained from literature (White et al., 2014, Zhao et al., 2013). Additionally, it is considered that there is no external boundary layer effect and the consumption of oxygen by the cells to be zero.

Boundary conditions:

\[ \begin{align*}
B.C.1: & \quad C_{O_2-Alg} = 0 \text{ at } t = 0 \\
B.C.2: & \quad C_{O_2-Alg} = C \text{ at } z = 0 \\
B.C.3: & \quad C_{O_2-Alg} = 0 \text{ at } z = R
\end{align*} \]

Where \( R \) is the radius of the alginate spheres.

\[ \frac{C_{O_2-Alg}}{C} = erf_c \left[ \frac{z}{\sqrt{4 \cdot D_e \cdot t}} \right] \]

\[ D_e = 1.4 \times 10^{-5} \text{ cm}^2/\text{s at 37° C} \]

\[ \Rightarrow C_{O_2-Alg} = erf_c \left[ 133.63 \cdot \frac{z}{\sqrt{t}} \right] \cdot C \]

To solve the system, the desired concentration inside the hydrogels is set in order to estimate the relationship between its radius (depth in direction \( z \)) and time (\( t \)). A plot can be drawn by setting the minimal oxygen concentration. The concentration was fixed at 80% of the surface concentration (medium concentration) and from the resulting plot,
it was found that for a distance $z = 1.3$ mm to be 80% saturated, approximately 40 minutes have to pass. Table 9 shows the time required for different percentages of saturation to be achieved by different diameters. Therefore, the depth from the surface to centre of the hydrogel can be set between 0.5 and 2.5 mm, and in less than 3 hours, 90% of the medium concentration would be attained.

Table 9: Variation of oxygen saturation times with diameter of hydrogel spheres.

<table>
<thead>
<tr>
<th>Diameter</th>
<th>70%</th>
<th>80%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,5 mm</td>
<td>3 min</td>
<td>5 min</td>
<td>23 min</td>
</tr>
<tr>
<td>1,0 mm</td>
<td>10 min</td>
<td>23 min</td>
<td>93 min</td>
</tr>
<tr>
<td>1,3 mm</td>
<td>17 min</td>
<td>40 min</td>
<td>158 min</td>
</tr>
<tr>
<td>1,5 mm</td>
<td>23 min</td>
<td>52 min</td>
<td>210 min</td>
</tr>
<tr>
<td>2,0 mm</td>
<td>40 min</td>
<td>93 min</td>
<td>370 min</td>
</tr>
<tr>
<td>2,5 mm</td>
<td>64 min</td>
<td>145 min</td>
<td>585 min</td>
</tr>
</tbody>
</table>

Computational Fluid Dynamics (CFD) analysis was used to simulate the transport of oxygen in the culture vessel to establish the time required to achieve saturation (Yeo, 2012). The model considered the oxygen supplied through the membrane and through perfusion of saturated culture media without cells-seeded hydrogels in the system. The boundary conditions used were the same as stated for the conceptual design. The results demonstrated that this setting can achieve an homogeneous ≈ 60% of saturation by 6 hours and after 18 hours saturation has reached the level of oxygen in the surface. The calculations done to design considered 3 day of oxygenation to achieve equilibrium with the environment. However, the dual-supply of oxygen through both the membrane and medium perfusion offers effective oxygenation and is able to reduce the time to achieve homogeneous equilibrium of oxygen tension with the incubator atmosphere in less than 24 hours.
Figure 37 Oxygenation within the culture vessel after A) 6 and B) 18 hours. The picture displays CFD models of oxygen concentration distributes in the culture vessel, the model describes the time required for medium inside the vessel to achieve saturation (Yeo, 2012).

The perfusion-RWV bioreactor was designed in a twin setting, and consist of 2 pre-sterilised hand-assembled culture vessels attached to a rotating system with 2 peristaltic pumps to drive the media from the oxygenator to the vessels. Sealed bottles contain sterile fresh media and waste media is disposed in sealed waste bottles to prevent the system of contamination. In Figure 38 a CAD model of the bioreactor can be seen.

The performance of the in-house designed perfusion-RWV bioreactor, has been compared with traditional two-dimensional culture for the expansion and osteogenic differentiation of ESCs. Results demonstrate enhanced undifferentiated expansion, as well as an improved osteogenic differentiation measured by an enhanced ALPase activity, mineralisation and gene expression for many important genes (Cha, 2010, Yeo, 2012, Randle et al., 2007a). However, our bioprocessing still remains to be tested with MSCs such as the hDPSCs.
5.2 Objectives
An efficient strategy for the reproducible scaling up of the production and osteogenic differentiation of hDPSCs for cell therapy, requires understanding the behaviour of cells under three-dimensional conditions for expansion and differentiation, by focusing on the three-dimensional undifferentiated expansion of hDPSCs and in the osteogenic differentiation of hDPSCs under perfusion flow, as the nutritional and environmental requirements of hDPSCs under this conditions.

The aims of this chapter are: 1) to establish long-term three-dimensional bioprocess in a perfusion-RWV bioreactor for the undifferentiated expansion of hDPSCs. 2) to establish the efficient long-term three-dimensional bioprocess in a perfusion-RWV bioreactor for directing the osteogenic differentiation of hDPSCs and compare it with three-dimensional static culture and a fed-batch RWV bioreactor.

5.3 Experimental Process

Previously it was demonstrated that hDPSCs can be long term grown undifferentiated under normal culture conditions and that passage 4 cells are able to produce the required number of cells for 3D culture without significant differences from the paternal cells (passage 1), these cells were used in this chapter. Prior to the osteogenic
differentiation, donor samples of hDPSCs were expanded undifferentiated until reaching the desired number by passage 4, these cells were encapsulated in an alginate/ gelatin scaffold with a density of 20,000 cells/bead. 250 beads were seeded and cultured in the perfusion-RWV bioreactor with growth medium and characterised through their ability to proliferate in suspended culture, by the presence of a consistent set of marker proteins on their surface and proliferative capabilities. Perfusion was set as 1 vessel volume per day (55 ml/day medium).

For the osteogenic differentiation, donor samples of hDPSCs were expanded undifferentiated until reaching the desired number by passage 4, these cells were encapsulated in an alginate/ gelatin scaffold with a density of 20,000 cells/bead. 250 beads were seeded and expanded in 175 T-flasks with expansion medium for 7 days, allowing cells to acclimatise to the new three dimensional environment. Following the process, beads were transferred to either, perfusion-RWV bioreactor, HARV bioreactor or 3D static culture and for 21 days osteogenic medium containing dexamethasone, β-GP, ascorbic acid and KH2PO4, was used to drive undifferentiated hDPSCs to osteogenic lineage. For the perfusion-RWV bioreactor, medium perfusion was set to 1 vessel volume per day (55 ml), for the HARV bioreactor, media was changed daily (55 ml) and for the static culture, media was changed daily (25 ml). Thus osteogenic differentiation was assessed using various techniques such as, proliferation measurement by Calcein AM/ ethidium homodimer, measurement of ATP content and MTS assay, characterisation of mineralised bone nodules by alizarin red and von Kossa, H&E, alkaline phosphatase expression, immunocytochemical characterisation, RT-PCR, scanning electron microscopy with X-ray microanalysis, ATR-FTIR and ATR-FTIR imaging. The detailed explanation of these techniques can be found in Chapter III. The overall experimental process is illustrated in Figure 39.
5.4 Results

5.4.1 hDPSCs cultured in three-dimensional environment for expansion and maintenance

It is an important factor to keep maintaining multipotency of hDPSCs in 3D culture during expansion and maintenance of the undifferentiated cells for bone tissue bioprocessing. Since we are developing a single step bioprocess, the cells are required to be expanded inside the bioreactor with the minimum disturbance before the osteogenic differentiation.

Cells were likely to show single cell suspension well distributed through the hydrogel, cell clumps can be seen. The hydrogels did not show any signal of mineralisation during the 12 days of culture. Pictures can be seen in Figure 40.

Figure 40 Morphology of hDPSCs encapsulated in alginate beads grown undifferentiated in perfusion-RWV bioreactor for A) 7 days and B) and C) day 12. Scale bar for picture A=100 µm and 200 µm for pictures B and C.
To assess the expansion of passage 4 hDPSCs-loaded hydrogels grown in the perfusion-RWV bioreactor, their multipotent immunophenotype was measured by flow cytometry during 12 days. Every three days, cells were stained with surface markers for CD44, CD73, CD90, CD105. In the last day of culture, the negative markers CD14, CD11b, CD34, CD45, CD79α, CD19 and HLA class II, were measured. As well, proliferation and ALPASE activity were assessed for 28 days by measuring ATP content and by MTS activity assay. Cells were found homogenously positive and indistinguishable from day 1 cells for the markers CD44, CD73 and CD90. However, cd105 demonstrated a tendency to decrease after day 6. The dot plots and percentage of expression of CD44, CD73, CD90 and CD105 every 3 days can be seen in Figure 41 and Figure 42 respectively.

Figure 41 Forward and side scatter plots top row, dot plots for the double positive markers CD105 vs CD90 mid row and dot plots for double positive markers CD44 vs CD73 down row measured by flow cytometry for A) day 3, B) day 6, C) day 9 and D) day 12 of culture in perfusion-RWV bioreactor, F) histogram of the negative grouped MSC markers by day 12, where orange represents the sample, red the unstained cells and violet the isotypes. 10,000 events were acquired per sample.
Figure 42 Percentage of cells with positive expression for the markers CD44, CD73, CD90 and CD105 measured every three days of culture in perfusion-RWV bioreactor for a total of 12 days by flow cytometry. 10,000 events were acquired per sample.

The growth kinetic of hDPSCs was measured by the ATP content (CelltiterGlo 3D assay) and MTS activity of beads (CCK-8 assay) (n=6). The ATP content increased 1.61 times fold by day 14, 6.74 times by day 21 and 13.33 by day 28, MTS followed a similar pattern increasing 2.47 times fold by day 14, 5.11 times by day 21 and 9.19 times by day 28. Charts that show these results can be seen in Figure 43 and Figure 44 for ATP content and MTS activity respectively. Both proliferation assays used have limitations as the 3D environment deviates from the real cell number. The relationship between seeded cell number and luminescent/ absorbance output after several days in culture is often curved due to the effects of contact inhibition on cell proliferation, as well as reduced metabolic activity and/ or necrosis in the centre of scaffolds. The non-linearity provides a semi-quantitative/ qualitative approximation to cell number rather than a quantitative measurement.
Figure 43 Measurement of hDPSCs proliferation by Celltiter-Glo 3D cell viability assay (ATP content) for 28 days of expansion in perfusion-RWV bioreactor. For each group n=6.

Figure 44 Measurement of hDPSCs proliferation by CCK-8 cell viability assay for 28 days of expansion in perfusion-RWV bioreactor. For each group n=6.

Alkaline phosphatase activity (ALPase) was low during all the culture, without significant differences between the time points, as cells did not start bone differentiation. It can be seen in Figure 45.
5.4.2 Comparison of 3D bioprocesses for osteogenic differentiation

The 3D expansion and acclimatising of the cells to the 3D environment (first 7 days of culture) was performed in 3D static culture (T175-flasks) placed in a shaker, for all the differentiation processes to start from the same conditions. However, since we are developing a single step bioprocess, in normal operation conditions, the bioreactors should be loaded from day 0 with the seeded hydrogels and cells should not be removed from the reactor or be disturbed in any possible manner. By day 7, cells were placed in one of the three bioprocesses in study and osteogenic differentiation of hDPSCs was performed by exposure to osteogenic media (Dex, ASAP, β-GP) for 21 more days. The best bioprocess for 3D osteogenic differentiation was established by measuring proliferation, gene expression, mineralisation through different techniques, and qualitatively by SEM microscopy, histology and immunostaining.

5.4.2.1 Morphology and cell viability of hDPSCs 3D osteogenic bioprocesses

Every hydrogels containing osteogenic cells after 28 days of culture showing changing colour from clear transparent becoming white, bone looking and appeared to be physically brittle in contrast to the initial alginate gel properties. As presented in Figure 46.
Figure 46 Images of hydrogels after 28 days of culture, A) perfusion-RWV bioreactor, B) HARV bioreactor and C) 3D static culture.

During the differentiation process, the morphology changes of the hydrogels were followed by light microscopy. By day 7 (expansion and acclimatising period), cells were likely to show single cell suspension well distributed through the hydrogel. The hydrogels did not show signal of mineralisation. By day 21 (day 14 of differentiation), mineralisation started conquering the hydrogels, and light almost could not travel across the hydrogels. By day 28 pictures showed black spheres, as light did not cross the hydrogels. From the three bioprocesses, mineralisation appears to be higher in the perfusion-RWV bioreactor, followed by the flask and the HARV bioreactor having similar results. Morphology of the beads can be seen in Figure 47 for days 7 and 28. The other days can be found in the supplementary information in the appendix.

The growth kinetics of hDPSCs seeded in beads (n=6) showed that by day 21, the perfusion-RWV bioreactor yielded significantly higher cell numbers than the other bioprocesses, with an enhancement of ≈7 times fold, compared with 5.8 and 5 times achieved by the HARV bioreactor and the 3D static culture respectively. Albeit by day 28 the MTS activity went down for the three bioprocesses, it’s believed that mineralisation of the beads or an enzymatic change related with differentiation, did not allow the reaction buffer to act on the cells causing the down expression of the MTS assay. Cells were shown to be alive and healthy by the end of the culture by Live/ dead assay, staining live cells with Calcein AM (green) and dead cells with ethidium homodimer (red). These results can be seen in Figure 48 and Figure 49.

Live/ dead assay shows that cells are very alive, the down expression of the MTS assay could be result of an enzymatic activity change after differentiation. The proliferation assay used have limitations as the 3D environment deviates from the real cell number. The relationship between seeded cell number and absorbance output after several days
in culture is often curved due to the effects of contact inhibition on cell proliferation, as well as reduced metabolic activity and/or necrosis in the centre of scaffolds. The non-linearity provides a semi-quantitative/qualitative approximation to cell number rather than a quantitative measurement.

Figure 47 morphology of hydrogels observed under light microscopy of A) Day 7, B) day 28 HARV bioreactor, C) day 28 static culture Flask, D) day 28 Perfusion-RWV bioreactor. Scale bar=200 µm.

Figure 48 Measurement of hDPSCs proliferation by CCK-8 cell viability assay scored by HARV bioreactor, perfusion-RWV bioreactor and 3D static culture through 28 days of culture. For each group n=3. * indicates statistical significance comparing between bioprocess for each time-point.
Figure 49 Live dead assay A) at day 0, B) at day 7, C) at day 28 for cells seeded in perfusion-RWV bioreactor, D) at day 28 for cells seeded in HARV bioreactor, E) at day 28 for cells seeded in 3D static culture. scale bar=200 µm.

In order to evaluate indirectly the proliferative capability of hDPSCs in 3D culture, the metabolic activity of hDPSCs cultured in perfusion-RWV bioreactor, HARV bioreactor or 3D static culture under osteogenic differentiation was evaluated by measuring for 28 days the time-course concentration of key substrates and metabolites in culture medium. Consumption of glutamine and glucose, production of ammonia and lactate, concentration of oxygen and detection of pH were acquired every 7 days with a bio-profile.

For the Perfusion-RWV bioreactor, medium perfusion was set to the equivalent of 1 vessel volume a day (55 ml of medium). For the HARV bioreactor and 3D static culture, media was exchanged daily with volumes of 55 and 25 ml respectively. Medium was measured before exchange, allowing to assess the metabolic stress that cells were suffering during an entire medium exchange cycle.

The glycolysis is a key metabolic pathway to produce energy for proliferative cells and as glucose is digested to produce ATP, it releases 2 molecules of Lactate. 20 [mmol/L] of lactate are limiting for the culture, above this level, lactate has a harmful effect (Ouyang et al., 2007). In the perfusion-RWV bioreactor, glucose was consumed from ≈7.1 [mmol/L] to ≈6 [mmol/L]. However, as lactate accumulation was too low, it did not reach
a measurable concentration. Glucose and lactate kinetics for the perfusion-RWV bioreactor can be seen in Figure 50A. In the HARV bioreactor, glucose was consumed from \( \approx 7.1 \) [mmol/L] to \( \approx 5.4 \) [mmol/L], lactate accumulation in the system was too low, and did not reach a concentration measurable by the bioprofiler. Glucose and lactate kinetics for the perfusion-RWV bioreactor can be seen in Figure 51A. In the 3D static culture, glucose was consumed from \( \approx 7.1 \) [mmol/L] to \( \approx 0 \) [mmol/L], and twice the number of molecules of lactate was produced. Lactate production rise from \( \approx 0 \) to \( \approx 12.3 \) [mmol/L]. Glucose and lactate kinetics for the perfusion-RWV bioreactor can be seen in Figure 52A.

The glutaminolysis is a key metabolic pathway to produce energy for proliferative cells and as glutamine is lysed into glutamate to produce ATP, it releases 1 molecule of ammonia, if ammonia concentration reaches 4 [mmol/L], it can have a toxic effect for the cells (Chaudhry et al., 2009). For the perfusion-RWV bioreactor, glutamine fluctuated from \( \approx 4 \) [mmol/L] and \( \approx 3 \) [mmol/L] and ammonia followed a similar trend, fluctuating between \( \approx 0.78 \) [mmol/L] and \( \approx 1.1 \) [mmol/L]. Glutamine and ammonia kinetics for the perfusion RWV-bioreactor can be seen in Figure 50B. For the HARV bioreactor, glutamine fluctuated from \( \approx 4 \) [mmol/L] and \( \approx 2.8 \) [mmol/L] and ammonia followed a similar trend, fluctuating between \( \approx 0.9 \) [mmol/L] and \( \approx 1.1 \) [mmol/L]. Glutamine and ammonia kinetics for the HARV bioreactor can be seen in Figure 51B. For the 3D static culture, glutamine fluctuated from \( \approx 4.1 \) [mmol/L] and \( \approx 2.39 \) [mmol/L] and ammonia followed a similar trend, fluctuating between \( \approx 0.8 \) [mmol/L] and \( \approx 1.3 \) [mmol/L]. Glutamine and ammonia kinetics for the static culture can be seen in Figure 52B.

For the perfusion-RWV bioreactor, the oxygen tension in the medium slowly decreased, fluctuating between 160 [mmHg] and 140 [mmHg]. The oxygen kinetics for the perfusion-RWV bioreactor can be seen in Figure 50C. For the HARV bioreactor, the oxygen tension in the medium slowly decreased, fluctuating between 160 [mmHg] and 130 [mmHg]. The oxygen kinetics for the HARV bioreactor can be seen in Figure 51C. For the 3D static culture, the oxygen tension in the medium slowly decreased, fluctuating
between 160 [mmHg] and 130 [mmHg]. The oxygen kinetics for the 3D static culture can be seen in Figure 52C.

The pH in the perfusion-RWV bioreactor culture, gradually decreased from 7.6 to 7.2 as culture time passed. The pH kinetics for the perfusion-RWV bioreactor can be seen in Figure 50D. The pH in the HARV bioreactor culture, gradually decreased from 7.6 to 7.08 as culture time passed. The pH kinetics for the HARV bioreactor can be seen in Figure 51D. The pH in the 3D static culture, gradually decreased from 7.6 to 7 as culture time passed. The pH kinetics for the 3D static culture can be seen in Figure 52D.

It is important to mention that in every time-point, 50 beads were removed for analysis. This disturbance inserted to the system implies that the resulting metabolite kinetics would be affected. If these samples were to be maintained in the system, the kinetics would reflect it and by the end of culture, 5 times more cells would be consuming medium, oxygen and producing waste. Lactate and ammonia wouldn´t reach harmful levels, they are limited by the glucose and glutamate concentration. Nevertheless, pH, glucose, glutamate and oxygen concentrations could reach low levels, and have a negative repercussion on the culture quality.

Figure 50 Change with culture time of key substrates and metabolites in culture medium for the perfusion-RWV bioreactor. A) Consumption of glucose and production...

Figure 51 Change with culture time of key substrates and metabolites in culture medium for the HARV bioreactor. A) Consumption of glucose and production of lactate. B) Consumption of glutamine and production of ammonia. C) pH. And D) oxygen.
Figure 52 Change with culture time of key substrates and metabolites in culture medium for the 3D static culture. A) Consumption of glucose and production of lactate. B) Consumption of glutamine and production of ammonia. C) pH. And D) oxygen

5.4.2.2 Histological analysis and immunostaining of hDPSCs 3D osteogenic bioprocesses
We addressed whether the hDPSCs were capable of forming mineralised bone nodule in 3D culture systems using alginate beads and any of the three bioprocesses being compared. In order to confirm whether these tissues were mineralised bone tissue, histological analysis and immunostaining was done to characterize the morphology and calcium deposits in tissues.

The resulting hydrogels from the 3 bioprocesses were cut in slices and histologically examined by hematoxylin and eosin staining (H&E). As shown in Figure 53, samples displayed a heterogeneous morphology and behave different. However, the three groups of osteogenic constructs showed gradual bone neo-tissue formation over the time period. At day 0, most cells appear scattered and spread homogenously through the beads, at this stage cells haven’t formed extracellular matrix (Figure 53A). After 7 days of culture in expansion medium, it can be seen that cells expanded and populated the whole hydrogels (Figure 53B). by day 14 (7 days in differentiation media), a decrease in cell number is observed, this is confirmed by the MTS measurements that show the same reduction. As time passed by, size and shape of cells, together with their surroundings changed, ECM was formed and approximately-round lacunae started showing around single cells, which is signal of cells differentiation into osteogenic lineage. These lacunae, when cells matured, became more elongated (osteocyte lacunae). The 3D static culture showed fair formation of round-shaped lacunae by day 21 and increased by day 28 (see Figure 53C). This suggests cell maturation. Interestingly, lacunae without cells started appearing by day 21 and cells attached to the flask surface began growing (see Figure 54). The HARV bioreactor showed less differentiation than the static culture. By day 21 poor lacunae formation was observed, suggesting poor maturation of the cells. Additionally, there were not many cells visible in the hydrogels. The perfusion-RWV bioreactor showed the production of more mature morphology than the other bioprocesses. By day 21, cells fully populated the scaffold and round lacunae was formed in all the beads except from the most inner area of the beads. By
day 28 elongated lacunae can be seen in the most external area of the beads. Nonetheless, the inner area was not as maturated, showing heterogeneity of the cell product. Figure 53 displays H&E pictures of day 0, 7 and 28. A complete scenario, including days 14 and 21, can be found in the supplementary images in the appendix.

Figure 53 H&E images showing the morphology of alginate beads: pictures A) and B) display cells grown in alginate beads in growth medium in static culture for acclimatisation to the 3D environment by day 0 and 7 respectively. Hydrogels cultured with osteogenic medium after 28 days C) in 3D static culture D) in HARV bioreactor and E) in perfusion bioreactor. Scale bar=200 µm.
Figure 54 hDPSCs adhered to culture flask by day 14 of culture in 3D static culture, these cells left the scaffold as consequence of mass transport limitations. Scale bar=200 µm.

Calcified deposition was widely spread in the osteogenic beads and was shown by alizarin red s staining (ARS) and von Kossa staining. ARS results displayed an increase of the nodules in time. 3D static culture and perfusion-RWV produced larger nodules through the 28 days, while HARV bioreactor produced small nodules in higher quantity. Negative controls are undifferentiated hDPSCs grown in alginate beads and a known to be negative tissue. Further in this chapter, quantitative analysis of ARS can be found. Figure 55 displays the results for 3D static culture, HARV bioreactor and perfusion-RVW bioreactor for days 14 and 28 of culture, additional pictures can be found in the appendix.

Von Kossa staining was done for day 28 and albeit the results do not show mass deposition (black colour), the beads produced by the 3 bioprocesses, specially the perfusion-RWV bioreactor, produced plenty of dispersed depositions of calcium (grey colour), compared with the negative sample that produced a pink stain without grey of black. These results can be seen in Figure 56
Figure 55 Alizarin red staining of beads produced by: 3D static culture, A) day 14, B) and C) day 28; HARV bioreactor, D) day 14, E) and F) day 28; perfusion-RWV bioreactor G) day 14, H) and I) day 28; F) undifferentiated hDPSCs, G) negative control. Scale bar=200 µm.

Figure 56 Von Kossa staining of beads after 28 day bioprocessed in: A) and B) 3D static culture, C) and D) HARV bioreactor, E) and F) perfusion-RWV bioreactor; G) negative control. Scale bar=200 µm.

For further confirmation of osteogenic neo-tissue formation, the hydrogels were stained with anti-osteocalcin antibody (Figure 57). The three bioprocesses by day 28 produced tissue positive for osteocalcin, with the perfusion-RWV bioreactor showing higher intensity. The other bioprocesses showed a comparatively weaker expression.
Figure 57 Fluorochrome labelling of bone constructs for Osteocalcin of beads differentiated after 28 days of culture of A) HARV bioreactor B) 3D static culture, C) perfusion-RWV bioreactor and D) negative control. Scale bar=200 µm.

5.4.2.3 Quantitative analysis of hDPSCs 3D osteogenic bioprocesses

The quantitative comparisons among the bioprocesses were undertaken by ALPase activity analysis, ARS-based quantification, chemical analysis with ATR-FTIR spectroscopy analysis and X-ray microanalysis and qPCR with various osteogenic genes.

ALPase activities in the 3 bioprocesses increased through the experiment, however, the differentiation in flasks, showed poor increase compared with the other bioprocesses, additionally, perfusion-RWV bioreactor achieved significantly higher activities than the other bioprocesses, scoring 7.3 times fold its initial activity. These results can be seen in Figure 58.

The level of mineralisation of the beads was measured by quantifying the ARS positive area in stained slides. The three bioprocesses demonstrated a similar trend, with a slow, but steady increase of the positive area through the 28 days. There was not a significant difference in the increase experienced by the 2 bioreactors. After 14 days of culture increasing from 5% to ≈32%. However, the 3D static culture had a positive surface significantly lower after the 28 days of culture. These results can be seen in Figure 59.
Figure 58 Measurement of Alkaline phosphatase activity by colorimetric assay, scored by HARV bioreactor, perfusion-RWV bioreactor and 3D static culture through 28 days of culture. For each group n=3. * indicates statistical significance between the three groups for each time-point.

Figure 59 Semi-quantitative indirect measurement of Alizarin red S stained slides: Percentage of mineralised area of hydrogels measured by image analysis software of stained slides (n=3). * indicates statistical significance between the three groups for each time-point.
Spectra from ATR-FTIR and X-ray microanalysis, confirmed that the comparison of mineralisation in this study was based on the true calcified hydroxyapatite components (Figure 60, Figure 61, Figure 65, Figure 65 and Figure 65). They were characterised in ATR-FTIR by the specific spectral bands at 1012 cm\(^{-1}\) and 1445 cm\(^{-1}\) which are comparable with pure hydroxyapatite and collagen of human bone respectively and in X-ray microanalysis by the ratio Ca/P, as previously reported hydroxyapatite has a ratio of 1.62 (Meyer et al., 1972, Figueiredo et al., 2012). It was seen that the perfusion-RWV bioreactor achieved better results in both analysis during the 28 days of culture. Having stronger signal in the mentioned bands and showing an average Ca/P ratio higher than the other bioprocesses. However, albeit Ca/P ratio scored by the static culture was in the same orders of magnitude as the perfusion-RVW bioreactor and higher than the resulting from the HARV bioreactor, the measurements in ATR-FTIR were opposite, scoring lower than HRV bioreactor.

![ATR-FTIR spectra comparison](image)

**Figure 60 ATR-FTIR spectra comparison for the three bioprocesses after 21 days of culture**
Figure 61 ATR-FTIR spectra comparison for the three bioprocesses after 28 days of culture.

Figure 62 ATR-FTIR spectra normal human bone tissue, obtained by Beasley et al. (Beasley et al., 2014). This image is used as control, the peaks for phosphate and carbonate can be seen in the wavenumbers 1035 and 1415 [cm⁻¹] respectively.

Figure 63 ATR-FTIR imaging of A) static culture, B) HARV bioreactor and C) perfusion bioreactor after 28 days of culture. These images where obtained with a field of view of 650x580 µm², Factor analysis was applied to the region of 1110-990 cm⁻¹. Scale bar=200 µm.
The C/P ratio is a characteristic ATR-FTIR measurement of bone. Samples were analysed measuring the absorbance of the samples for the wavenumbers 1415 (for C) and 1035 [cm\textsuperscript{-1}] (for P), the resulting values can be seen in the Figure 64.

**Figure 64** Relationship between the organic and inorganic fraction of bone constructs. C/P ratio of hydrogels for different sampling days. For each group n=3. * indicates statistical significance between the three groups for each time-point.

![Image](image1.png)

Following the evaluation of the enzymatic and chemical characteristics of the biomaterial constructs formed by the three bioprocesses, osteogenic differentiation was also characterized by distinct cellular phenotypes. Osteogenic differentiation of hDPSCs was evaluated by measurement of gene expression level using qPCR. The expression of
genes Runx2/CBFA1, osteocalcin (BGLAP), alkaline phosphatase (ALPL), collagen type I (COL1A1) and sclerostin (SOST) can be seen in Figure 66.

The positive expression for the five genes in the three bioprocesses, indicates successful osteogenic differentiation. Especially, when going from day 21 of induction to day 28. An enhanced or steady expression was seen in most of the genes. Perfusion culture had a steady expression of Runx2 and produced an enhancement in the expression of COL1A1, BGLAP, ALPL and SOST in the last 7 days of culture. HARV culture had a steady expression of Runx2 and produced a soft enhancement in all the analysed genes in the last 7 days of culture. Static culture had a steady expression of ALPL and COL1A1 and produced an enhancement in the expression of Runx2, BGLAP and SOST in the last 7 days of culture. These results indicate the progression of the osteogenic differentiation.

Figure 66 Relative gene expression for the osteogenic markers A) RUNX2, B) BGLAP, C)COL1A1, D) ALPL and D) SOST, for the three bioprocesses for 28 days of culture under
5.4.2.4 **Surface analysis by scanning electron microscopy of hDPSCs 3D osteogenic bioprocesses**

When viewed by SEM, many mineralised deposit were observed within alginate hydrogels after osteogenic culture. Additionally, the lacunae seen in the histology are clearly viewed in these pictures, along with the morphogenesis of the osteoblastic cells. In the first days of culture, cells were seen scattered homogenously, additionally, as ECM was still not produced, the hydrogels were weak and difficult to process for SEM. After, scaffolds started getting rigid and pictures showed the presence of non-stoichiometric mineralised deposits. These nodules were mainly composed of calcium and phosphorous (proved by X-ray microanalysis). By day 28 the lacunae were distributed in large portions of the scaffolds and in the pictures of the perfusion-RWV bioreactor, osteocytes can be morphologically recognised. The morphology of the bone constructs produced by the 3D static culture can be seen in Figure 67 A to C, where the maturation of the tissue can be seen. However, osteocyte lacunae were found empty (in accordance with H&E pictures). The morphology of the bone constructs produced by the HARV bioreactor can be seen in Figure 67 D to F, where cell heterogeneity and less mature neo-tissue can be seen. The morphology of the bone constructs produced by the perfusion-RWV bioreactor can be seen in Figure 67 G to L. In these SEM pictures it can be seen that cells in the surface of the beads resemble osteocytes showing canaliculi and their surroundings osteocytes lacuna. The cells in the inner area of the scaffolds, look immature, implying cell heterogeneity.

5.5 **Discussions**

Tissue engineering holds the keys for the production of neo-tissue-equivalents with clinical use. Cells, scaffolds and bioreactors are the tools it uses, in hope to produce fully functional living tissue-mimicking biomaterials. In due process, it can provide the basis for systemic and controlled in vitro studies of tissue development and function. To date, several studies in 2D and 3D static platforms have been done, using different cell sources and scaffold materials with the aim to produce bone-like constructs in a laboratory scale (Zhao et al., 2011, Karbanova et al., 2010, Jensen et al., 2015, Heng et al., 2015).
Additionally, in the last years, there have been numerous research to develop three-dimensional *in vitro* bone neo-tissue constructs using MSCs, 3D scaffolds, growth factors/chemical cues and bioreactors, showing the potential use of MSCs in bone regeneration (Sikavitsas et al., 2002, Stiehler et al., 2009, Hwang et al., 2008, Hwang et al., 2009, Randle et al., 2007b, Diederichs et al., 2009, Jung et al., 2012a). All these studies had in common the use of encapsulated cells (or cells seeded into a scaffold), a semi-batch feeding strategy and shear stress modulation, showing successful approaches for osteogenic differentiation of MSCs. Nonetheless, these efforts have been insufficient and the production of cellular constructs with heterogeneous populations of cells is a trademark of these bioprocesses. The *in vivo* microenvironment is a rich in complexity and dynamic setting, in which transport of nutrients and physical stimuli happen naturally and optimally. In 2D/3D static culture, this does not happen and limited nutrients and oxygen transport from culture medium to the innermost layer of the scaffolds, along with lack of proper physical cues represent the standards. The histological pictures provided, can support these idea, as density of cells in the core of the beads is low, and as we get closer to the surface, it reaches its highest (see Figure 53). These limitations hinder the cell culture outcome and produce this heterogeneity.
Figure 67 SEM pictures of cultured scaffolds in differentiation medium after 28 days showing characteristic bone features: Pictures A to C Display cell constructs produced by 3D static culture. Pictures D to F display cell constructs produced by HARV bioprocessing. Pictures G to L display pictures of cell constructs produced by perfusion-RWV bioprocessing.

To overcome these strains, medium perfusion has been introduced to the design of culture vessels. This new layer of bioreactor design, provides the culture systems with dynamic exchange of nutrients and oxygen, together with a new source of controlled shear stress and has been developed in an attempt to close the gap between the in vitro platforms and the in vivo natural environment. Therefore, we developed a culture system to create three-dimensional bone-like tissues based on hDPSCs, alginate/gelatin bead, and perfusion-RWV bioreactor. To the best of our knowledge, this is the first study demonstrating the three dimensional bone-like tissue formation from hDPSCs in
alginate/gelatin scaffolds, grown suspended in a perfusion bioreactor system. Thus, could have widespread applications in bone tissue engineering.

In this study, hDPSCs were homogenously encapsulated within 1.1% alginate mixed with 0.1% gelatin hydrogels, afterwards the encapsulated hDPSCs were placed to culture in suspension environment within vessels of perfusion-RWV bioreactor, whereas the bioreactor maintains controlled in vitro culture conditions that permit tissue growth and development.

This in-house designed perfusion-RWV bioreactor produce a suspension culture by providing laminar flow of culture media and rotation of the vessel. Hence minimising cell exposure to shear and turbulence which can be detrimental for cell growth and differentiation, while providing sufficient nutrients and oxygenation to support 3D neo-tissue growth and removing the undesirable cellular by-products. In our study, a protocol for cell expansion previously defined by our group for mESC was used to expand undifferentiated cells in 3D environment. However, the need to design a specific expansion protocol for hDPSCs, considering seeding density, culture time and a thorough follow up of undifferentiated state, still remains to be done. The perfusion bioprocess demonstrated to be good for the expansion of hDPSCs, providing with ≈13.5 times fold expansion after 28 days of culture. A significant decrease in CD105 (endoglin) marker after 6 days of culture, giving rise to 2 subpopulations of hDPSCs, one CD15+ and another CD105-. This heterogeneity was believed to be due to different stages of multi-lineage differentiation (Jin et al., 2009). However, more resent studies have found that CD105+ and CD105- MSCs represent independent subpopulations that maintain their properties upon several passages. These subpopulations have similar growth potential and express almost identical MSC markers, but their differentiation capabilities may differ. Interestingly, CD105- MSCs were found to be more prone to differentiate into osteoblastic and adipocytic lineages and suppressed the proliferation of CD4+ T cells (less immune response) more efficiently compared to the CD105+ subpopulations (Anderson et al., 2013). If we consider ALPase activity as a measurement of differentiation, our results suggest that through the long term culture, there was no differentiation and it could be thought to be in front of a multipotent CD105- population. A redefinition of the MSCs characteristic phenotype is due to happen and this CD105-
could represent an interesting source of cells for bone tissue engineering and adipose tissue engineering, particularly for hDPSCs bioprocessing, as they have been proven to have troubles with the adipogenic lineage differentiation (as discussed in chapter 4). However, more research is required to establish the capabilities of CD105- MSCs. In the mean time, more than 1 week of expansion prior to the differentiation process should not be recommended, as it is known with certainty that 1 week of culture can produce hDPSCs with multipotent features ready for 3D differentiation and with high cell viability.

Morphological assay showed increased cell aggregation size in accordance with culture time and finally well developed cells within alginate beads.

The bioprofile measurements of the expansion media, demonstrated cells growing and that the stress produced by nutrients exhaustion and metabolites accumulation was limited with perfusion of pre-oxygenated fresh media. With a positive influence in proliferation of hDPSCs.

In the comparison study between the differentiation capability of perfusion-RWV bioreactor, HARV bioreactor and 3D static culture of hDPSCs within alginate beads, the perfusion-RWV bioreactor demonstrated much higher cell proliferation and viability. The differences found between the systems, may have their roots in the mass transfer characteristics of every system as well as the mechanical stimulation caused by hydrostatic pressure and shear stress. In case of the static culture, the comparatively lower cell viability might be produced by mass transfer limitations. Including external (culture medium) and internal (hydrogel) mass transfer restriction with regard to the supply of oxygen and nutrients. If the rate of nutrient consumption from the immediate vicinity of the cell-construct is faster than the replacement rate of those nutrients, then a nutrient concentration gradient is generated on the scaffold's surface and the seeded cells may experience malnutrition or decreased concentrations of growth factors vital for their long-term growth and differentiation. In a static culture the only transport mechanism of nutrients is molecular diffusion, which cannot achieve the metabolic requirement of hDPSCs, while in both bioreactors, the low physiological fluid shear conditions enhance nutrient transport, thereby decreasing the existing nutrient
concentration gradients (Sikavitsas et al., 2002, Mueller et al., 1999). In case of the HARV bioreactor, significant differences with static culture were not found, the low shear condition alone did not produce the physiologically relevant microenvironment for cells to grow, hence perfusion could deliver key mechanical stimuli for the cells to expand properly and differentiate properly.

Cell growth and expression of the osteoblastic phenotype in 2D static culture has three distinct phases: 1) High proliferation and development of extracellular collagenous matrix (10 to 12 days). 2) Matrix maturation, down-regulation of proliferation and up-regulation of ALPase activity (12<sup>th</sup> to 18<sup>th</sup> day). 3) High mineralisation and further decrease of proliferation, declination of ALPase activity and the induction of osteocalcin expression (16<sup>th</sup> to 20<sup>th</sup> day). Similar behaviour can be seen in the proliferation of cells in the three platforms being assessed with a time delay, this could be associated with the 3D environment. The decreased cellularity observed at the end of the experiment on the hydrogels cultured in the three bioprocesses, is in accordance with the apoptotic behaviour observed of mature osteoblasts residing in mineralised nodules (Sikavitsas et al., 2002). Although the live/dead assay showed that by day 28, the scaffolds were fully populated of living cells, which suggests that the down expression of the MTS assay could be result of the mentioned decrease in proliferation and enzymatic activity.

The morphological analyses by light microscopy showed how ECM formation and mineral deposition happen through the culture. At the beginning of the culture, beads where transparent and cells were visible with light microscopy. However, through the culture, for the three bioprocesses, it was seen how ECM formation and mineralisation started blocking the light until light transmission through the beads was not possible.

The bone neo-tissue formed by cell growth within alginate/ gelatin hydrogels consisted of viable and metabolically active cells, which were evaluated by measuring pH, glucose, glutamine, lactate, ammonia and oxygen kinetics in osteogenic medium during cultures. It has been reported that the profile of pH and these metabolites can be used to modulate cell metabolism, for instance ECM deposition and neo-tissue progress for developing optimal culture condition for SCs culture (Obradovic et al., 1999). Animal cells utilize glucose and glutamine as vital energy sources. Through glycolysis, 1 molecule
of glucose is metabolised and produces 2 molecules of lactate as a by-product under anaerobic condition, yielding 2 mole of adenosine triphosphate (ATP) per mole of glucose, or CO$_2$ and H$_2$O through the TCA cycle in aerobic condition, yielding 36 mole of ATP per mole of glucose. Through the TCA cycle, 1 molecule of glutamine is lysed into glutamate and produces 1 molecule of ammonia as by-product. Hence, the amount of ammonia can be used as an indicator of glutamine utilization and TCA cycle activity. In our study of 3D osteogenesis. Glucose consumption kinetics showed both bioreactors had similar profiles, a slow but steady decrease through the culture, implying the adequate feeding strategy to produce metabolically active cells in sustained growth. However, the 3D static culture showed a rapid decrease in glucose concentration of the medium to below measurable level, this might be due to a low initial concentration of glucose in the medium. A high-glucose minimum essential medium (mem), could replace the usage of the low-glucose mem utilised in the formulation of the osteogenic medium. This change could represent a more adequate substrate for this bioprocessing. However, measurements should be taken if this strategy was to be taken, as higher glucose would produce more lactate. In lactate kinetics, both bioreactors produced below measurable levels, this reflects the slow but steady glucose consumption seen. 3D static culture accumulated lactate fast, reaching a maximum of 12 [mmol/L], this was the limiting concentration achievable, as all glucose was consumed. A high glucose medium could help cell proliferation, but as well would increase the concentration of lactate. As concentrations of lactate above 16 [mmol/L] can be harmful for the cells (Ouyang et al., 2007), lactate kinetics should be followed carefully. Glutamine showed a similar profile for the three bioprocesses, going down for the first 21 days and then up again, ammonia had a behaviour that mirrors glutamine consumption. We believe the decrease in the consumption of glutamine reflects the amount of cells sampled rather than the cells consumption. Ammonia did not reach toxic levels through the whole culture for the three bioprocesses, with values between 1 and 2 [mmol/L] (less than 4 [mmol/L] (Chaudhry et al., 2009)). Additionally, pH and oxygen concentration are other parameters to consider as these affect cell viability, growth, differentiation and metabolic activity. pH in osteogenic medium decreased with culture time and followed the increase of lactate and ammonia concentration, as it is known that these metabolites affect medium pH (Barngrover et al., 1985). pH change was within the
proper pH range (7.0-7.4) of cell culture media, implying no strong influence on cell growth and function. Oxygen has a major role in SCs growth and differentiation, hypoxic conditions favouring undifferentiated expansion and normoxic conditions, differentiation. For the three bioprocesses, oxygen fluctuated between normoxic values through the whole culture.

For validation of the 3D osteogenic differentiation achieved by the three bioprocesses, the cell constructs during differentiation were characterised by histological, immuno-staining and SEM microscopy. Histological H&E staining of sectioned hydrogels at day 0, showed most cells appear scattered in the bead without having form extracellular matrix. After, displayed well distributed tissue growth for both bioreactors and heterogeneity for the static culture. Morphogenesis of the osteogenic cells until achieving maturation, can be seen in both the perfusion and the static culture by the emergence of osteocyte lacunae. This is not so clear in the HARV bioreactor culture. However, the presence of vacant lacunae in static culture, demonstrates that cells are leaving the scaffold. Prove of this, is seen in Figure 54, where approximately confluent cells growing attached to the flask can be found. A probable reason for the cells to leave the beads, would be poor diffusive transport of oxygen and nutrients from the media to the inner area of the hydrogels, causing an impediment for growth and differentiation and as a consequence, forcing cells from the inner area of the scaffold to migrate to the surface, and further to the flask surface (Sikavitsas et al., 2002). This could be another probable reason behind the low MTS and ALPase activity achieved at the end of the static culture. ARS staining indicated a progressive differentiation through time, a gradual enhancement in mineral deposition for the cell construct produced by the three bioprocesses was seen. The ARS quantitative analysis is consistent with the qualitative results and both bioreactors show better performance than the static culture, von Kossa confirmed the mineralisation by showing disperse deposits of calcium through the hydrogels for the three bioprocesses. Immunostaining for Osteocalcin was positive for the three bioprocesses, but perfusion culture had clearly higher expression. The surface of the cell constructs was analysed with SEM microscopy, here the morphogenesis of the hDPSCs can be perceived in different levels for the three bioprocesses, the perfusion-RWV bioreactor showed higher neo-tissue maturation, seen by the many
hydroxyapatite crystals found, the osteocyte lacunae and the presence of osteocytes with developed canaliculi. Static culture showed more maturation than the HARV bioreactor. However most of the quantitative analysis suggests that this maturation is only in the surface of the construct, whereas the HARV bioreactor produces more homogeneous constructs.

ATR-FTIR of human bone shows carbonate bands at wavenumbers 870, 1415 and 1470 [cm⁻¹], and phosphate bands at wavenumber 565, 605 and 1035 [cm⁻¹]. Carbonate bands are distinctive of the bone organic component (mainly collagen) and phosphate bands of the inorganic fraction (hydroxyapatite), and the ratio of organic and inorganic component, C/P is characteristic biogenic signature for human bone. However, it is recommended to use the phosphate peak of 1035 and carbonate peak of 1415 to calculate C/P ratio. When these wavenumbers are used, the human bone C/P ratio is 0.23 (Beasley et al., 2014). When differentiation starts, the C/P ratio is high, and it decreases with differentiation and hydroxyapatite formation. Static culture produced a high C/P value, showing poor differentiation. Both bioreactors scored very well, with HARV culture achieving ≈0.28 and perfusion culture achieving ≈0.23, the exact value described for human bone in literature. ATR-FTIR imaging confirms these results, with yellow being positive and blue negative.

X-ray microanalysis (SEM/EDS) of hydroxyapatite shows the elemental composition of the analysed materials, it has been seen that the Ca/P ratio represents a characteristic biogenic signature of the inorganic fraction of bone (hydroxyapatite). Pure hydroxyapatite has a Ca/P ratio of 1.67 (Medina Ledo et al., 2008). In our experiments it can be seen how values increase with time. All the samples start in a virtually null value, after 28 days of culture, perfusion reaches 1.23, static culture 1.14 and HARV 0.77. Although, all bioprocesses produced calcium deficient hydroxyapatite (non-stoichiometric), the increase is clear and more research should be done until the proper ratio is reached.

3D osteogenesis was characterised at a genetic level, examining the expression of Runx2/CBFA1, osteocalcin (BGLAP), alkaline phosphatase (ALPL), collagen type I (COL1A1) and sclerostin (SOST), these genes are representative of the entire MSCs to
osteocyte ontogenesis. Osteogenic ontogenesis requires the precise and orchestrated activity of several genes and signals (extracellular and intracellular). Runx2 is a transcription factor and the central control of osteogenic differentiation. Its upregulation happens early in the ontogenesis, and is believed to have a constant expression through the whole differentiation (Shui et al., 2003). ALPL is a ubiquitous cellular protein, and its function is ill-defined. However, it is known that its action starts as early as 2 days after the beginning of the osteogenic drive. Its expression is increased steadily through the differentiation. Collagen I (Col1a1), is an important component in the bone ECM, with a role in cell adhesion, proliferation and osteoblast phenotype, its upregulation is characteristic of osteogenic differentiation and can be considered an early marker of differentiation. Osteocalcin (BGLAP), is an osteoblast specific gene and one of the most abundant proteins in bone, its expression is significantly upregulated in matrix synthesis and mineralisation (Ryoo et al., 1997). Sclerostin (SOST) is a known specific gene for mature osteocytes, and it is integral to osteocyte function as a signal to damp the action of osteoblast bone deposition and to control bone metabolism (Compton and Lee, 2014).

MSCs, preosteoblasts and osteoblasts express Runx2 together with secreting osteoid, when osteoblasts mature, they express alkaline phosphatase. When mature osteoblasts become embedded in osteoid, they express osteocalcin and start producing dendritic projections characteristic of osteocytes. The only known marker unique for osteocytes is sclerostin (Compton and Lee, 2014). Perfusion culture showed low and constant expression of RUNX2 along with high and almost constant expression of osteocalcin. The expression of these two genes, could signify most of the cells already went through the transition from immature to mature osteoblast. The high increase of SOST in the last week of culture could represent the further transition from mature osteoblasts to osteocytes. During all this period, Col1a1 and ALPL were active and positive. These genes are related with ECM formation and mineralisation, and are indicative of a well developed in vitro osteogenesis. These results suggest that a homogeneous population of cells was produced with cells in the last stages of maturation. HARV culture showed low enhancement in expression of RUNX2 along with high increase in expression of osteocalcin. The expression of these two genes, could signify cells are going through the
transition of immature cells to mature osteoblast. The increase of SOST in the last week of culture could represent the cells transitioning from mature osteoblasts to osteocytes. During all this period, Col1a1 and ALPL are active and positive. These genes are related with ECM formation and mineralisation, and are indicative of a well developed *in vitro* osteogenesis. These results suggest that a heterogeneous population of cells was produced with cells undergoing maturation to osteoblasts and osteocytes. 3D static culture showed high increase in expression of RUNX2 along with a relatively constant expression of osteocalcin. The expression of these two genes, could signify the presence of significant amount of immature cells. The high increase of SOST in the last week of culture could represent that most of the cells that matured into osteoblasts have further differentiated to osteocytes. During all this period, col1a1 and ALPL are active and positive. These genes are related with ECM formation and mineralisation, and are indicative of a well developed *in vitro* osteogenesis. These results suggest that, a highly heterogeneous population of cells was produced with cells in the early, middle and final stages of maturation.

Perfusion bioreactor showed a higher proliferation, calcification and a more homogeneous bone tissue, the HARV bioreactor showed a lower differentiation in comparison with the perfusion system and the static culture showed good differentiation in the surface of the constructs, but poor in the inner part of the hydrogels, additionally, cells left the construct to start growing on the flask surface.

Low proliferation of cells in HARV culture (compared with perfusion culture) could have damped the differentiation, the morphogenetic analysis validates this idea, other authors have argued this could happen as a consequence of the collision of the beads with the wall of the reactor vessel, traumatising the cells in the surface of the scaffold and retarding differentiation (Sikavitsas et al., 2002). However, this is not our case, as cells are grown in a free fall state. Static culture produces a highly heterogeneous cell construct, with limited mineralisation compared with the other bioprocesses, it is believed that mass transfer impediments produce poor nutrition of the cells and finally hinders the quality of the cell construct.
The enhanced proliferation, AP activity, OC secretion, and calcium deposition observed in the perfusion culture indicates a positive influence of the continuous feeding strategy, and potential mitigation of external mass transport limitations on the growth and differentiation of MSCs towards the osteoblastic phenotype.

5.6 Conclusions
This study demonstrated that the bioprocessing providing perfusion flow of medium in a suspended environment is successful in the mitigation of nutrient and oxygen transport limitations, external to three-dimensional cell/alginate constructs and is more efficient that the HARV bioreactor and the 3D static culture, positively influencing the proliferation, differentiation, mineralisation and expression of osteoblastic markers of hDPSCs cultured in the presence of osteogenic media, ultimately enabling the formation of 3D bone-like constructs for clinical purposes. Progressive differentiation was confirmed for the three different bioprocesses by the gene expression pattern, the increase and further decrease in ALPase activity, the gradual decrease of the C/P ratio measured by ATR-FTIR, the increase in the Ca/P ratio, measured by SEM/EDS and the ARS positive area, measured by software analysis; and was qualitatively validated by immunostaining, H&E and von Kossa staining, SEM pictures and ATR-FTIR. The perfusion system achieved better results in most of the analysis, producing a homogeneous cell construct with regenerative medicine potential. On contrary, static culture produced a highly heterogeneous cell construct and due to mass transfer limitations, proportioning an unfavourable environment for cells to grow and differentiate. The HARV demonstrated slower proliferation than the perfusion culture, which may have delayed or dampened the normal differentiation pattern of osteoblastic cells.

The perfusion culture system described here would provide an efficient and easy culture system for applications in bone tissue engineering in the context of macroscopic bone formation.
6 Enhancing the three-dimensional perfusion bioprocessing for osteogenic differentiation: Bone morphogenetic protein-2 and Simvastatin
6.1 Introduction

The scaled-up, *ex vivo* production of three-dimensional bone-like constructs, to be utilised in clinical settings, to augment bone repair and regeneration in patients, is on demand. The tissue engineering (TE) approach, provides the toolset to tackle with the production of these bone mimics. By employing autologous bone-forming cells, three-dimensional osteoconductive scaffold materials and bioreactor vessels, has been extensively working in producing the homogeneous, reproducible and functional artefact, required to fulfil this clinical need (Gaspar et al., 2012, Godara et al., 2008, Hidalgo-Bastida et al., 2012, Qiu et al., 1999, Zhang et al., 2009, Grayson et al., 2011, Yeatts and Fisher, 2011, Salter et al., 2012). Cells provide the machinery to produce bone-tissue, being able to produce ECM/ osteoid and mineralise its surrounding. However, they require a 3D environment to produce the adequate histoarchitecture. Scaffold provide the 3D environment for the cells to attach, spread and transform into bone-tissue, and in due process being resorbed and replaced by ECM and calcifications for further produce a full neo-tissue. Nonetheless, scaffolds provide an impeded mass transfer for cell nutrition. Bioreactors provide the means to deliver/ fulfil nutritional requirements of the cells, by enhancing transport properties inside and outside the scaffold, while removing the cellular waste. A variety of dynamic 3D bioreactor concepts mimicking the native microenvironment in bone tissue, for example, spinner flasks, rotating wall vessel constructs, perfusion bioreactors, and systems based on mechanical or electromagnetic stimulation of cell/scaffold composites, have been developed (Rauh et al., 2011). These novel setting, in different levels being successful in reproducing specific aspects of the *in vivo* milieu, and producing mineralised constructs, with positive expression of osteogenic genes and proteins, whilst partially mimicking bone microstructure. Additionally, bioreactors allow automation and make up for limitations of the conventional cell handling manual and static technique (Wendt et al., 2009). However, these setup alone produces heterogeneous constructs, with mixture of mature and immature cells and distinguishable different microscopic neo-tissue anatomy (Gaspar et al., 2012, Godara et al., 2008, Hidalgo-Bastida et al., 2012, Qiu et al., 1999, Zhang et al., 2009, Grayson et al., 2011, Yeatts and Fisher, 2011, Salter et al., 2012, Park et al., 2008).
The engineering parameters occurring and controlled in bioreactors are equally important as the biological parameters associated with differentiation. Hence, the adequate cues/ signalling for bone differentiation drive are required to be studied together with the 3D bioprocessing. The bone morphogenetic proteins (BMPs) signalling pathway has widely been recognized as the main player in bone developmental processes, and exhibit versatile and efficacious regulatory functions in the body. The disruption of them has been connected to a number of bone diseases (Chen et al., 2012), They Induce and are essential for cartilage, tooth and bone formation, crystallization and maturation.

BMPs have major relevance in the differentiation of SCs into osteogenic lineage, causing the induction of the Runx2 gene expression in early stage osteogenesis (preosteoblasts) and subsequently the expression of Osterix (OSX) in late state osteogenesis (osteoblasts) (Wozney, 2002, Chen et al., 2004, Bei and Maas, 1998).

The BMPs family is a secreted cytokine/ growth factor subfamily of the transforming growth factor-β (TGF-β) superfamily. They are multifunctional cytokines regulators of development, proliferation, differentiation, adhesion and apoptosis in different cell types at embryonic and adult ages (Kirkbride et al., 2008, Miyazono, 2000).

BMP2 has been approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for the use in certain types of bone fracture, making this growth factor really important in the regenerative medicine field (Ghodadra and Singh, 2008, Starman et al., 2012).

BMP signal transduction produces a cascade of genes that will lead to osteogenesis. It starts inducing Runt-related transcription factor 2 (Runx2, CBFA1), followed by collagen I (Col1), osteopontin (OPN or Spp1), osterix (OSX) in the preosteoblast stage (Komori, 2010, Matsubara et al., 2008). Further, SP7 and Wnt signalling direct the preosteoblast to a mature stage (Komori, 2010). Mature osteoblasts keep expressing Col1 and start expressing osteocalcin (Bglap), Runx2 is kept constant.

Committed cells express different bone matrix protein genes, depending on the stage of the cell development. Immature preosteoblasts weakly express collagen I (Col1) and in
a higher level osteopontin (OPN or Spp1) and osterix (OSX), forming unpacked woven bone (Komori, 2010, Matsubara et al., 2008). Mature osteoblasts strongly express Col1 and protein 1/ osteocalcin (Bglap), forming densely packed lamellar bone. When osteoblasts become osteocytes, will express Dmp1, sclerostin and podoplanin (E11).

Despite the fact that BMPs have been widely researched and recognised as key factors for osteogenic differentiation. The supra-physiological doses used in cell therapy, are known to produce ectopic bone formation, increase risk of cancer and evidence suggests that BMP2s not only decide cancer cell fate, but they stimulate angiogenesis in existing tumours. Langenfeld and colleagues demonstrated in vitro, how using Noggin, a BMPs antagonist, inhibited proliferation of cancer cells (Carragee et al., 2013b, Langenfeld and Langenfeld, 2004). The harmful effect produced by BMPs may be dose dependant (DeVine et al., 2012). Hence, BMPs based cell therapeutics, are required to utilise minimal dose possible. In addition to the side effects, the use and costs of rhBMPs are also to be considered. BMPs are delicate to handle, expensive to manufacture and supra-physiological doses are used, resulting in high costs. The physiological level of BMP2 in humans is of ≈300 [pg/ml] (Tosovsky et al., 2014). Nonetheless, in standard conditions of culture and in clinical applications, the concentrations utilised, range from 50 to 1000 times fold. The impact of pioneer bioprocesses in the efficacy of BMP2 physiological concentration, reminds to be assessed and is a key factor to reduce the risks and costs of applications employing BMPs.

Alternatively, osteogenic differentiation pharmacologic enhancers able to enhance the action of the naturally occurring cellular BMPs, could circumvent these difficulties.

A less direct mechanism, is followed by statins. Statins are inhibitors of cholesterol synthesis. However, they have been demonstrated to have cholesterol-independent anabolic effects on bone metabolism and fracture healing (Pagkalos et al., 2010b). By interacting with both canonical and non canonical BMP pathways, they upregulate BMP2 expression, along with the cascade of genes associated with BMP2 (Chen et al., 2010). Several inexpensive statins are available with different molecular and pharmacokinetic properties. Simvastatin, is more lipophilic than pravastatin and has higher affinity to phospholipid membranes than atorvastatin,
allowing its passive diffusion through the cell membrane (Pagkalos et al., 2010b). Hence Simvastatin represent an interesting alternative to direct BMP stimuli of SCs.

6.2 Objectives
In previous chapters, hDPSCs were shown to be multipotent highly proliferative stem cells with potential for bone tissue engineering and regenerative medicine. Further, the establishment of the efficient and reproducible long-term three-dimensional bioprocess in a perfusion-RWV bioreactor for directing the osteogenic differentiation of hDPSCs was developed. The expansion and osteogenic differentiation of hDPSCs seeded in alginate/gelatine scaffolds in a suspended environment and with media perfusion was studied and compared with commonly used bioprocesses, demonstrating a better performance. However, the cell construct produced by this bioprocess can be additionally improved by the use of osteoinductive supplements as bone morphogenetic proteins (BMP). BMPs are expensive and are required in high concentrations to have an osteogenic effect in common culture systems, in the order of 100-1000 times higher than physiological level of human plasma (≈300 [pg/ml]) (Tosovsky et al., 2014). In this study, the effect of supplementing with BMP2 at physiological level the designed bioprocessing, was assessed and compared with a commonly used osteogenic supplement, Simvastatin.

The aims of this chapter are: 1) to pre-assess the effect of BMP2 at a physiological level and compare with simvastatin as osteo-inducers in the perfusion three-dimensional bioprocess previously designed. 2) to establish the efficient long-term 3D bioprocess in a perfusion-RWV bioreactor for directing the osteogenic differentiation of hDPSCs supplemented with minimal concentrations of BMP2, and compare it with the effect of simvastatin.

The pilot study of the supplements in growth medium allowed to determine if these supplements had osteogenic effect under 3D conditions, then the two supplements were used in long term culture to assess the differentiation in full osteogenic medium to develop an enhanced bioprocess for osteogenic differentiation.
6.3 Experimental Process

Previously we demonstrated that hDPSCs long-term cultured in 3D scaffolds in a suspended environment with perfusion flow of culture medium could produce high quality bone constructs for therapeutic purposes. For the pilot study of the osteogenic supplements, donor samples of hDPSCs were expanded undifferentiated until reaching the desired number by passage 4, these cells were encapsulated in an alginate/ gelatin scaffold with a density of 40,000 cells/bead. 250 beads were seeded and cultured in the perfusion-RWV bioreactor with growth medium supplemented with either 300 [pg/ml] of BMP2 (experimental group BMP2-1), or 10 uM of simvastatin (experimental group SIMV-1) for 14 days. Perfusion was set as 1 vessel volume per day (55 ml/day medium). Osteogenic differentiation was assessed using various techniques such as, proliferation measurement by Calcein AM/ ethidium homodimer, measurement of ATP content and MTS assay, characterisation of mineralised bone nodules by alizarin red and von Kossa, H&E, alkaline phosphatase expression, immunocytochemical characterisation, scanning electron microscopy with X-ray microanalysis, ATR-FTIR. The detailed explanation of this techniques can be found in Chapter 3: Materials and methods.

For the differentiation in full media, donor samples of hDPSCs were expanded undifferentiated until reaching the desired number by passage 4, these cells were encapsulated in an alginate/ gelatin scaffold with a density of 20,000 cells/bead. 250 beads were seeded and expanded in 175 T-flasks with expansion medium for 7 days, allowing cells to acclimatise to the new three dimensional environment. Following the process, beads were transferred to perfusion-RWV bioreactor and for 21 days osteogenic medium containing dexamethasone, β-GP, ascorbic acid and KH$_2$PO$_4$, and either 300 [pg/ml] of BMP2 (experimental group BMP2-2), or 10 uM of simvastatin (experimental group SIMV-2) was used to drive undifferentiated hDPSCs to osteogenic lineage. Medium perfusion was set to 1 vessel volume per day (55 ml). Thus osteogenic differentiation was assessed using various techniques such as, proliferation measurement by Calcein AM/ ethidium homodimer, measurement of ATP content and MTS assay, characterisation of mineralised bone nodules by alizarin red and von Kossa, H&E, alkaline phosphatase expression, immunocytochemical characterisation, RT-PCR, scanning electron microscopy with X-ray microanalysis, ATR-FTIR and ATR-FTIR imaging.
The detailed explanation of this techniques can be found in Chapter III. The overall experimental process is illustrated in Figure 39.

Figure 68 Experimental process for the enhancement of perfusion bioprocess for 3D osteogenic differentiation.

6.4 Results

6.4.1 Pilot study of osteogenic supplements.
For the pilot study of the supplements, no acclimatising of the cells to the 3D environment was performed and the cells started the differentiation straight after being encapsulated. Two groups were studied, BMP2-1 in which cells were grown in expansion medium supplemented with 300 pg/ml of BMP2 for 14 days and SIMV-1, in which cells were grown in expansion media supplemented with 10 uM of simvastatin for 14 days. The osteogenic capability of the supplements was established by measuring proliferation, mineralisation through different techniques, and qualitatively by SEM microscopy, histology and immunostaining.

The growth kinetic were measured by the ATP content and MTS activity of beads. The ATP content for BMP2-1 increased 1.079 times fold by day 14 and for SIMV-1, 1.175 times, MTS followed a similar pattern increasing for BMP-2 1.47 times fold by day 14,
and for SIMV-1 2.18 times. Charts that show these results can be seen in Figure 69A and can be seen in Figure 69B for ATP content and MTS activity respectively.

Alkaline phosphatase activity (ALPase) can be seen in Figure 69C, was positive for both samples, with SIMV-1 showing a higher expression. However, the difference was not significantly different.

Figure 69 Measurement of proliferation and Alkaline Phosphatase activity of hDPSCs in growth media supplemented with BMP2 or simvastatin measured by CCK-8, Celltitter-Glo 3D and ALPase colorimetric assay respectively: A) Celltiter-Glo 3D, B) CCK-8 and C) ALPase activity (n=3). * indicates statistical significance when comparing between day 0 and 14 for proliferation and between treatments for ALPase activity.

Cells were shown to be alive, healthy and well distributed by the end of the 14 days of culture by Live/ dead assay, staining live cells with Calcein AM (green) and dead cells with ethidium homodimer (red). Live/ dead assay shows that cells are very alive, well
distributed and healthy, it is hard to find dead cells in the samples, these results can be seen in Figure 70.

Figure 70 Live dead assay A) BMP-2 at day 14 of differentiation, B) SIMV-1 at day 14 of differentiation. Scale bar= 200 µm.

6.4.1.1 **Histological analysis, immunostaining and SEM microscopy of hDPSCs 3D osteogenic differentiation, pilot study.**

We addressed whether the hDPSCs were capable of forming mineralised bone nodule in 3D culture systems using alginate beads and growth media supplemented with BMP2 at physiological level or simvastatin. In order to confirm whether these tissues were mineralised bone tissue, histological analysis and immunostaining was done to characterize the morphology and calcium deposits in tissues.

The resulting hydrogels from from groups BMP2-1 and SIMV-1 were cut in slices and histologically examined by hematoxylin and eosin staining (H&E). As shown in Figure 71, samples displayed a heterogeneous morphology. However, the two groups of osteogenic constructs showed bone neo-tissue formation over the time period. At day 1, most cells appear scattered and spread homogenously through the beads (Figure 71H). After 14 days of culture in supplemented medium, it can be seen that cells expanded and populated the whole hydrogels and differentiation started (Figure 71). Size and shape of cells, together with their surroundings changed, ECM was formed and approximately-round lacunae started showing around single cells, which is signal of cells differentiation into osteogenic lineage. SIMV-1 showed less differentiation than BMP2-1. By day 14 of culture, less lacunae formation was observed and mostly in the surface
area, suggesting a lower degree of maturation of the cells. Interestingly, the BMP2-1 group showed the appearance of lacunae in the inner area of the beads, meanwhile the perfusion group with osteogenic media from the previous chapter by day 14 of differentiation had lacunae allocated from the middle to the periphery of the beads (Figure 71G).

Calcified deposition started spreading in beads produced by both experimental groups and was shown by alizarin red s staining (ARS) and by a soft von Kossa staining. ARS results displayed an increase of the nodules in time. Both treatments displayed similar deposition. Negative controls are undifferentiated hDPSCs grown in alginate beads and a known to be negative tissue. Figure 72 shows ARS results for both treatments and Figure 73, shows von Kossa results for both treatments.

Von kossa staining was done for day 14 of differentiation and albeit the results do not show mass deposition (black colour), the beads produced by both treatment, especially BMP2-1, produced dispersed depositions of calcium (grey colour), compared with the negative sample that produced a pink stain without grey of black.
Figure 71 H&E images displaying the morphology of alginate beads: A, B and C) are pictures of group BMP2-1 at day 14 of culture. D, E and F) are pictures of group SIMV-1 at day 14 of culture, G) cells grown in osteogenic media by day 14 and H) undifferentiated day 3 cells. Scale bar=200 µm.

Figure 72 ARS staining images showing the mineral deposition on alginate beads: A, B and C) are pictures of group BMP2-1 at day 14 of culture. D, E and F) are pictures of group SIMV-1 at day 14 of culture, G) cells grown in osteogenic media by day 14, H) undifferentiated day 1 cells and I) control. Scale bar=200 µm.
Figure 73 Von Kossa staining images calcium deposition on alginate beads: A, B and C) are pictures of group BMP2-1 at day 14 of culture. D, E and F) are pictures of group SIMV-1 at day 14 of culture, G) cells grown in osteogenic media by day 28, H) undifferentiated day 1 cells and I) control. Scale bar=200 µm.

For further confirmation of osteogenic neo-tissue formation, the hydrogels were stained with anti-osteocalcin, anti-osteopontin, anti-sclerostin and anti-podoplanin antibodies (Figure 74 and Figure 75). The two experimental groups by day 14 of culture under differentiation medium, produced tissue positive for osteopontin, osteocalcin, podoplanin and sclerostin, with the BMP2-1 group showing higher intensity for osteocalcin and podoplanin, but showing weak expression of osteopontin. SIMV-1 showed a comparatively weaker expression of osteocalcin and podoplanin, but showing higher osteopontin. The intensity of sclerostin expression was similar in both groups.
Figure 74 Fluorochrome labelling of bone constructs for Osteocalcin (green) and Osteopontin (red): A, B and C) are pictures of group BMP2-1 at day 14 of culture. D, E and F) are pictures of group SIMV-1 at day 14 of culture. Scale bar=200 µm.

Figure 75 Fluorochrome labelling of bone constructs for Sclerostin (green) and Podoplanin (red): A and B) are pictures of group BMP2-1 at day 14 of culture. C and D) are pictures of group SIMV-1 at day 14 of culture. Scale bar=200 µm.

When observed by SEM, many mineralised deposit were detected within the alginate hydrogels of both experimental groups after osteogenic culture. Additionally, the lacunae seen in the histology are seen in these pictures, along with the morphogenesis of the osteoblastic cells. Pictures showed the presence of non-stoichiometric mineralised deposits. By day 14 the lacunae were distributed in some areas of the scaffolds and in the pictures of both groups, osteocytes can be morphologically
recognised. The morphology of the bone constructs produced by group BMP2-1 and SIMV-1 by day 14 can be seen in Figure 76, where the maturation of the tissue can be seen. The morphology of the bone constructs produced by both experimental groups appears immature. In some pictures, it can be seen that cells in the surface of the beads resemble osteocytes and their surroundings osteocytes lacuna.

Figure 76 SEM pictures of cultured scaffolds in differentiation medium after 28 days showing characteristic bone features for experimental groups: A), B) and C) BMP2-1, D), E) and F) SIMV-1 by day 14 of culture.

6.4.2 Enhancing the three-dimensional perfusion bioprocessing for osteogenic differentiation: Bone morphogenetic protein 2 and Simvastatin
For the enhancement of the previously established perfusion bioprocess, expansion and acclimatising of the cells to the 3D environment was performed for 7 days in static culture placed in a shaker. By day 7, hydrogels were placed in the perfusion-RWV bioreactor. Following the pilot study, two new experimental groups were established and studied, BMP2-2 in which osteogenic differentiation of hDPSCs was performed by exposure to osteogenic medium (Dex, ASAP, β-GP) supplemented with 300 pg/ml of BMP2 for 21 more days and SIMV-2, in which osteogenic differentiation of hDPSCs was performed by exposure to osteogenic medium (Dex, ASAP, β-GP) supplemented with 10 uM of simvastatin for 21 more days. The osteogenic capability of the supplemented osteogenic media was established by measuring proliferation, gene expression,
mineralisation through different techniques, and qualitatively by SEM microscopy, histology and immunostaining.

Every hydrogels containing osteogenic cells after 28 days of culture showing changing colour from clear transparent becoming white, bone looking and appeared to be physically brittle in contrast to the initial alginate gel properties. As presented in Figure 77.

During the differentiation process, the morphology changes of the hydrogels were followed by light microscopy. By day 7 (expansion and acclimatising period), cells were likely to show single cell suspension well distributed through the hydrogel. The hydrogels did not show signal of mineralisation. By day 21 (day 14 of differentiation), mineralisation started conquering the hydrogels, and light almost could not travel across the hydrogels. By day 28 pictures show black spheres, as light did not cross the hydrogels. From the two experimental groups, mineralisation appears to be higher in the BMP2-2 group. Morphology of the beads can be seen in Figure 78.

The growth kinetics of hDPSCs seeded in beads (n=6) for both experimental groups followed a sigmoid growth profile with the appearance of distinct lag, exponential and stationary phases. They showed that by day 28, the experimental group SIMV-2 yielded higher cell numbers than group BMP2-2, with an increase of ≈ 6.3 times fold in ATP content, compared with 5.6 times achieved by the experimental group BMP2-2. MTS assay followed a similar trend, with SIMV-2 having 2.92 times fold increase and BMP2-2, a 2.61 increase. Albeit the difference between the results for the two groups was not significant for the ATP content, it was for the MTS assay. By day 21 of culture, cells reached a plateau in both groups and expansion was decelerated. Cells were shown to
be alive and healthy by the end of the culture by Live/ dead assay, staining live cells with Calcein AM (green) and dead cells with ethidium homodimer (red). These results can be seen in Figure 79 and Figure 80. Both proliferation assays used have limitations as the 3D environment deviates from the real cell number. The relationship between seeded cell number and luminescent/ absorbance output after several days in culture is often curved due to the effects of contact inhibition on cell proliferation, as well as reduced metabolic activity and/ or necrosis in the centre of scaffolds. The non-linearity provides a semi-quantitative/ qualitative approximation to cell number rather than a quantitative measurement.

Figure 78 Morphology of hydrogels observed under light microscopy of A) Day 7, B) day 28 BMP2-2 and C) day 28 SIMV-2 group. Scale bar=100 µm.

Figure 79 Measurement of proliferation for the enhanced osteogenic differentiation of hDPSCs, where A) CelltiterGlo 3D and B) CCK-8 assay results scored by the two experimental groups (n=6). * indicates statistical significance between groups for each time-point.
In order to evaluate indirectly the proliferative capability of hDPSCs in the enhanced 3D bioprocess, the metabolic activity of experimental groups BMP2-2 and SIMV-2 was evaluated by measuring for 28 days the time-course concentration of key substrates and metabolites in culture medium. Consumption of glutamine and glucose, production of ammonia and lactate, concentration of oxygen and detection of pH were acquired every 7 days with a bio-profiler. For both experimental groups, medium perfusion was set to the equivalent of 1 vessel volume a day (55 ml of medium) and samples were taken from the waste medium line, hence not disrupting the culture.

The glycolysis is a key metabolic pathway to produce energy for proliferative cells and as glucose is digested to produce ATP, it releases 2 molecules of Lactate. 20 [mmol/l] of lactate are limiting for the culture, above this level, lactate has a harmful effect. In the experimental group BMP2-2, glucose was consumed from $\approx 6.7$ [mmol/L] to $\approx 1.6$ [mmol/L] and approximately twice the number of molecules of lactate was produced. Lactate accumulation was low through the experiment, only achieving readable measurements by last week of culture, reaching $\approx 10.1$ [mmol/L]. Glucose and lactate kinetics for experimental group BMP2-2 can be seen in Figure 81A. In the experimental group SIMV-2, glucose was consumed from $\approx 6.7$ [mmol/L] to $\approx 1.7$ [mmol/L] and approximately twice the number of molecules of lactate was produced. Lactate accumulation was low through the experiment, only achieving readable measurements by last week of culture, reaching $\approx 10$ [mmol/L]. Glucose and lactate kinetics for experimental group SIMV-2 can be seen in Figure 81A.
The glutaminolysis is a key metabolic pathway to produce energy for proliferative cells and as glutamine is lysed into glutamate to produce ATP, it releases 1 molecule of ammonia, if ammonia concentration reaches 4[mmol/L], it can have a toxic effect for the cells. For BMP2-2 group, glutamine fluctuated from ≈4 [mmol/L] and ≈1 [mmol/L] and ammonia followed a similar trend, fluctuating between ≈0.9 [mmol/L] and ≈1.7 [mmol/L]. Glutamine and ammonia kinetics for the BMP2-2 group can be seen in Figure 81B. For SIMV-2 group, glutamine fluctuated from ≈4 [mmol/L] and ≈0.89 [mmol/L] and ammonia followed a similar trend, fluctuating between ≈0.9 [mmol/L] and ≈1.68 [mmol/L]. Glutamine and ammonia kinetics for group SIMV-2 can be seen in Figure 82B.

For BMP2-2, the oxygen tension in the medium slowly decreased, fluctuating between ≈160 [mmHg] and ≈107 [mmHg]. The oxygen kinetics for BMP2-2 can be seen in Figure 81C. For SIMV-2, the oxygen tension in the medium slowly decreased, fluctuating between ≈160 [mmHg] and ≈106 [mmHg]. The oxygen kinetics for SIMV-2 group can be seen in Figure 82C.

The pH in experimental group BMP2-2, gradually decreased from ≈7.6 to ≈7.1 as culture time passed. The pH kinetics for BMP2-2 group can be seen in Figure 81D. The pH in experimental group SIMV-2, gradually decreased from ≈7.6 to ≈7 as culture time passed. The pH kinetics for the HARV bioreactor can be seen in Figure 82D.

As seen in Figure 81 and Figure 82, both groups followed a very similar bioprofile.
Figure 81 Change with culture time of key substrates and metabolites in culture medium for the experimental group BMP2-2. A) Consumption of glucose and production of lactate. B) Consumption of glutamine and production of ammonia. C) pH and D) oxygen.

Figure 82 Change with culture time of key substrates and metabolites in culture medium for the experimental group SIMV-2. A) Consumption of glucose and

6.4.2.1 Histological analysis and immunostaining of hDPSCs 3D osteogenic bioprocesses
We addressed whether the hDPSCs were capable of forming mineralised bone nodule in 3D culture systems using alginate beads and supplemented differentiation medium. In order to confirm whether these tissues were mineralised bone tissue, histological analysis and immunostaining was done to characterize the morphology and calcium deposits in tissues.

The resulting hydrogels from two experimental groups were cut in slices and histologically examined by hematoxylin and eosin staining (H&E). As shown in Figure 83, samples from both experimental groups displayed a more homogeneous morphology compared to the un-supplemented samples (Figure 83F), however, the morphology looks less mature. Both groups showed gradual bone neo-tissue formation over the time-period. As time passed by, size and shape of cells, together with their surroundings changed, ECM was formed and approximately-round lacunae started showing around single cells, which is signal of cells differentiation into osteogenic lineage. These lacunae, when cells matured, became more elongated (osteocyte lacunae).

Our previous experiments demonstrated that un-supplemented samples differentiated from the surface to the inner area of the beads. However, both SIMV-2 and BMP2-2 showed lacunae starting to form from the core area to the surface, suggesting that differentiation started earlier in the centre of the beads. BMP2-2 resulted in homogeneous beads, totally populated by cells and their lacunae. By day 21, fair formation of round-shaped lacunae was seen, and increased by day 28 (see Figure 83). This suggests cell maturation. SIMV-2 group showed less differentiation than group BMP2-2. By day 21 less lacunae formation was observed, suggesting inferior maturation of the cells. Additionally, hydrogels appear fully and homogeneously populated with cells. Group BMP2-2 showed the production of mature morphology, however, most of the lacunae is circle-shaped and elongated lacunae was scarce.

Calcified deposition was widely spread in the osteogenic beads and was shown by alizarin red S staining (ARS) and von Kossa staining. ARS results displayed an increase of
the nodules in time. Through time nodules were growing in size and number for both experimental groups. Negative controls are undifferentiated hDPSCs grown in alginate beads and a known to be negative tissue. Further in this chapter, quantitative analysis of ARS can be found. Figure 84 shows the results for both experimental groups for day 14, 21 and 28 of culture.

Von kossa staining was done for day 28 and albeit the results do not show mass deposition (black colour), the beads produced by the two groups, produced visible dispersed depositions of calcium (grey colour), compared with the negative sample that produced a pink stain without grey of black. These results can be seen in Figure 85.

Figure 83 H&E images display the morphology of alginate beads: pictures A) and B) experimental group BMP2-2 at day 28, C) and D) experimental group SIMV-2 at day 28, E) day 28 differentiation in regular osteogenic medium, F) undifferentiated. Scale bar=200 µm.
Figure 84 Alizarin red staining of beads, A) day 14 BMP2-2, B) day 28 BMP2-2, C) day 14 SIMV-2, D) day 28 SIMV-2, E) undifferentiated hDPSCs and F) negative control. Scale bar=200 µm.

Figure 85 Von Kossa staining of beads by day 28. A), B) and C) BMP2-2 group, D), E) and F) SIMV-2, G) day 28 osteogenic differentiation without supplements, H) undifferentiated hDPSCs and I) negative control. Scale bar=200 µm.

For further confirmation of osteogenic neo-tissue formation, the hydrogels were stained with anti-osteocalcin, anti-osteopontin, anti-sclerostin and anti-podoplanin antibodies (Figure 86 and Figure 87). The two experimental groups by day 28 of culture under
differentiation medium, produced tissue with positive expression for anti-osteopontin, anti-osteocalcin, anti-podoplanin and anti-sclerostin antibodies, with the BMP2-2 group showing higher intensity for all antibodies. SIMV-2 showed a positive but comparatively weaker expression for all the antibodies. The negative controls can be found in Figure 88.

Figure 86 Fluorochrome labelling of bone constructs obtained by experimental group BMP2-2 A) Anti-osteocalcin (green) and anti-osteopontin (red) and B) anti-sclerostin (green) and anti-podoplanin (red). Scale bar=200 µm.

Figure 87 Fluorochrome labelling of bone constructs obtained by experimental group SIMV-2 A) anti-osteocalcin (green) and anti-osteopontin (red), B) anti-sclerostin (green) and anti-podoplanin (red). Scale bar=200 µm.
6.4.2.2 Quantitative analysis of hDPSCs 3D osteogenic bioprocesses

The quantitative comparisons among the supplements was undertaken by ALPase activity analysis, ARS-based quantification, chemical analysis with ATR-FTIR spectroscopy analysis and X-ray microanalysis and qPCR with various osteogenic genes.

ALPase activities in both groups increased through the experiment. Additionally, both groups showed a similar increase, with the BMP2-2 group having higher expression, but without significant differences, by day 28, BMP2-2 achieved an increase of 14.6 times fold and SIMV-2, 12.6 times fold. Both treatments seem to have reached a plateau by day 21 of differentiation. These results can be seen in Figure 89.

The level of mineralisation of the beads was measured by quantifying the ARS positive area in stained slides. Experimental group BMP2-2 showed higher expression in the sample times, achieving a significantly higher positive stained area of ≈30 percent,

Figure 88 Immunostaining for controls A) anti-osteocalcin (green) and anti-osteopontin (red) undifferentiated beads, B) anti-sclerostin (green) and anti-podoplanin (red) of undifferentiated beads and C) anti-osteocalcin (green) and anti-osteopontin (red) negative control, B) anti-sclerostin (green) and anti-podoplanin (red) of negative control.
whereas SIMV-2 only achieved ≈20 percent. Both groups showed a big increase between
day 21 and 28 of culture. These results can be seen in Figure 90.

**Figure 89** Measurement of Alkaline phosphatase activity by colorimetric assay, scored
by experimental groups BMP2-2 and SIMV-2 through 28 days of culture. For each
group n=3. * indicates statistical significance between the three groups for each time-
point.

**Figure 90** Semi-quantitative indirect measurement of Alizarin red S stained slides:
Percentage of mineralised area of hydrogels achieved by experimental groups BMP2-
2 and SIMV-2, measured by image analysis software of stained slides (n=3). * indicates statistical significance between the three groups for each time-point.

Spectra from ATR-FTIR, ATR-FTIR imaging and X-ray microanalysis, confirmed that the comparison of mineralisation in this study was based on the true calcified hydroxyapatite components (Figure 91, Figure 92, Figure 93, Figure 94 and Figure 95). They were characterised in ATR-FTIR by the specific spectral bands at 1012 cm\(^{-1}\) and 1445 cm\(^{-1}\) which are comparable with pure hydroxyapatite and collagen of human bone respectively and in X-ray microanalysis by the ratio Ca/P, as previously reported hydroxyapatite has a ratio of 1.62 (Meyer et al., 1972, Figueiredo et al., 2012). It was seen that BMP2-2 experimental group achieved better results in both analysis during the 28 days of culture, having a significantly stronger signal in the mentioned bands and showing an average Ca/P ratio higher than SIMV-2. However, Ca/P ratios scored by both groups were lower than values achieved by the perfusion bioprocessing under normal differentiation conditions. These low ratios represent non-stoichiometric hydroxyapatite formation. ATR-FTIR has a penetration depth between 0.5 to 5 µm depending on the wavenumber, which allows a spectral contribution to be measured and seen from subsurface material, while elemental analysis has a penetration depth of 1-2 µm. The lower Ca/P values obtained for the supplemented groups in comparison to the unsupplemented, could be a result of a cell-dense surface producing a more crystallised surface in the unsupplemented group. However, the better C/P ratios, suggest the more homogeneous calcification of the supplemented groups, as ATR-FTIR is able of subsurface measurements and X-ray microanalysis is not.
Figure 91 ATR-FTIR spectra comparison for the two experimental groups 21 days of culture.

Figure 92 ATR-FTIR spectra comparison for the two experimental groups after 28 days of culture.
Figure 93 ATR-FTIR imaging comparison for A) SIMV-2 and B) BMP2-2, after 28 days of culture. Scale bar=200 µm.

The C/P ratio is a characteristic ATR-FTIR measurement of bone, with values of 0.23. Samples were analysed measuring the absorbance of the samples for the wavenumbers 1415 (for C) and 1035 (for P) [cm\(^{-1}\)], the resulting values can be seen in the Figure 64.

![Figure 93 ATR-FTIR imaging comparison](image)

Figure 94 Relationship between the organic and inorganic fraction of bone constructs. C/P ratio of hydrogels for for different sampling days. For each group n=3. * indicates statistical significance between the three groups for each time-point.

![Figure 94 Relationship between organic and inorganic fraction of bone constructs](image)

Figure 95 Measurement of hydroxyl apatite quality by X-ray microanalisis. Standardised Ca/P ratios scored by Experimental groups BMP2-2 and SIMV-2 by day 28 days of culture (n=3). * indicates statistical significance comparing the experimental groups.

![Figure 95 Measurement of hydroxyl apatite quality by X-ray microanalisis](image)
Following the evaluation of the enzymatic and chemical characteristics of the biomaterial constructs formed by the two experimental groups, osteogenic differentiation was also characterized by distinct cellular phenotypes. Osteogenic differentiation of hDPSCs was evaluated by measurement of gene expression level using qPCR. The expression of genes Runx2/CBFA1, osteocalcin (BGLAP), alkaline phosphatase (ALPL), collagen type I (COL1A1) and sclerostin (SOST) can be seen in Figure 96.

The positive expression for the five genes in the two experimental groups, indicates successful osteogenic differentiation. Particularly, when going from day 21 of induction to day 28. An enhanced or steady expression was seen in most of the genes. BMP2-2 experimental group had a steady expression of Runx2 and produced an enhancement in the expression of COL1A1, BGLAP, ALPL and SOST in the last 7 days of culture. SIMV-2 group produced a enhancement in all the analysed genes in the last 7 days of culture. These results indicate the progression of the osteogenic differentiation.
Figure 96 Relative gene expression for the osteogenic markers A) RUNX2, B) BGLAP, C) COL1A1, D) ALPL and D) SOST, for the two experimental groups for 28 days of culture (n=3). * indicates statistical significance comparing day 21 and day 28 of culture.

6.4.2.3 **Surface analysis by scanning electron microscopy of hDPSCs 3D osteogenic bioprocesses**

When observed by SEM, many mineralised deposits were detected within the alginate hydrogels of both experimental groups after osteogenic culture. Additionally, the lacunae seen in the histology are clearly viewed in these pictures, along with the morphogenesis of the osteoblastic cells. In the first days of culture, cells were seen scattered homogenously, additionally, as ECM was still not produced, the hydrogels were weak and difficult to process for SEM. After, scaffolds started getting rigid and pictures showed the presence of non-stoichiometric mineralised deposits. These nodules had an important proportion of calcium and phosphorous (proved by X-ray microanalysis). By day 28 the lacunae are distributed in large portions of the scaffolds and in the pictures of both groups, osteocytes can be morphologically recognised. The morphology of the bone constructs produced by group BMP2-2 can be seen in Figure 97 A to C, where the maturation of the tissue can be seen. A) displaying whole differentiated beads, B) and C) mineralisation, lacunae and mineralising osteocytes.

The morphology of the bone constructs produced by group SIMV-2 can be seen in Figure 97 D to F. Where, D) shows whole beads, E) and F) mineralisation, osteoid osteoblasts, osteocytes and lacunae being mineralised (mineralising osteocytes).

The morphology of the bone constructs produced by both experimental groups appears immature. In these SEM pictures, it can be seen that cells in the surface of the beads resemble osteocytes and their surroundings osteocytes lacuna.
Figure 97 SEM pictures of cultured scaffolds in differentiation medium after 28 days showing characteristic bone features for experimental group BMP2-2 A), B), C), D), E), F), G), H) and I) day 28 and J) day 7.

6.5 Discussions
The standard procedure for the osteogenic differentiation of multipotent stem cells has been treatment of a confluent monolayer with a cocktail of dexamethasone (Dex), ascorbic acid (Asc) and β-glycerophosphate (β-GP) (Langenbach and Handschel, 2013). However, monolayer culture has been demonstrated to confront severe difficulties, the *in vivo/in vitro* environment disparity, the mismatching cell phenotypes produced, and the lack of adaptability to the three dimensional environment, mainly because of mass transfer limitations. Tissue engineering drives the revolution to close the gap between the *in vivo-in vitro* environments, for the production of neo-tissue-equivalents. Its strategy involves studying cells, 3D scaffolds/biomatrixes and the design of bioreactors. In due process, elaborating the framework for systemic and controlled *in vitro* studies of tissue development and function. To date, several studies in 2D and 3D platforms have been done, refining the technique to a complex bioprocess to produce bone-like constructs in a laboratory scale, involving different MSCs, 3D scaffolds, growth factors/chemical cues and bioreactors (Zhao et al., 2011, Karbanova et al., 2010, Jensen et al., 2015, Heng et al., 2015, Sikavitsas et al., 2002, Stiehler et al., 2009, Hwang et al., 2008, Hwang et al., 2009, Randle et al., 2007b, Diederichs et al., 2009, Jung et al., 2012a), showing the potential use of MSCs in bone regeneration. The different strategies,
encapsulated cells (or cells seeded into a scaffold), semi-batch or perfused feeding strategies and shear stress modulation, showing successful results for osteogenic differentiation of MSCs. Nonetheless, these efforts have been insufficient and the production of cellular constructs with heterogeneous populations of cells is a trademark of these bioprocesses. With the novelty of perfused vessels, the gap between in vivo and in vitro has never been closer to be tight. Bioprocesses with medium perfusion have been introduced, providing culture systems with dynamic exchange of nutrients and oxygen, together with a new source of controlled shear stress. Previously, we developed a culture system to create three-dimensional bone-like tissues based on hDPSCs, alginate/gelatin bead, and perfusion-RWV bioreactor and tested its performance with regular osteogenic differentiation medium. Its comparative results exceeded the performance obtained with static culture and a commercially available HARV bioreactor. However, the transition from laboratory to the clinic, requires the production of the homogeneous and constant production of cellular constructs, cell therapy requires the highest quality production. Therefore, we studied the capability of supplement BMP2 at physiological level and compared it with simvastatin, a commonly used bone differentiation supplement.

To the best of our knowledge, this is the first study demonstrating the three dimensional bone-like tissue formation from hDPSCs in alginate/gelatin scaffolds, grown suspended in a perfusion bioreactor system using BMP2 at a physiological level. Most of the studies on BMPs use 100 to 1000 times higher concentrations. Thus, could have widespread applications in bone tissue engineering.

In this chapter, a pilot study was done, hDPSCs were homogenously encapsulated within 1.1% alginate mixed with 0.1% gelatin hydrogels, afterwards the encapsulated hDPSCs were placed to culture in suspension environment within vessels of perfusion-RWV bioreactor for 14 days of culture, whereas the bioreactor maintains controlled in vitro culture conditions that permit tissue growth and development. Each day of culture, BMP2 and simvastatin were supplemented to the perfused culture media to avoid degradation of the supplements.
This bioprocess provides a suspension culture. The laminar flow of medium, minimise cell exposure to shear and turbulence which can negatively affect the cell construct produced, while providing sufficient nutrients and oxygenation, as well as osteogenic supplements to support three dimensional bone neo-tissue growth and removing the undesirable cellular by-products.

In the performed pilot study of the supplements, both supplements, BMP2 (experimental group BMP2-1) and simvastatin (experimental group SIMV-1) in expansion medium, demonstrated to be able to produce bone differentiation. Experimental group SIMV-1 revealed higher cell proliferation and viability. Simvastatin has been seen to protect cells from cellular senescence induced by long-term cultures/passages in vitro (Zanette et al., 2015), and this effect could be the underlying cause of the differences in viability. Implying that although differentiation inhibits proliferation, simvastatin could yield more cells than other bone differentiation supplements.

Cell growth and expression of the osteoblastic phenotype in 2D static culture has three distinct phases: 1) High proliferation and development of extracellular collagenous matrix (10 to12 days). 2) Matrix maturation, down-regulation of proliferation and up-regulation of ALPase activity (12th to 18th day). 3) High mineralisation and further decrease of proliferation, declination of ALPase activity and the induction of osteocalcin expression (16th to 20th day). Similar behaviour can be seen in the proliferation of cells in the two experimental groups (BMP2-1 and SIMV-1) being assessed with a time delay, this could be associated with the 3D environment.

The bone neo-tissue formed by cell grown within alginate/ gelatin hydrogels consisted of viable and metabolically active cells, which were evaluated by The live/dead assay and ALPase activity. It showed that by day 14, the scaffolds produced by the two experimental groups were fully populated of living cells and presented positive ALPase activity. The two groups did not present significant differences in their level of ALPase activity. However, SIMV-2 had slightly higher activity. Both groups shown higher expression compared with the unsupplemented system, SIMV-2 presented 98% higher ALPase activity and BMP2 71% higher.
For validation of the 3D osteogenic differentiation achieved by the two experimental groups, the cell constructs during differentiation were characterised by histological, immuno-staining and SEM microscopy. Histological H&E staining of sectioned hydrogels at day 0, showed most cells appear scattered in the bead without having form extracellular matrix. After, displayed well distributed tissue growth for both experimental groups. Morphogenesis of the osteogenic cells until achieving maturation, can be seen in both experimental groups by the emergence of osteocyte lacunae. ARS staining indicated positive differentiation after the 14 day-long culture, a gradual enhancement in mineral deposition for the cell constructs produced by both experimental groups was seen. Von Kossa staining was poor but slightly positive, and confirmed the mineralisation by showing disperse deposits of calcium through the hydrogels for both groups. Immunostaining for osteopontin, osteocalcin, podoplanin and sclerostin was positive for both groups, showing cells undergoing bone ontogenesis from early osteoblasts to mature osteocytes.

The surface of the cell constructs was analysed with SEM microscopy, here the morphogenesis of the hDPSCs can be perceived in different levels for the two experimental groups, BMP2-1 showed higher neo-tissue maturation, seen by the hydroxyapatite crystals found, the osteocyte lacunae and the presence of immature osteocytes.

The results of the pilot study suggest that both supplements have osteogenic effect. Hence, their differentiability in full osteogenic media was further assessed.

Further to the short pilot study, we investigated the capability of supplement BMP2 at physiological level (experimental group BMP2-2) and compared with simvastatin (experimental group SIMV-2) in full osteogenic medium for long-term culture. hDPSCs were homogenously encapsulated within 1.1% alginate mixed with 0.1% gelatin hydrogels, afterwards the encapsulated hDPSCs were placed to culture in suspension environment within vessels of perfusion-RWV bioreactor for 28 days, whereas the bioreactor maintains controlled in vitro culture conditions that permit tissue growth and development.
Both supplements, BMP2 and simvastatin in osteogenic medium, demonstrated to be able to produce bone differentiation. Experimental group SIMV-2 revealed higher cell proliferation and viability, similar to what was seen in the pilot study. However, the difference was significant only in MTS assay, but not in ATP content, and after 21 days of culture. As previously stated, simvastatin has been seen to protect cells from cellular senescence induced by long-term cultures/passages in vitro (Zanette et al., 2015), this could be the underlying cause of the difference. Simvastatin could yield more cells than other bone differentiation supplements by partially abrogating the negative effect of differentiation in proliferation.

Cell growth and expression of the osteoblastic phenotype in 2D static culture follows a three staged pattern previously described, and a similar behaviour was seen in the proliferation of cells in both experimental groups, but with a time delay, this could be associated with the 3D environment. The plateau observed in the cell proliferation profiles at the end of the experiment, is in accordance with a typical cell culture sigmoidal curve, and cells have reached a stationary phase, with the apoptotic behaviour observed of mature osteoblasts residing in mineralised nodules (Sikavitsas et al., 2002). The live/dead assay showed that by day 28, the scaffolds were fully populated of living cells.

The morphological analyses by light microscopy showed how ECM formation and mineral deposition happen through the culture. At the beginning of the culture, beads where transparent and cells were visible with light microscopy. However, through the culture, it was seen how ECM formation and mineralisation began blocking the light until light transmission through the beads was not possible.

The bone neo-tissue formed by cell growth within alginate/ gelatin hydrogels consisted of viable and metabolically active cells, which were evaluated by measuring pH, glucose, glutamine, lactate, ammonia and oxygen kinetics in osteogenic medium during cultures. It has been reported that the profile of pH and these metabolites can be used to modulate cell metabolism, for instance ECM deposition and neo-tissue progress for developing optimal culture condition for SCs culture (Obradovic et al., 1999). Animal cells utilize glucose and glutamine as vital energy sources. Through glycolysis, 1 molecule
of glucose is metabolised and produces 2 molecules of lactate as a by-product under anaerobic condition, yielding 2 mole of adenosine triphosphate (ATP) per mole of glucose, or CO₂ and H₂O through the TCA cycle in aerobic condition, yielding 36 mole of ATP per mole of glucose. Through the TCA cycle, 1 molecule of glutamine is lysed into glutamate and produces 1 molecule of ammonia as by-product. Hence, the amount of ammonia can be used as an indicator of glutamine utilization and TCA cycle activity. In our study of supplemented 3D osteogenesis. Glucose consumption kinetics showed both experimental groups had similar profiles, a slow but steady decrease through the culture, implying the adequate feeding strategy to produce metabolically active cells in sustained growth. In lactate kinetics, both groups produced concentrations below toxic levels, reaching a maximum of 10 [mmol/L], this reflects the slow but steady glucose consumption seen. As metabolites are removed, if glucose were to reach zero, the limiting concentration of lactate achievable, would be 12 [mmol/L], never reaching toxic levels (>16 [mmol/L]). A high glucose medium could help cell proliferation, but as well would increase the concentration of lactate produced and with it, the risk of damaging the cells would arise. Glutamine showed a similar profile for both groups, going down for the first 14 days and then rising up again, ammonia had a behaviour that mirrors glutamine consumption. We believe the decrease in the consumption of glutamine reflects proliferation reaching stationary phase and the amount of cell constructs, sampled rather than the cells consumption. Ammonia did not reach toxic levels through the whole culture for both groups, with values between 1 and 2 [mmol/L] (less than 4 [mmol/L]). Additionally, pH and oxygen concentration are other parameters to consider as these affect cell viability, growth, differentiation and metabolic activity. pH in osteogenic medium decreased with culture time and followed the increase of lactate and ammonia concentration, as it is known that these metabolites affect medium pH (Barngrover et al., 1985). pH change was within the proper pH range (7.0-7.4) of cell culture media, implying no strong influence on cell growth and function. Oxygen has a major role in SCs growth and differentiation, hypoxic conditions favouring undifferentiated expansion and normoxic conditions, differentiation. For both experimental groups, oxygen fluctuated between normoxic values through the whole culture.
For validation of the 3D osteogenic differentiation achieved by both experimental groups, the cell constructs during differentiation were characterised by histological, immuno-staining and SEM microscopy. Histological H&E staining of sectioned hydrogels, showed how undifferentiated cells at the beginning of the culture, appear scattered in the beads without having form extracellular matrix. After, displayed well distributed and homogeneous tissue growth for both experimental groups. Morphogenesis of the osteogenic cells until achieving maturation, can be seen by the emergence of osteocyte lacunae. Interestingly, BMP2-2 group shows better neo-tissue formation, with lacunae spreading homogeneously through the whole bead. SIMV-2 shows lacunae forming from the core to the surface, but not reaching the surface, as opposed with the unsupplemented culture which produced lacunae from the surface to the core (see Figure 53). ARS staining indicated a progressive differentiation through time, a gradual enhancement in mineral deposition for the cell construct produced by both experimental groups. The ARS quantitative analysis is consistent with the qualitative results and BMP2-2 group shows better performance than SIMV-2 group. Von Kossa confirmed the mineralisation by showing scarce but positive disperse deposits of calcium through the hydrogels. Immunostaining for osteopontin, osteocalcin, podoplanin and sclerostin was positive for both groups, showing cells undergoing bone ontogenesis from early osteoblasts to mature osteocytes. BMP2-2 group had clearly higher expression. Meanwhile osteopontin (marker of early osteoblasts) and osteocalcin (marker of mature osteoblasts) positive regions can be seen through the whole bead, podoplanin (marker of early osteocyte) and sclerostin (marker of mature osteocyte) are positive selectively in the inner or outer areas of the scaffold respectively, showing how osteocyte maturation is dependant of the surrounding osteoid and hydroxyapatite. The surface of the cell constructs was analysed with SEM microscopy, here the morphogenesis of the hDPSCs can be perceived in different levels for both groups, where osteoid osteoblasts and mineralising osteocytes are clearly distinguished, but not mature osteocytes with dendritic processes (canaliculi). Both groups showed high neo-tissue maturation, seen by the many hydroxyapatite crystals found, the osteocyte lacunae and the presence of osteoid osteoblasts and mineralising osteocytes, however, the unsupplemented culture, showed more mature structure and terminal osteocytes.
ATR-FTIR of human bone shows carbonate bands at wavenumbers 870, 1415 and 1470 [cm$^{-1}$], and phosphate bands at wavenumber 565, 605 and 1035 [cm$^{-1}$]. Carbonate bands are distinctive of the bone organic component (mainly collagen) and phosphate bands of the inorganic fraction (hydroxyapatite), and the ratio of organic and inorganic component, C/P is characteristic biogenic signature for human bone. However, it is recommended to use the phosphate peak of 1035 and carbonate peak of 1415 to calculate C/P ratio. When these wavenumbers are used, the human bone C/P ratio is 0.23 (Beasley et al., 2014). When differentiation starts, the C/P ratio is high, and it decreases with differentiation and hydroxyapatite formation. BMP2-2 group produced a significantly higher C/P value by day 21, but by day 28, it decreased to a significantly lower value, achieving ≈0.23, the exact value described for human bone in literature, showing good differentiation. SIMV-2 culture achieving ≈0.3. ATR-FTIR imaging confirms these results, with yellow being positive and blue negative.

X-ray microanalysis (SEM/EDS) of hydroxyapatite shows the elemental composition of the analysed materials, it has been seen that the Ca/P ratio represents a characteristic biogenic signature of the inorganic fraction of bone (hydroxyapatite). Pure hydroxyapatite has a Ca/P ratio of 1.67 (Medina Ledo et al., 2008). In our experiments, both groups scored low ratios, demonstrating the production of calcium deficient hydroxyapatite (non-stoichiometric). BMP2-2 group scored a significantly higher value. Further analysis demonstrated the quality of the bone construct achieved, and the low scores for Ca/P ratio, could be product of the more homogeneous cell construct, while values presented in the previous chapter, show a surface differentiation front with highly calcified surface, less calcified interior. More research should be done until the proper ratio is reached. This statement can be confirmed with the ATR-FTIR results, which measure with a depth of penetration of up to 5 [um], against the 0.1-2 [nm].

3D osteogenesis was characterised at a genetic level, examining the expression of Runx2/CBFA1, osteocalcin (BGLAP), alkaline phosphatase (ALPL), collagen type I (COL1A1) and sclerostin (SOST), these genes are representative of the entire MSCs to osteocyte ontogenesis. Osteogenic ontogenesis requires the precise and orchestrated activity of several genes and signals (extracellular and intracellular). Runx2 is a transcription factor and the central control of osteogenic differentiation. Its
upregulation happens early in the ontogenesis, and is believed to have a constant expression through the whole differentiation (Shui et al., 2003). ALPL is a ubiquitous cellular protein, and its function is ill-defined. However, it is known that its action starts as early as 2 days after the beginning of the osteogenic drive. Its expression is increased steadily through the differentiation. Collagen I (Col1a1), is an important component in the bone ECM, with a role in cell adhesion, proliferation and osteoblast phenotype, its upregulation is characteristic of osteogenic differentiation and can be considered an early marker of differentiation. Osteocalcin (BGLAP), is an osteoblast specific gene and one of the most abundant proteins in bone, its expression is significantly upregulated in matrix synthesis and mineralisation (Ryoo et al., 1997). Sclerostin (SOST) is a known specific gene for mature osteocytes, and it is integral to osteocyte function as a signal to damp the action of osteoblast bone deposition and to control bone metabolism (Compton and Lee, 2014).

MSCs, preosteoblasts and osteoblasts express Runx2 together with secreting osteoid, when pre-osteoblasts go through maturation, the expression alkaline phosphatase is increased. When mature osteoblasts become embedded in osteoid, they express osteocalcin and start producing dendritic projections characteristic of osteocytes. Through all the ontogenesis, collagen 1 is secreted. BMP2-2 experimental group showed a constant expression of Runx2 along with high 10 times fold increase in expression of osteocalcin. The expression of these two genes, could signify most of the cells already went through the transition from immature to mature osteoblast. The increase of SOST in the last week of culture could represent the further transition from mature osteoblasts to osteocytes. During all this period, Col1a1 and ALPL were active and positive with a 6-time fold and a 3 times fold increase. These genes are related with ECM formation and mineralisation, and are indicative of a well developed in vitro osteogenesis. These results suggest that a homogeneous population of cells was produced with cells in a mature osteoblastic and early osteocytic stage. SIMV-2 experimental group showed high increase in expression of Runx2 (10 times fold), Galindo and coleagues sugest that an increase in Runx2 is related with the the cessation of cell growth (i.e. \( G_0/G_1 \) transition) (Galindo et al., 2005), this could be linked to cell growth achieving stationary phase. A high expression of osteocalcin was seen by day 21,
but by day 28, the increase was low. The expression of these two genes, could signify cells are going through late stages of maturation and are becoming osteocytes. The high increase of SOST in the last week of culture could sustain this idea of maturation to osteocytes. During all this period, Col1a1 was low positive, meanwhile ALPL was highly expressed. These genes are related with ECM formation and mineralisation, col1a1 being an early marker, and ALPL a more mature and are indicative of late stage differentiation. These results suggest that a homogeneous mostly osteocytic population of cells was produced.

BMP2-2 group showed lower proliferation, higher calcification and a more homogeneous bone tissue with what we believe to be population of mature osteoblasts and a smaller fraction of osteocytes. SIMV-2 group showed a higher proliferation, less calcified, with a less homogeneous construct, populated with what we believe is a mixture of osteoblasts and higher fraction of early osteocytes.

The viability and proliferation, AP activity, gene expression, immunostaining, and calcium deposition observed in the BMP2-2 group, along with the morphological analyses done, suggest that this group produced a homogeneous construct of mature osteoblasts. these cells are active cells capable of proliferate, secrete osteoid, calcify and merge/ consolidate with the in vivo environment. The more mature cell construct product of SIMV-2 group, are a functional cell construct rather than a developmental cell construct. Hence, the merge/ consolidation with in vivo tissue would be more difficult to achieve. Sclerostin producing osteocytes have a negative effect in osteoblast proliferation and increase bone resorbing osteoclast presence and activity in the surrounding in vivo areas. However, the SIMV-2/osteocyte construct would be very useful to research osteocyte phenotype and function.

6.6 Conclusions
This study demonstrated that the bioprocessing providing perfusion flow of medium in a suspended environment is successful in the mitigation of nutrient and oxygen transport limitations, external to three-dimensional cell/alginate constructs, positively influencing the proliferation, differentiation, mineralisation and expression of
osteoblastic markers of hDPSCs cultured in the presence of osteogenic media, and the supplementation with either BMP2 or SIMV, provide successful enhancing strategies for the homogeneous production of bone mimics, ultimately enabling the formation of 3D bone-like constructs for clinical and research purposes. Additionally, we demonstrated that this bioprocessing positively influences the proliferation, differentiation, mineralisation and expression of osteoblastic markers of hDPSCs cultured in the absence of osteogenic osteoinductive supplements, apart from BMP2 or SIMV. Through the analyses followed to characterise these constructs, the capability of the bioprocess to reproduce the whole osteogenic lineage ontogenesis was established.

Progressive differentiation was confirmed for both experimental groups, by the gene expression pattern, the ALPase activity, the gradual decrease of the C/P ratio measured by ATR-FTIR and the ARS positive area, measured by software analysis. However, the increase in the Ca/P ratio, measured by SEM/EDS was poor and demonstrated the production of non-stoichiometric hydroxyapatite, previously we showed better results with the unsupplemented bioprocess, this constructs had a surface differentiation front, and produced a highly mineralised surface. Both experimental groups presented a homogeneous differentiation front, hence producing less mineralised surface. These were qualitatively validated by immunostaining, H&E and von Kossa staining, SEM pictures and ATR-FTIR imaging. Experimental group BMP2-2 achieved better results in most of the analysis, producing a well mineralised homogeneous cell construct, mainly populated by osteoblasts, hence showing big potential in regenerative medicine and clinic. Interestingly, experimental group SIMV-2 produced a mineralised homogeneous cell construct, mainly populated by osteocytes, hence showing big potential for research in bone functionality. The perfusion culture system supplemented with either BMP2 or simvastatin described here, would provide an efficient and easy culture system for applications in bone tissue engineering in the context of macroscopic bone neo-tissue formation. In particular, the successful use of physiological BMP2 promises to have an impact in cell therapy, as this low dosage, could help to avoid the negative dose dependent risk product of BMP2 therapeutics.
7 Summary and conclusions
Mesenchymal stem cells from most tissues of the body have arisen as attractive therapeutic tools with high potential in regenerative medicine. Their capability to give raise to a variety of cell types, made them the focus of extensive research to produce biological substitute devices. hDPSCs are a particular source for adult mesenchymal stem cells. They present ease in access and extraction, high proliferative characteristics, bone tissue differentiability and they do not elicit ethical considerations, as their extraction is one of the less invasive between SCs.

Tissue engineering encompasses the required toolset to process SCs into tissue constructs. Through the crosstalk between cellular biology and engineering, it attempts to discover the missing link between cells and the in vivo environment. Ultimately, to generate a bridge for ex vivo cellular constructs to fuse with living organisms. Its approach has been to find the proper tools to bioprocess SCs and produce neo-tissue able to mimic and replace their in vivo counterparts. Tissue engineering researches: a) the cellular factor, their interactions and the developmental processes they follow to produce neo-tissue. b) the bio-signalling factor, comprising the cues and stimuli to sustain cells and drive their differentiation to the adequate lineage. C) the environmental factor, the three dimensional area, where cells inhabit and develop, able to be converted into the tissue itself, with all the extracellular components and structures found in a regular tissue, along with the functionality required. and finally, d) the bioprocessing factor, the design of bioreactors able provide the platform to deliver nutrients and oxygen for the cells to develop appropriately. Additionally, the physical stimuli, along with the macro environment for all the previous factors to interact.

Bone tissue engineering, would be the particular field researching bone healing. The cells able to differentiate into functional osteogenic cells, producing the characteristic extracellular matrix. The required physical and chemical cues to drive bone differentiation. The fabrication of resorbable scaffolds displaying suitable mechanical and biological properties to replace large bone defects, and able to undergo neo-vascularisation when implanted in vivo. The design and fabrication of tailor-made bioreactors able to supply homogeneous medium environment, efficient mass transport characteristics, together with the physical stimuli, during the culture process.
Before the bioreactor era, most research was done in 2D monolayered static culture. However, this system was not appropriate for tissue development. Bone cells are enclosed in a 3D complex histoarchitecture not reproducible in 2D. Thus, \textit{in vivo} reproducibility of the 2D \textit{in vitro} development is unfeasible. To confront the adimensionality of this system, 3D scaffolds where incorporated into the system and TE continued to evolve towards the production of functional tissue substitutes. Nonetheless, this setting provided the cells, with nutritional diffusion constrains, hence requiring an adequate delivery system. Dynamic suspended culture bioreactors, wherein the cell/ scaffold constructs are processed under controlled conditions, such as nutritional requirements, temperature, pH and dissolved gases, appear to be the solution to this hindrance and to long-term stem cell culture.

In this thesis, a bioprocess for the homogeneous undifferentiated expansion and further bone differentiation was established. The in-house designed perfusion system incorporated to a RWV unit provided: a convenient gas-permeable membrane vessel casing and an oxygenator to ensure the homogeneous, efficient and constant supply of oxygen, medium perfusion to ensure adequate nutrient supply and metabolic waste removal, and the avoidance of experimental error from manual operation by an automation system. These novel features, consequently, resulted in an effectively maintained culture condition throughout the entire long-term culture. Additionally, showing to be a convenient adaptation culture environment by controlling culturing factors at real time.

The bioprocess has successfully proven to be a significant improvement for maintenance, expansion and osteogenic differentiation of hDPSCs, in comparison to fed-batch bioreactors and 2D static culture. It was effective in producing the complete osteogenic lineage ontogenesis, producing cells ranging from the early and mature stage osteoblasts, to further produce the early and mature stage osteocytes. Additionally, the characteristic bone-extracellular matrix and mineral deposition produced, was found to successfully replace the scaffold architecture. Hence, producing a bone-mimicking construct. Ultimately, a bioprocess for the production of homogeneous reproducible 3D bone neo-tissue was established.
Hence, it was concluded that this bioprocessing, providing perfusion flow of medium in a suspended environment is successful in the mitigation of nutrient and oxygen transport limitations, external to three-dimensional cell/alginate constructs and is more efficient that the HARV bioreactor and the 3D static culture, positively influencing the proliferation, differentiation, mineralisation and expression of osteoblastic markers of hDPSCs cultured in the presence of osteogenic media, ultimately enabling the formation of 3D bone-like constructs for clinical purposes. However, this setup alone still produces a level of heterogeneity in the constructs, with mixture of mature and immature cells and distinguishable different microscopic neo-tissue anatomy.

These enhanced culture conditions successfully proven to significantly improve the designed three-dimensional hDPSCs bioprocess. Consequently, resulted in further enhanced maintenance condition throughout the entire long-term culture. Additionally, both biologics showing to be an effective solution to the homogeneous production of bone cell constructs.

Studies describing the 3D undifferentiated expansion of hDPSCs in 3D environment, under long-term culture have not been found in Pubmed, EBSCO or Google scholar. Albeit this was not the main scope of this research project, a first approximation has been done and it could lead to further research. There is the need to design the appropriate protocol specifically for hDPSCs expansion, addressing cell seeding density that would yield the optimal number of cells while maintaining their multipotency, as well as the seeding density for the better differentiation is still on demand. The mass production of undifferentiated hDPSCs for its further banking could provide ready-availability of cells for any cell therapy when required, and the impact in the medical market could be significant. Additionally, the proper control of cell density for cell differentiation could produce the rich in cells cell-construct required for a fast integration when implanted in vivo.

Prior to this project, similar settings have been used to bioprocess bone-like constructs with interesting results (Cha, 2010, Bancroft et al., 2003, Janssen et al., 2006, Qian et al., 2013, Gardel et al., 2014, Li et al., 2014, Sinlapabodin et al., 2016, Kleinhans et al., 2015, Wang et al., 2003, Gomes et al., 2006, Hosseinkhani et al., 2006, Gao et al., 2016).
However, the positive results shown, were limited by the difficulties inherent to SCs processing, heterogeneous production of cells and an ill-characterised bone construct (Sheehy et al., 2015). Most of the studies done in this field, procure to demonstrate osteogenic differentiation with a limited number of widespread analyses: ALPase, proliferation, SEM microscopy, OC expression and histology and occasionally, mechanical loading and; but lack the thorough characterisation a product of medical nature requires. Producing bone implants for humans, requires the fully characterisation of the cellular biomaterial produced. Not only is it required to assess biological aspects, as cell types with the analysis of a number of protein and gene expression; but also by the composition and physical aspects of the biomaterial. X-ray microanalysis and FTIR are effective for characterising composition of the biomaterial. Mechanical loading testing should be performed in vitro and in vivo to assess the materials response to vibration, fluid shear, substrate deformation and compressive loading compared to regular bone. These aspects of the biomaterial should match the bone tissue they would replace. Bone tissue, as previously described, is comprised by bone-forming cells, bone-resorbing cells and functional cells. It is required to be able to describe the ontogenesis of the produced tissue, which stage of differentiation is accomplished and how homogeneous the cell constructs are. The cell-types will be determining of the applicability of the therapy. In this project, that extra layer of characterisation was applied to the validation of the cell-constructs produced by the in-house designed bioreactor. Spotting structural features, as well as specific markers (genes and proteins) of bone turnover unique to each stage/ cell type, measuring viability, enzymatic activity and composition of the cell constructs. Additionally, the nutritional aspects of the system were assessed following metabolic kinetics of key nutrients and cellular by-products. Mechanical testing was not performed, but future work should include its measurement. By following this characterisation, the production of homogeneous mineralised tissue, rich in either osteoblasts or osteocytes was established. Cell construct Homogeneity plays a key role, as fully matured osteocytes, would bring difficulties for the cell construct to fuse with the treated bone defect. To accomplish these homogenous histo-constructs, the use of minimal growth factors was assessed. Utilising BMP2 at physiological level, as well as a common osteogenic signal, simvastatin. To our knowledge, this is the only successful study showing positive effect of BMP2 at
such low level (300 pg/ml), demonstrating significant differences with regular differentiation media. Normal studies involving BMP2, utilise concentrations 50-1000 times higher to be able to obtain comparable results (Nguyen et al., 2016, Lysdahl et al., 2014). Supra-physiological doses have been proved to produce ectopic bone formation. Additionally, there is a significant association between treatment with BMP2 and incidence of cancer (Carragee et al., 2013b). Ultimately, the expensive administration makes low dosing not only valuable for stem cell bioprocessing, but a requirement.

If the variability of MSCs-constructs was controlled, 3D bioprocessing could score better results. Hence, the knowledge obtained over this investigation puts the clinical setting at a glance. However, more validation is required for a safety product to be achieved. Good manufacturing practices (GMP) should be adopted and in vivo validation should be followed. GMP require quality control of collection, processing, storage and release of cell therapy products and of the facilities, equipment, materials and quality assurance: control, validation, qualification and documentation (Giancola et al., 2012). It would require for the culture media to be fully-defined and not containing any exogenous materials, a single step closed setup bioprocess in a clinical setup.

For validation and assessment of the neotissue constructs efficacy and safety, an in vivo model should be done. A preclinical study to assess bone union after implantation (osseointegration), osteoconduction, vascularisation and the physical properties of the resulting implant should be performed. Critical-size defects should be treated with cell therapy and then assessed for: biochemical markers of bone turnover, bone/mass density measurement, analysis of bone architecture/histology, and biomechanical testing of bone strength. Preclinical studies in animal models are key to ensure the quality required to start clinical studies and essential in the medical devices production pipeline. Without fulfilling these requirements, the product would fail at the arrival to the clinical platform. Furthermore, in vivo models represent the only true assessment to compare cell constructs between different bioprocess platforms.

Research in perfusion is taking new directions, from perfusing media directly through scaffolds, with 3D printed microfluidic systems (Mohanty et al., 2015), to scaffold free 3D systems with cell aggregates (Ong et al., 2008). Although these strategies promise to
provide new advantages in the field, it is arguable that they would bring higher processing complexity for the operators and set back the possibility of automation.

In the future, the adequate bioprocess would be a single step closed setup. Able to process patient cells in a fully automated routine. Starting from scaffold seeding, until the fully differentiated bone neo-tissue construct is ready to be transplanted. This strategy, would allow minimal handling of operators, avoiding disruptions, variability, contamination of the samples. The system should allow the production of critical size implants able to vascularise when transplanted, here microfluidics will play an important role. Additionally, producing tissue implants in hospitals would elude logistical complication, as transferring specimens amid different locations. Good manufacturing practices should be tightly followed. Additionally, the tissue mimics should be amenable for long term storage, allowing the prompt availability of tissue for emergency transplants when a patient custom made cell construct is not possible, or is not timely feasible. Research is still on demand to reach a point where safety cell constructs are fabricated and cell therapy becomes the standard procedure in hospitals. Until then, development will keep pioneering new bioprocesses.
8 Future work
Although numerous major progresses have been introduced in the bone tissue engineering field, current bioprocessing still faces many shortcomings, and no adequate substitutes have been developed. Bone tissue is complex, its development and healing involves an entangled network of growth factors, working in joint effort with multipotent cells in a characteristic environment. Knowledge is limited and unknown factors yet need to be discovered.

Development in cell sources, signalling molecules, scaffolds and bioreactor could provide, the means to close the in vitro/ in vivo gap, and help the transfer of the mimicking devices developed, to a clinical setting. Additionally, the tight good manufacturing practices are required to be followed.

Future studies in our perfusion system, to produce the homogeneous bone replacement while shortening culture time could be achieved by the incorporation of biologically active molecules into the scaffold structure (rather than in the culture medium). Hence, providing more cues to cell attachment, expansion and differentiation. BMP2, TGF-β1, PDGF, VEGF, or other known osteogenic factors could be dispersed or covalently linked to alginate/ gelatin hydrogels. Pharmacokinetics, life-span of the biologics in use, and the interactions with the cells, would require to be studied. Long-term culture would affect integrity, effectiveness and the availability of growth factors for bone differentiation in long periods of culture.

Determining the optimal operational conditions of the perfusion-RWV bioreactor. Different stages of differentiation could require different perfusion rates. The metabolic activity of undifferentiated cells, immature cells and mature cells, are different. Hypoxic culture has been reported to yield higher proliferation, keeping cells undifferentiated, even under confluent conditions. On the contrary, normoxia has been seen to support cell differentiation. These conditions are easily modified with the appropriate incubator where the perfusion system resides. Finding the adequate strategy for hypoxic expansion and normoxic differentiation, could yield a better bone construct. Additionally, osteogenic tissue is acknowledged to be sensitive to mechanical stimuli in vivo and in vitro, numerous studies have incorporated mechanical stimuli to their designs, with interesting results. Suggesting that incorporating a convenient ultrasound
prove to the existing perfusion-RWV system, could yield enhanced bone construct production. Alternatively, it has been reported that low level light irradiation (LLLI) and pulsed electro-magnetic field (PEMF) have an effect on the proliferation and osteogenic differentiation of MSCs. Attaching a red-light light emitting diode (LED) irradiation system to the vessel wouldn’t be difficult, as the membrane case is suitably transparent and radiation could travel through. Two round solenoid electromagnets forming a Helmholtz coil could be allocated surrounding the vessel to produce an electro-magnetic field. Both systems should be accounted for their pulsed strategy effect.

Figure 98 Ultrasound probe perfusion-RWV bioreactor vessel

Figure 99 Red LED perfusion-RWV bioreactor vessel
Serum free, xeno free and chemically defined medium experiments should be performed. Bioprofiling of different culture conditions should be taken, to further develop computational models of the culture, to assist and optimise the supplements to be added in the medium. Serum and other culturing reagents of animal origin, part of traditional undefined culture media, pose numerous safety issues in clinical therapy (e.g. infections, immune reactions). Serum and xeno components are not allowed when working under GMP conditions. This technology requires the use of fully defined and non animal materials to reach clinical relevance. The perfusion-RWV system is a suitable platform to establish the required parameters to achieve GMP conditions.
Donor/ cell dependent computational models should be developed. Clinical efficacy is variable and it’s unclear how the phenotypes defining MSCs as well as donor characteristics (e.g. age, gender) affect their functional properties. Studying the intrinsic variability produced by donor specific characteristics, should be assessed and bioprocessing, determining the parameters to be controlled to minimise heterogeneous production.

All the mentioned parameters would help to improve the bioprocessing of MSCs and aid the optimal production of clinically utilisable bone cell devices. However, the ready-availability of cellular constructs is as vital as the quality of the constructs. Hence, studying the long term storage of scaffolds loaded with cells undifferentiated and differentiated, would pose invaluable. Methodologies of storage, along with viability and maintenance of undifferentiated and differentiated phenotype of cells should be studied. Currently there are different storage methodologies, some use cryopreservation (e.g. in serum and DMSO, in sperm freezing medium, vitrification). Hence, freezing and thawing processes have to be accounted. others do not (e.g. sub-zero non freezing storage, cryoprotectants).

Finally, more focus on in vivo studies is unavoidable and there is need for more research on composite scaffolds in realistic biological systems. All the mentioned settings should reach in vivo pre-clinical and clinical testing, to ensure safety of the produced constructs.

All these studies would be expected to produce the homogeneous, highly mineralised and functional tissue. However, cell proliferation, quality and time of culture would be modified. Probably getting us closer to the day when bone TE bioprocessing reaches the clinical field and synthetically produced osteo-bio-neo-tissues start aiding people.
9 Appendix
Figure 102 morphology of hydrogels observed under light microscopy of A) Day 7, B) day 28 HARV bioreactor, C) day 28 static culture Flask, D) day 28 Perfusion-RWV bioreactor. Scale bar=200 µm.

Figure 103 Live dead assay A) at day 0, B) at day 7, C) at day 28 for cells seeded in perfusion-RWV bioreactor, D) at day 28 for cells seeded in HARV bioreactor, E) at day 28 for cells seeded in 3D static culture. scale bar=200 µm.
Figure 104 Alizarin red staining of beads produced by 3D Static culture A) day 14, B) day 21, C) and D) day 28

Figure 105 Alizarin red staining of beads produced by HARV bioreactor A) and D) day 14, B) and E) day 21, C) and F) day 28
Figure 106 Alizarin red staining of beads produced by perfusion-RWV bioreactor A), B) and C) day 14, D) and E) day 21, G) and H) day 28, F) undifferentiated hDPSCs, G) negative control
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