GENERATION OF MINERALISED CELLULAR CONSTRUCTS USING MOUSE EMBRYONIC STEM CELLS ENCAPSULATED IN ALGINATE HYDROGELS AND CULTURED WITHIN A CUSTOM-MADE ROTATING WALL VESSEL PERFUSION BIOREACTOR

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Declaration of Originality

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Copyright Declaration

Conferences and publications

- D. Yeo, **A. Georgiou**, S. Tangviriyasirikul, JM. Cha, A. Mantalaris "Production of 3D Mineralised Cellular Implants from mESCs in a Novel Perfusion Scalable & Automatable Bioreactor" Oral, Musculoskeletal Technology Network, South Kensington campus, Imperial College London, Feb 2011, London, United Kingdom
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- **Anastasia Georgiou**, "Generation of 3D Mineralised Cellular Constructs from mESCs in a Perfusion Bioreactor Using Simvastatin", Poster, Chemical Engineering PhD Symposium, South Kensington campus, Imperial College London, July 2013, London, United Kingdom
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ABSTRACT

The prevalence of musculoskeletal disorders is a major burden for modern societies. Due to the increasing aging population and the lifestyle changes, a significant number of people are severely affected worldwide. The important issue with these diseases is the fact that they cause pain and disability on a person's physical functioning for long time, thus diminishing the quality of life of the individual. Moreover, they are accompanied by a high financial cost for the society and the healthcare system. Current ways of treatment do not provide optimum therapy. They employ high concentration of growth factors and they are expensive, inefficient and many times exhibit various side effects. For this reason, an alternative solution is needed.

Tissue engineering (TE) strategies offer a novel approach to the problem. The combination of the appropriate cell source with the essential scaffold leads to the formation of three-dimensional (3D) constructs, which can be subsequently, cultured within a bioreactor, with the employment of proper osteoinductive factors. This process leads to the generation of high number of efficiently differentiated cells, which are needed for cellular therapies.

In this project, the generation of 3D mineralised cellular constructs was performed using mouse Embryonic Stem Cells (mESCs) encapsulated in alginate hydrogels. The novelty of this project lied on two components; the employment of a custom-made rotating wall vessel (RWV) perfusion bioreactor, which had been shown previously to exhibit advantageous properties regarding the efficient differentiation of high cell numbers needed for potential therapeutic applications and the use of simvastatin (Sim) in the culture media, acting as an osteoinductive substance in very low concentration of the nanomolar scale. Sim had been previously employed to induce osteoblast differentiation. The novelty lied on the total combined configuration with the low concentration of Sim and the perfusion bioreactor used for cell culture and differentiation.

Evaluation of cell proliferation and osteogenic differentiation was performed through several analyses. Extended gene expression was tested and obtained results were also compared with those acquired previously by the currently used protocol with dexamethasone (Dex). Acquired results indicated that the favorable environment of the perfusion bioreactor culture could support higher cell number sand more efficient osteogenic differentiation in comparison to static configuration. Sim was more efficient when supplied in the culture at the appropriate time point, after two weeks of initiation of the experiment. Sim and Dex indicated similar outcome in biochemical analysis. Osteogenic gene expression was strongly induced after Dex treatment while Sim supported the generation of higher cell numbers. These findings suggested the generation of a more progenitor cell type after Sim treatment and a more mature phenotype after Dex treatment.

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LIST OF ABBREVIATIONS

α-MEM	alpha Minimal Essential Medium	EPL	early primitive ectoderm-like
AA	Ascorbic acid	ESC	Embryonic stem cell
AFS	amniotic fluid–derived stem cells	FBS	Foetal bovine serum
ALP	Alkaline phosphatase	FDA	Food and Drug Administration
aMSCs	amniotic fluid mesenchymal stem cells	FGF	Fibroblast growth factor
ANOVA	analysis of variance	FGFR3	Fibroblast growth factor receptor
ARS	Alizarin Red S staining	FITC	Fluorescein isothiocyanate
ASCs	Adult stem cells	FSCs	Foetal stem cells
ATF4	Activating Transcription Factor 4	FTIR	Fourier-transform infrared
ATR-FTIR	Attenuated Total Reflection -Fourier Transform Infrared	HA	Hydroxyapatite
b-GP	b-glycerophosphate	HAC	Hydroxyapatite ceramic
BMCs	bone marrow cells	HARV	High Aspect Ratio Vessel
BMD	bone mineral density	hBMSCs	Human bone marrow stromal cells
BMP	Bone morphogenetic protein	HepG2	Human hepatocarcinoma cell line
BMPR	BMP receptor	HepG2-CM	HepG2 conditioned media
BSP	Bone sialoprotein	HESC	Human embryonic stem cells
BTE	Bone Tissue Engineering	hiPSCs	Human induced pluripotent stem cells
Ca	Calcium	HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
Cbfa-1	Core-binding factor subunit alpha-1	HSC	Hematopoietic stem cell
cDNA	complementary DNA	ICM	Inner cell mass
CNS	Central nervous system	IGF-1	Insulin-like growth factor-1
COL 1	Collagen I	IPSC	Induced Pluripotent Stem Cell
COL 10	Collagen 10	LDL	Low-density lipoprotein
DAPI	4', 6’ diamidino-2- phenylindole	LIF	Leukemia inhibitory factor
Dex	Dexamethasone	MESCS	Mouse embryonic stem cells
Dlx5	distal-less homeobox 5	miRNAs	microRNAs
dsDNA	Double-stranded DNA	miPSCs	mouse induced pluripotent stem cells
EB	Embryoid body	mPCL	medical-grade poly-e-caprolactone
ECM	Extracellular matrix	MSC	Mesenchymal stem cell

Msx2	Msh homeobox 2
n-HA	nano-hydroxyapatite
OBL	Osteoblast
OCN	Osteocalcin
Oct-4	Octamer binding protein-4
OI	Osteogenesis Imperfecta
ON	Osteonectin
OPN	Osteopontin
OSX	Osterix
P	Phosphate
Pax6	Paired box protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDFG	platelet-derived growth factor
PLAGA	poly (lactic acid glycolic acid)
PLLA	Poly L lactic acid
PTH	Parathyroid Hormone
PTHrP	parathyroid hormone - related protein
PTH1R	Parathyroid hormone 1 receptor
qRT–PCR	Quantitative real-time PCR
rhBMP-2	recombinant human bone morphogenetic protein-2
rhBMP-7	recombinant human bone morphogenetic protein-7
rhOP-1	recombinant human osteogenic protein-1
RM	Regenerative `medicine
RNA	Ribonucleic acid
rNPP	r-nitrophenol phosphate
Rpl27	ribosomal protein L27
RT-PCR	Real-time polymerase chain reaction
Runx2	Runt-related transcription factor2
RWV	Rotating Wall Vessel
SCID	Severe combined Immunodeficient
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SIBLING	Small Integrin Binding Ligand N- linked Glycoprotein
Sim	Simvastatin
Sox2	SRY - box2
Sox5	SRY - box5
Sox6	SRY - box6
Sox 9	SRY - box9
Sox17	SRY - box17
SPARC	Secreted protein, acidic, rich in Cysteine
SRY	sex determining region Y
SSCs	Somatic stem cells
SSEA-1	Stage-specific embryonic antigen-1
SSEA-3	Stage-specific embryonic antigen-3
SSEA-4	Stage-specific embryonic antigen-4
STLV	Slow Turning Lateral Vessel
TE	Tissue Engineering
TE buffer	Tris-EDTA buffer
TGF-β	Transforming growth factor-β
VEGF	Vascular endothelial growth factor
vitD3	1,25-dihydroxyvitamin D3
2D	Two-dimensional
3D	Three-dimensional
3DP	Three-dimensional printing

1ST CHAPTER

1. LITERATURE REVIEW

1.1 Bone Biology

Bone is one of the most important dynamic tissues in the human body. It receives blood supply and it has the unique capacity to regenerate and the ability to self-repair and to heal small-scale defects after an injury. It has major role in several significant functions in human body.

Due to the increasing aging population, musculoskeletal diseases affect significantly the quality of life of millions of people around the world. Very common are metabolic bone diseases, which are disorders of bone remodeling, exhibiting an imbalance between bone formation and resorption and resulting in an increased risk of fracture. The high clinical need for bone repair is the motivation of the project.

The literature review follows a specific organization pattern starting with important information regarding the biology of bone tissue. Description of the main structure, the important functions of this tissue, the cell types it contains and the essential role of the extracellular matrix, are presented. Bone development and the role of gene markers contributing to the differentiation process and the timeline of their expression are also described.

Following this introductory fundamental information, the current problem and the clinical need for bone formation and regeneration are highlighted through presentation of several major clinical diseases affecting significantly the life of many people. Data are presented specifically for the UK population using information obtained from charities and organizations dedicated to help and support people with these diseases.

It also includes the description of currently employed therapeutic strategies and presents the appeared and observed limitations of their application. It mainly refers to grafting procedures followed and the use of growth factors to enhance bone formation.

In addition, the literature review focuses on new approaches and alternative ways to treat these problems in the near future. The focus is TE and analysis of each of its main components. Examples of current applications are also presented.

The literature review is finishing by highlighting the novelty and the importance of this project. The next chapter will state the aim and objectives of this research project.

1.1.1 Structure/Function

Bone tissue in the adult skeleton consists of two major types with different architectural forms: the cortical or compact bone and the cancellous or trabecular bone (Figure 1.1).

Cortical bone is a dense tissue, which is organized in cylindrical structures known as osteons or Haversian systems. These contain blood vessels, lymphatics, nerves and connective tissue. Cortical bone forms the outer shell of long bones and the outer surface of the small and flat bones. It consists 80% of the skeleton, but it is characterized by low porosity. It provides mechanical and protective function (Sikavitsas et al., 2001).

Cancellous bone is a highly porous structure composed of many interconnecting spaces, which contain the bone marrow (Brydone et al., 2010). It is organized in trabecules. This type of bone is found at the ends of the long bones and inside flat bones (Athanasiou et al., 2000; Yaszemski et al., 1996). It represents approximately 20% of the skeleton. Cancellous bone is a very active metabolically tissue (Buckwalter et al., 1996a; Buckwalter et al., 1996b) and constitutes the major site of bone remodeling (Feng and McDonald, 2011).

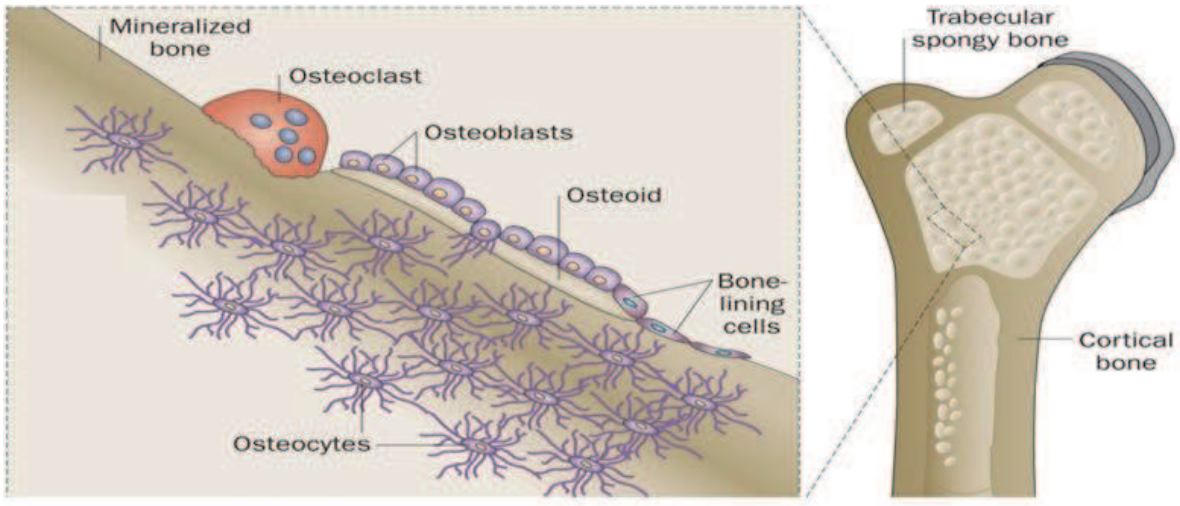


Figure 1.1: Cortical and trabecular bone morphology. Adapted from (DiGirolamo et al., 2012)

Bone is a unique tissue, which contributes to a number of significant functions and can affect either in a positive or a negative way the quality of life of an individual. The main role of bone is to provide structural support to the skeleton. It also protects vital internal organs, such as the heart, the brain, the lungs and the spinal cord. Bone constitutes a mineral storage for the body containing calcium (Ca) and phosphate (P), which are released in the blood stream when necessary. Moreover, it has attachment points for skeletal muscles and supports locomotion

(Salgado et al., 2004). Bone also provides the environment for hematopoiesis (the production of all blood cells) within the marrow spaces (Bowman and Zon, 2009; Rafii et al., 1997). It also contains the yellow bone marrow, which is composed mainly of adipose cells and constitutes storage of chemical energy for the body (Gurevitch et al., 2009).

1.1.2 Cells and Extracellular matrix

Bone is composed of a cellular component and an extracellular matrix (ECM). The cellular part consists of three cell types: the bone-forming osteoblasts, the bone-resorbing osteoclasts, and the osteocytes, which are inactive osteoblasts trapped in the ECM. Osteoblasts and osteocytes originate from mesenchymal precursors named osteoprogenitors, whereas osteoclasts are derived from hematopoietic stem cells (HSCs) that give rise to monocytes and macrophages (Boyle et al., 2003). Osteoclasts are multinucleated cells formed by the fusion of mononuclear precursors (Udagawa et al., 1990).

The principal function of the osteoblasts is to synthesize the proteins of the bone ECM. They are also responsible for the expression of genes necessary for calcification and secrete important molecules that regulate the generation and the function of osteoclasts (Cohen, 2006; Rucci, 2008). Osteoblasts have an essential role in mineralisation. They promote hydroxyapatite (HA) formation by regulating the release of matrix vesicles containing Ca and P (Clarke, 2008). They also express high level of alkaline phosphatase (ALP), which is anchored to the external surface of the plasma membrane and has been indicated to play an important role in bone mineralisation (Manolagas, 2000). Their membrane has been also shown to possess receptors for parathyroid hormone (PTH). When bone formation is completed, a high percentage of osteoblasts undergo apoptosis and the remaining become osteocytes or bone-lining cells. When preosteoblasts stop proliferating, osteoblast precursors change shape from spindle-shaped osteoprogenitors to large cuboidal differentiated osteoblasts. It is characteristic the fact that osteoblasts never appear or function individually but they always appear in as clusters of cuboidal cells along the bone surface (~100-400 cells per bone forming site) (Hadjidakis and Androulakis, 2006). Moreover, mature osteoblasts have large nuclei, enlarged Golgi structures and extensive endoplasmic reticulum, which support them in synthesizing bone matrix and secrete mainly collagen type I (Col I) (Clarke, 2008).

Osteocytes act mainly as mechanosensors by recognizing mechanical stress signals received by the cell and turn them into biologic activity. They also contribute to homeostasis by maintaining the right oxygen and mineral levels. Osteocytes are considered an important regulator of bone

mass and a key endocrine regulator of phosphate metabolism (Dallas and Bonewald, 2010). Osteocytes are the most abundant cell type in bone, being 10 times more than osteoblasts. They are usually located throughout the mineralised matrix and communication with each other and with cells on the bone surface is performed via multiple extensions of their plasma membrane (Manolagas, 2000). Osteoclasts are the cells responsible for resorption of bone matrix through the process of bone remodeling. Their function is regulated from signals received from osteoblasts (Boyle et al., 2003).

It has been indicated that growth factors and cytokines produced in the bone marrow microenvironment control the development and differentiation of both osteoblasts and osteoclasts. Moreover, it has been shown that various hormones and mechanical stimulus also exert significant effects and participate in this process. Finally, there are several adhesion molecules that mediate cell-cell and cell-matrix interactions (Manolagas, 2000).

Osteoblasts and osteoclasts play a significant role in coordinating the bone remodeling. Bone remodeling is a complex process, which occurs throughout life and by which old bone is continuously replaced by new tissue in order to maintain bone strength, mineral homeostasis and adapt to changing biomechanical forces (Clarke, 2008), following many cycles of bone resorption and subsequent new bone formation (Hadjidakis and Androulakis, 2006).

ECM constitutes the second element, which is the cornerstone of bone tissue. The matrix, which is responsible for the mechanical strength of the bone tissue, is formed by an organic and a mineral phase. The organic phase is mainly composed of collagen fibers and few non-collagenous proteins whereas the mineral phase consists of HA crystals (Frohlich et al., 2008). ECM offers several significant functions to the cells. One of its major roles is that it acts as a 3D scaffold for the cells allowing them to attach and grow on it by offering them the required environment for the development of the specific tissue (Huang and Ingber, 1999). It also provides structural support and regulates intercellular communication and the dynamic behavior and nature of the cells. Moreover, it separates tissues from each other acting as a borderline (Baptista et al., 2009). Finally, ECM contains important bioactive molecules such as water, nutrients, cytokines and growth factors, which regulate cell behavior.

ECM is considered as a dynamic network of cell-secreted molecules that regulate cell behavior by modulating the proliferation and differentiation. To summarize, bone development and regeneration are complex processes regulated by a number of different growth and transcription factors, which have been indicate to coordinate the interaction of cells and matrix in response to external or internal stimuli (Kneser et al., 2006).

1.1.3 Bone development

Bone development takes place through two mechanisms which both begin with mesenchymal stem cell (MSC) proliferation and condensation (Goldring et al., 2006; Karsenty and Wagner, 2002). The first mechanism is called endochondral ossification (Figure 1.2b). During this process, MSCs differentiate initially into proliferating chondrocytes and form a cartilage template. In the next step, they further differentiate into hypertrophic chondrocytes which are subsequently invaded by blood vessels and finally, they are replaced by bone. This process takes place in the development of long bones and the majority of bones of the skeleton (Kelly and Jacobs, 2010). The second mechanism is called intramembranous ossification (Figure 1.2a) and is the direct differentiation of the condensed MSCs into osteoblasts (Frohlich et al., 2008). This process mainly occurs in the head region to generate the flat bones of the skull (Kelly and Jacobs, 2010).

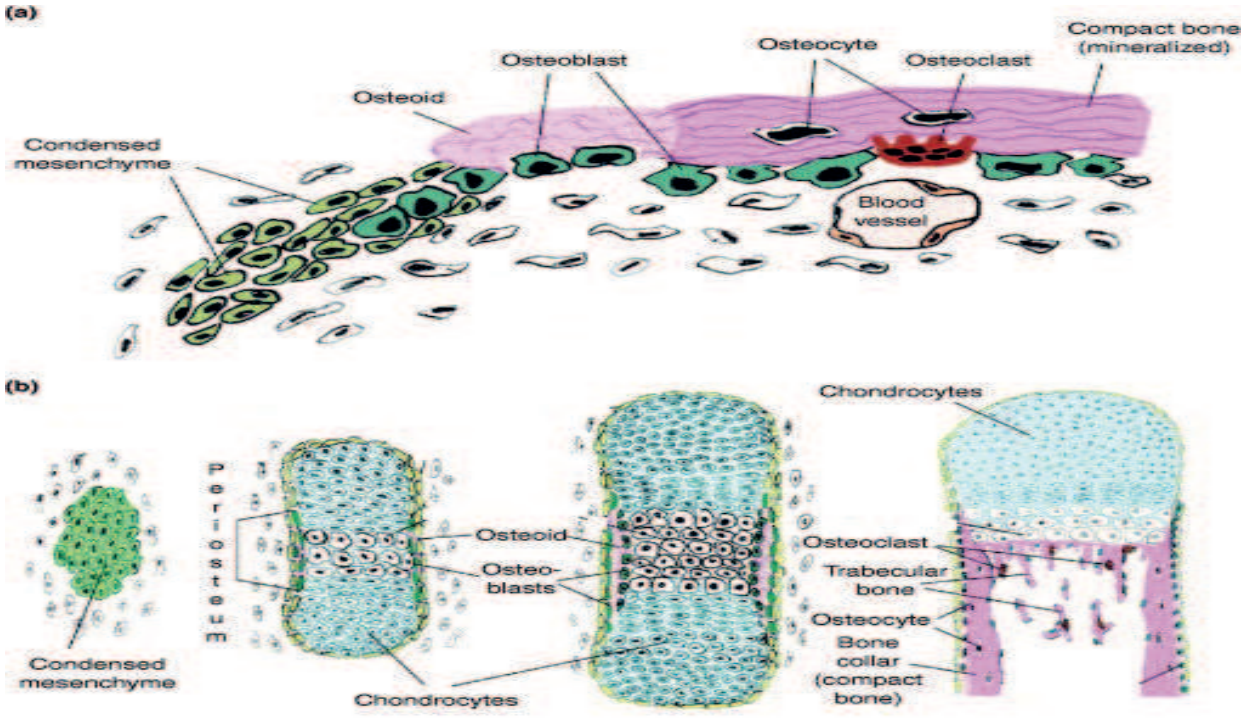


Figure 1.2: Intramembranous (a) and Endochondral (b) ossification. Adapted from (Hartmann, 2006; Hartmann, 2009)

Several important gene markers are expressed during different stages of bone development and can be used in order to identify the status of the cell at a specific time point. A number of essential bone markers are usually tested to confirm osteogenic differentiation. Markers of

cartilage and hypertrophic chondrocytes can also be tested in order to identify the ossification process followed. Finally, gene markers of the three germ layers can also be evaluated in order to follow the process of differentiation.

1.1.4 Gene expression markers of bone development, ossification pathway and three germ layer formation

ESCs have the capacity to differentiate to all cell types consisting the embryo apart from the placenta. In order to follow the differentiation pathway towards a specific cell type, ESCs go through the stage of the three germ layer formation and they first need to commit to a specific germ layer before proceeding to the differentiation towards the desired cell type. Gene expression markers are presented in the following section according to the stage of differentiation. Initially, the expression of markers from each of the three germ layers was evaluated in order to get more insight information regarding the differentiation process and the efficiency of the employed protocol. Starting with ectoderm, the expression of the transcription factor Pax6 and of Nestin protein was evaluated.

- It has been previously indicated that expression of homeobox transcription factor **Pax6** in uncommitted ESCs favored their differentiation towards neuroectodermal lineage. In particular, it has been proposed that in mouse cells Pax6 expression was implicated in the progression of neuroectoderm toward radial glia-derived early neurons (Suter et al., 2009). It is well known that Pax6 is highly conserved among vertebrate and invertebrate species and is crucial for the development of the eye, pancreatic islet cells and the central nervous system (CNS). The same group indicated that there is enhanced Pax6 expression during induction of neural progenitor cells from ESCs (Gao et al., 2011).

- **Nestin** is a well-known neuroectoderm marker (Mansergh et al., 2009) identified on neural progenitor cells and specifically a marker of the central nervous system (Jin et al., 2009). Earlier research suggested that nestin may be a common marker of multi-lineage progenitor cells as it has been demonstrated that it is abundant in early embryonic stem-derived progenitor cells that proliferate and differentiate into the neuroectodermal, mesodermal and endodermal lineages. It has been also proposed that nestin-expressing cells may be directly implicated in tissue regeneration (Wiese et al., 2004).

The next germ layer to be evaluated was the endoderm. The expression of the transcription factors Gata4 and Sex Determining Region Y- Box17 (Sox17) was tested.

- During early mouse development, **Gata4** is expressed in the primitive endoderm, the first germ layer to be formed from the pluripotent inner cell mass (ICM) of the early blastocyst (Arceci et al., 1993; Morrisey et al., 1996). Its expression is restricted to the primitive endoderm and the derived visceral and parietal endoderm of the extraembryonic tissues (Arceci et al., 1993; Koutsourakis et al., 1999; Morrisey et al., 1998). Apart from endoderm formation, Gata4 is considered a key regulator of cardiogenesis (Pikkarainen et al., 2004) and heart development (Afouda et al., 2008) and essential for cardiomyocyte gene expression and differentiation (Grepin et al., 1995). Gata4 has been shown to function in both mesoderm and endoderm, consistent with a role in regulating the emerging lineages derived from a common precursor germ-layer, referred to as the mesendoderm (Loose and Patient, 2004).

- **Sox17** is expressed in mouse visceral endoderm and parietal endoderm as well as in definitive endoderm. It has been suggested to have a crucial role in the differentiation of the definitive endoderm in the mouse, thus indicating the conservation of the mechanism of endoderm development between vertebrates (Kanai-Azuma et al., 2002).

Finally, the expression of markers from the germ layer of interest, mesoderm in that case, was evaluated. The protein Desmin and the transcription factors BMP4 and Hand1 were tested.

- **Desmin** has indicated strong expression from mesodermal precursors (Torres et al, 2012). It has been shown to regulate the expression of transcription factor genes important for mesodermal and early myocardial differentiation. Moreover, it promotes proliferation of differentiating cardiomyocytes and also has a well-known function in the maintenance of the muscle cell phenotype. These data suggest its supportive role during commitment and early differentiation of cardiomyocytes (Hofner et al., 2007).

- **BMP4** has been indicated to play a significant role in fate decision in ESCs as it has been demonstrated to reduce neuroectoderm differentiation and to induce mesoderm formation (Johansson and Wiles, 1995; Lindsley et al., 2006; Ying et al., 2003). In particular, during ESC differentiation, BMP4 has been shown to enhance the expression of posterior and extraembryonic mesoderm genes at the expense of anterior primitive streak patterning (Willems and Leyns, 2008). BMP4 has been indicated to participate in the differentiation of ES cells toward cardiac or hematopoietic fates (Kennedy et al., 2007; Laflamme et al., 2007).

- The transcription factor **Hand1** has been indicated to possess an important role in extraembryonic mesodermal and heart development (Firulli et al., 1998). It is expressed in neural crest derivatives and lateral mesoderm (Cserjesi et al., 1995; Thomas et al., 1998). Recently, it has been suggested as one of the key cardiac transcription factors (Mendjan et al., 2014).

Following germ layer formation, the expression of a hypertrophic chondrocyte marker and a couple of cartilage markers was also examined in order to evaluate if endochondral or intramembranous ossification was followed.

- **Collagen 10 (COL10)** is the only known hypertrophic chondrocyte–specific marker. It has been shown that Runx2 can directly transactivate COL10 promoter both *in vitro* and *in vivo* via Runx2 binding sites found in the promoter region (Zheng et al., 2003).
- The first transcription factor to be tested was **Sex Determining Region Y-Box9 (Sox9)**. Sox9 expression has been indicated in all chondroprogenitor cells in mouse embryos and at higher levels in chondrocytes but its expression is completely shut off in hypertrophic chondrocytes. Research has also indicated that Sox9 together with Sox5 and Sox6, are responsible for activating the chondrocyte-specific genetic program that produces the characteristic cartilage ECM (de Crombrugghe et al., 2000). Sox9 is particularly needed for formation of mesenchymal condensations (de Crombrugghe et al., 2001).
- **Aggrecan** is a large chondroitin sulfate proteoglycan and constitutes the major structural component of the articular cartilage ECM (Kiani et al., 2002). It has been demonstrated that Sox9 activates and enhances the transcriptional activity of the aggrecan promoter (Sekiya et al., 2000).

Finally, the expression of a number of important bone genes was examined in order to evaluate *in vitro* bone formation. These osteogenic markers are only expressed in particular stages during bone formation.

- **Alkaline phosphatase (ALP)** is known to be an early marker of the osteoblast phenotype. It is upregulated at the onset of differentiation and subsequently decreased as mineralisation occurs (Jia et al., 2014; Rawadi et al., 2003).

- **Collagen 1 (COL 1)** is the most abundant protein in mineralised tissues (Young, 2003a; Young, 2003b) and is widely distributed in almost all connective tissues with the exception of hyaline cartilage. It is the major protein in bone, skin, tendon, ligament, sclera, cornea, and blood vessels (Viguet-Carrin et al., 2006). COL 1 comprises approximately 95% of the entire collagen content of bone and about 80% of the total proteins present in bone (Niyibizi and Eyre, 1994).

- **Bone Morphogenetic proteins (BMPs)** are considered the most potent inducers of osteoblast differentiation. They stimulate osteoprogenitors to differentiate into mature osteoblasts but also induce nonosteogenic cells to differentiate into osteoblast lineage cells (Gersbach et al., 2007; Nakamura et al., 2005; Wang et al., 1993). BMPs are members of the Transforming Growth Factor-β (TGF-β) superfamily and play critical roles as soluble mediators of tissue morphogenesis. They have a significant role in embryonic development and the induction of bone formation in postnatal life. It has been indicated that these molecules activate the cascade of bone differentiation and induce bone formation when implanted in heterotopic extraskeletal sites (Reddi, 1998; Reddi, 2005; Ripamonti, 2006; Ripamonti et al., 2006). **BMP-2** in particular has been shown to stimulate the expression of ALP through activation of the BMP receptor (BMPR) (Nohe et al., 2002). It is also involved in the expression of the nuclear transcription factors Cbfa-1/Runx2 (Jadlowiec et al., 2003) and Dlx5 (Kim et al., 2004). BMP-2 has been indicated to participate in the commitment of multipotent stromal cells (MSCs) towards the osteogenic lineage and to induce new bone formation (Riley et al., 1996).

- **Runt-related transcription factor2 (Runx2), also known as Core-binding factor subunit alpha-1 (Cbfa-1)**, is a transcription factor essential for osteogenic cell differentiation (Ziros et al., 2008). It is required for osteoblast commitment and for both intramembranous and endochondral bone development (Toth et al., 2010). At the early stage of embryogenesis, Runx2 determines the osteoblast lineage from multipotent mesenchymal stem cells, whereas inhibits it at the late stage (Ducy et al., 1999). Runx2 is initially detected on day 9.5 in the notochord and on day 10.5 is strongly expressed in all developing skeletal elements (Otto et al., 1997). Ectopic expression of Runx2 in mesenchymal cell lines leads to up-regulation of osteoblast-specific genes like OCN, ALP, BSP and COL1a1 (Ducy et al., 1997).

- The zinc-finger-containing transcription factor **Osterix (OSX)** acts downstream of Runx2 and has been indicated to be essential for osteoblast differentiation and bone formation during embryonic development (Nakashima et al., 2002). OSX is specifically expressed in osteoblast

lineage cells and, at lower levels, in prehypertrophic chondrocytes (Zhou et al., 2010). It has been shown to be essential for activation of bone-specific genes that support bone formation in embryos and adults (Sinha and Zhou, 2013).

- **Osteonectin (ON), also known as secreted protein acidic and rich in cysteine (SPARC),** is the major non-collagenous bone matrix protein. It is highly expressed early in osteogenic differentiation and decreases when cells acquire more mature phenotype (Delany and Hankenson, 2009; Kusafuka et al., 1999).

- **Osteopontin (OPN)** is considered a mid-stage marker (Frank et al., 2002). It is a protein secreted by osteoblasts throughout the differentiation process, but is up regulated at the onset of matrix mineralisation (Hughes and Aubin, 1998).

- **Osteocalcin (OCN)** is a protein secreted by osteoblasts and signals terminal osteoblast differentiation (Paredes et al., 2004).

- **Bone sialoprotein (BSP)** is a noncollagenous small integrin-binding ligand N-linked glycoprotein (SIBLING) in mineralised tissues such as bone, dentin, cementum, and calcified cartilage (Fisher et al., 1990). During bone morphogenesis, BSP is produced by osteoblasts, osteoclasts, osteocytes and hypertrophic chondrocytes. Through binding specific integrins, BSP mediates cell attachment and signaling, HA nucleation with subsequent mineral deposition and binding of Col I with high affinity (Fisher and Fedarko, 2003; Ganss et al., 1999; Kruger et al., 2013; Masi et al., 1995; Tye et al., 2005).

- A recently discovered bone marker is Activating Transcription Factor 4 **(ATF4)**, which has been shown to regulate terminal differentiation and function of osteoblasts. It also determines the synthesis of Col I, the most abundant bone ECM protein by favoring amino acid import (Yang et al., 2004). The same group discovered that ATF4 osteoblast-specific accumulation is controlled by a post-translational mechanism. Moreover, they indicated that ATF4 could induce osteoblast-specific gene expression in non-osteoblastic cells (Yang and Karsenty, 2004). It has been also shown that there is a functional relationship between ATF4 and Runx2. They form a complex (Dobreva et al., 2006) and cooperatively regulate OCN mRNA expression by binding to specific sites on its promoter (Xiao et al., 2005).

In Figure 1.3, there is a graphical representation indicating the process starting from three germ layer formation, following endochondral ossification and subsequent osteogenic differentiation.

Gene markers associated with each stage are presented in order to help characterize cell status based on gene expression.

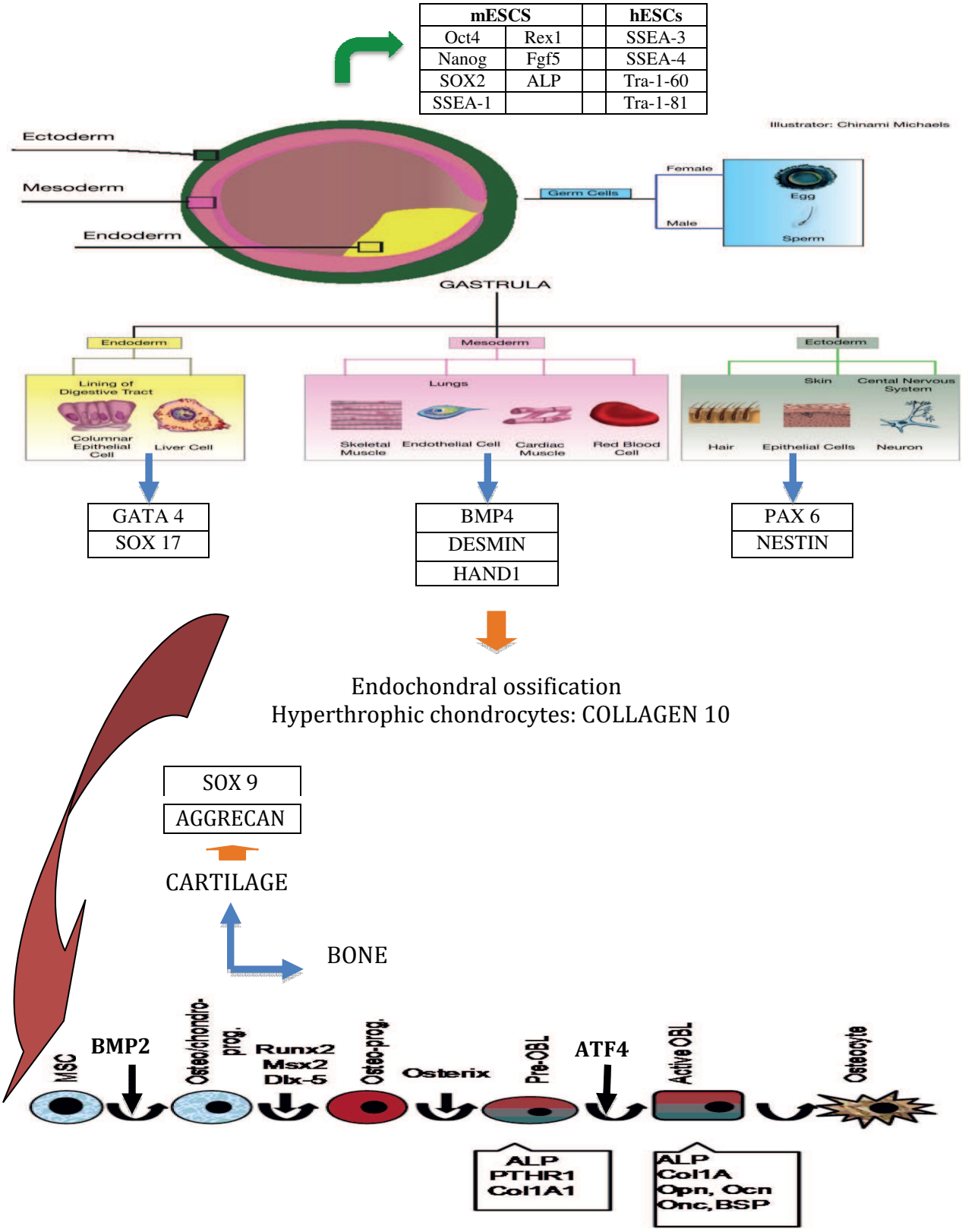


Figure 1.3: Gene expression markers characterizing pluripotency, germ layer formation, endochondral ossification and osteogenic differentiation. Adapted from (Chinami, 2014; Rucci et al., 2009).

Apart from the above-mentioned and widely used bone markers to evaluate gene expression and osteogenic differentiation, there are also some recently discovered transcription factors. In particular, members of the Msx and Dlx family have been indicated to play an important role in patterning and formation of skeletal structures during embryogenesis and are acting as upstream regulators of Runx2, a key activator of osteogenesis (Hassan et al., 2006; Samee et al., 2007).

- Specifically, **Dlx5** and **Msx2** proteins have been shown to antagonize each other during osteoblast differentiation by competing for binding to common response elements in bone-specific marker genes such as OCN (Hassan et al., 2004; Newberry et al., 1998; Ryoo et al., 1997) and ALP (Kim et al., 2004). It has been suggested that this regulation takes place at the transcription level with Msx2 inhibiting Dlx5 expression and vice versa. Results regarding the exact role of these proteins are controversial within research groups but in general, the majority suggest that Dlx5 functions at later stages (Ryoo et al., 1997) and stimulates osteoblast differentiation by activating the promoter of bone marker genes (Bendall and Abate-Shen, 2000; Kim et al., 2004; Newberry et al., 1998) whereas Msx2 has an opposite role and stimulates cell proliferation while inhibits osteogenic differentiation (Dodig et al., 1999; Hu et al., 2001) and in particular osteoblast terminal differentiation since it is expressed before OCN (Hoffmann et al., 1994). More recent studies indicated once more the significant role of Dlx5 in controlling the expression of bone-related genes (Samee et al., 2007; Samee et al., 2008).

- **Type 1 PTH receptor (PTHR1)** has being recognized as an important regulator of bone remodeling (Datta and Abou-Samra, 2009). It is expressed at later stage of differentiation by committed preosteoblasts (Moseley et al., 1991; Suda et al., 1996).

Deciphering the transcriptional mechanisms underlying osteogenic differentiation of ESCs is crucial in order to get deeper understanding of the followed process and try to ameliorate it.
Finally, another widely used indicator of bone formation is matrix mineralisation. Matrix mineralisation is considered a late-stage marker of the osteoblastic phenotype. It is measured by the amount of calcium deposition onto the scaffolds over the culture period (Sikavitsas et al., 2003).

1.1.5 Common bone disorders and data from the UK population

Osteoporosis is one of the most commonly occurred metabolic bone diseases. It affects mostly women and is characterized by increased bone resorption, meaning excessive loss of calcified matrix, bone mineral and collagenous fibers, which gradually results in reduction in total bone mass. It is often referred to as the **'fragile bone disease'.** Based on current data obtained from National Osteoporosis Society, each year in UK, there are about 300.000 fractures incidents due to osteoporosis. These incidents have a substantial financial cost for the National Health Service, which estimates the total cost for hospital and social care for patients with a hip fracture to more than £2.3billion per year. Nowadays, **it is estimated that around 3 million people in the UK have osteoporosis** and this leads to bones becoming fragile and breaking easily, resulting in pain and disability (http://www.nos.org.uk).

Another common metabolic disease is **Paget's disease** characterized by increased activity of osteoclasts, which break down bone more rapidly. Osteoblasts respond with depositing bone at an increased rate. This unbalanced remodeling causes bones to become thickened and enlarged but also brittle due to abnormal structural development. Based on current data from the Paget's Association charity, the disease in UK is happening to approximately 8% of men and 5% of women, by the age of 80 years (http://www.paget.org.uk).

Osteogenesis imperfecta (OI) or **brittle bone disease** is another common bone disorder. It is caused by a genetic mutation that affects the body's production of collagen, the main protein that forms the bone structure. Collagen produced is usually of poor quality or not enough to support its role, resulting in fragile bones, which is the hallmark of OI. This also causes increased fracture incidents. OI is difficult to diagnose. However, based on current data from The Brittle Bone Society, it is estimated that approximately 1 in every 15,000 people in the UK have OI (http://www.brittlebone.org).

Osteoarthritis is a very common disease affecting the joints. A joint is located between two or more bones and allows them to move in a freely but controlled way. The knees have also layers of cartilage between the bones acting as shock absorbers to spread the load more evenly across the joint. In osteoarthritis, there is incident of wear and tear of the surface within the joints resulting in difficulty to move smoothly. Cartilage covering the end of the bones becomes rough and thin while bone underneath gets thick. Osteoarthritis is causing pain and stiffness in the joints. Recent data from Arthritis Research UK suggest that **the disease affects approximately 8 million people in the UK** (http://www.arthritisresearchuk.org).

Another type of prevalent bone disease that affects many people is **bone cancer** and its various types. Resection of musculoskeletal tumors, like malignant bone and soft tissue sarcoma, results in large bone defects where bone regeneration is compromised or the quantity needed is far beyond the normal potential of self-healing. Reconstruction of these defects is still one of the most demanding procedures in orthopaedic surgery associated with high socioeconomic costs and highly influences patients' quality of life. The constraints of common treatment strategies have triggered a need for new therapeutic concepts to design and engineer functional bone grafts. The use of TE strategies together with a more biological approach can help satisfy the need for long-term repair and good clinical outcome (Holzapfel et al., 2013). Based on recent data from Cancer Research UK, around 560 people were diagnosed with bone sarcoma in 2011 in the UK, that's more than one person every day. Moreover, there were 246 deaths from bone sarcoma in 2012 (http://www.cancerresearchuk.org).

The above-presented information is summarized in Table 1.1.

Table 1.1: Common bone disorders affecting UK population

COMMON BONE DISORDERS	DATA FROM UK POPULATION
Osteoporosis	3 million people suffer from the disease
Paget's disease	Affects 8% of men and 5% of women
Osrteogenesis imperfecta	1 in 15.000 people have the disease
Osteoarthritis	8 million people are affected
Bone cancer	Deaths from prevalence of bone sarcoma

1.2 Current clinical treatment and observed limitations

Bone is a unique tissue with the ability to regenerate and self-repair small injuries. However, the case is different when dealing with large bone fractures, where injury exceeds a critical size and cannot heal by itself within the lifetime of the individual or in the event of unhealthy patients with pre-existent problems affecting bone formation. These incidents and their effective treatment are still considered a challenge for orthopaedic surgeons.

Different types of grafting procedures are usually followed to treat these defects (Brydone et al., 2010). The ideal graft in order to successfully regenerate bone should possess four specific features. The first one is osteoconductivity, meaning the ability to provide a platform, which facilitates the migration of osteoblasts and allows them to grow. The second one is osteoinductivity, indicating the capacity to induce osteogenic differentiation of undifferentiated or progenitor cells. The third characteristic is osteogenicity, which refers to the ability to form new bone using the cells seeded on the construct. Finally, a new requirement, which was recently added to the definition, is osteointegrativity, meaning the ability to incorporate with the surrounding native tissue (Greenwald et al., 2001).

Currently, gold standard treatment for osteogenic bone replacement in osseous defects is **autologous bone grafting** or **autograft.** This is performed by using bone obtained from another part of the patient, usually from the iliac crest. Autograft is considered ideal for grafting procedures, providing osteoinductive growth factors, osteogenic cells, an osteoconductive scaffold and the ability to integrate very well with the surrounding host tissue. Moreover, autologous transplantation has the advantage of not posing a risk for immune rejection as it comes from the patient' own body but has the drawbacks of donor site morbidity, limited material supply and the fact that is an invasive procedure (Amini et al., 2012). An alternative solution is **allograft** transplantation using cadavers or demineralised bone matrices, which both do not contain living cells and the material comes from another human donor. This approach has inherent risk of disease transmission and immune rejection. A third possible solution is **xenograft** where the material comes from a non-human donor posing a number of risks, such as immune rejection or cross species contamination (Oryan et al., 2014).

Apart from the grafting solutions, another way of treatment incorporates the use of **Bone-Graft Substitutes**, which consist of scaffolds made of natural or synthetic biomaterials with or without the combination of osteoinductive agents (Nandi et al., 2010).

Synthetic bone grafts are widely used because of their high availability but compared to an ideal bone graft; they possess only osteoconductive and osteointegrative properties while lacking osteoinductivity and osteogenesis (Giannoudis et al., 2005; Miron and Zhang, 2012). For example, Poly L lactic acid (PLLA) is a biocompatible polymer which is Food and Drug Administration (FDA) approved to be used for bone reconstruction due to the fact that is highly biodegradable (Schofer et al., 2011a; Schofer et al., 2011b). There are also composite graft substitutes combining two different biomaterials. Usually composites include an osteoconductive matrix with bioactive agents that provide osteoinductive and osteogenic properties (Malhotra et al., 2013).

It is of outstanding importance to mention that an ideal graft should be easy to use and readily available at low cost. Moreover, it is essential to be biocompatible, to provide biomechanical support and eventually to degrade in non-toxic byproducts (Giannoudis et al., 2005; Janicki and Schmidmaier, 2011). The above-mentioned information on the current grafting options and the existing limitations are summarized in Table 1.2.

Table 1.2: Current grafting options and the existing limitations

GRAFTING TREATMENT	LIMITATIONS	REFERENCES
Autograft	Donor site morbidity Limited material supply Invasive procedure	(Amini et al., 2012)
Allograft	Disease transmission Immune rejection	(Oryan et al., 2014)
Xenograft	Immune rejection Cross-species contamination	(Oryan et al., 2014)
Bone-Graft substitutes	Lack osteoinductivity Lack osteogenesis	(Nandi et al., 2010)

The majority of bone fractures heal well after treatment by bone grafting, distraction osteogenesis or the use of bone replacement materials. However, large bone defects, which cannot heal by themselves within the lifetime of the individual, are a challenge for orthopaedic surgeons. Millions of people around the world suffer from large bone fractures caused by trauma, infection and diseases such as osteoporosis or cancer like osteosarcoma (Calori et al., 2011). Currently applied treatments are neither effective nor sufficient to completely treat these defects and they usually lead to poor quality of life of the patient after few years. This fact makes urgent the need for the discovery of a novel and effective treatment strategy aiming to improve patient's life due to the increasing aging population and the lack of donor tissue.

1.2.1 Use of Osteoinductive molecules/Growth factors

One interesting strategy to improve the osteoinductive properties of the scaffolds used for bone regeneration is to incorporate the application of biologically active molecules, such as the growth factors (Jabbarzadeh et al., 2008; Janicki and Schmidmaier, 2011). The most important and widely used osteoinductive growth factors are analyzed in the following section.

1.2.2 Bone Morphogenetic Proteins (BMPs)

In 1965, Marshall Urist demonstrated that demineralised, lyophilized segments of bone were capable of inducing new bone formation when implanted into ectopic sites (Urist, 1965). Responsible for the ectopic bone formation was proven to be some low molecular weight bone glycoproteins named BMPs. BMPs are pleiotropic and multifunctional growth factors, which belong to the transforming growth factor b (TGF-b) superfamily. Several cell types, such as osteoprogenitor cells, osteoblasts, chondrocytes and platelets have been observed to synthesize these proteins. BMPs participate in several different functions, most of which are related with bone tissue. They are considered potent regulators of bone and cartilage formation and repair (Garrett, 2007; Reddi, 2001a; Reddi, 2001b).

During embryonic development, they support cell proliferation while in the adult life, they contribute to maintenance of bone homeostasis (Chen et al., 2004). They have been proved to be, through various *in vitro* and *in vivo* studies, the strongest osteoinductive molecules able to regulate mesenchymal cell proliferation and differentiation to osteoblasts. They actively regulate both endochondral and intramembranous ossification and support bone formation and skeletal integrity (Carreira et al., 2014a; Carreira et al., 2014b). They also support the development of many cell types in various tissues and participate in a cascade of events starting from chemotaxis, continue with regulation of growth and differentiation, to finish with angiogenesis and apoptosis (Lissenberg-Thunnissen et al., 2011).

Nowadays, there are about 20 different human BMPs that have been discovered and grouped in subfamilies (Miyazono et al., 2005; Wu et al., 2007). Treatment with these proteins is used all over the world for spinal fusions, non-union fractures, bone defects, osteoporosis and maxillofacial reconstruction. In 2002, the U.S. FDA approved the use of two recombinant human BMPs for specific clinical applications. The first one was recombinant human BMP-2 with the commercial name INFUSE Bone Graft (McKay et al., 2007) which received approval for fusion of the lumbar spine in skeletally mature patients with degenerative disc disease and for certain oral and maxillofacial uses. The other was recombinant human osteogenic protein-1 (rhOP-1) or recombinant human BMP-7 with the commercial name OP-1 implant or Osigraft which received approval for an alternative to autograft in recalcitrant long bone nonunions (White et al., 2007).

BMPs are promising molecules for TE and bone therapy. However, there are several significant drawbacks regarding their clinical application to humans. The first issue is their high cost. Recombinant technology products like the BMPs require a high financial cost for their production. Another disadvantage of these molecules is their short half-life. They also need

appropriate carriers for efficient delivery. BMPs, in order to be effective for *in vitro* applications, are used in very high superphysiological concentrations in the experimental procedures. Furthermore, these cytokines and their receptors are regularly found within tumors. The last two observations, regarding the location and the application of these molecules, raise suspicions for carcinogenicity of these compounds and there is literature evidence supporting this idea and observing such outcome (Laitinen et al., 1997; Poynton and Lane, 2002; Soda et al., 1998; Yoshikawa et al., 1994).

1.2.3 Parathyroid Hormone (PTH)

Another FDA approved anabolic agent for treatment of Osteoporosis is PTH. Human PTH is an 84-amino acid, single-chain polypeptide with a molecular weight of 9,425 Da. The section of human PTH between the N terminus and amino acid 34 (PTH1–34) retains the biological activity of full-length PTH (Morley et al., 2001) and is marketed as a drug. PTH is secreted from the parathyroid gland and has an important role in regulating calcium levels in the blood. When calcium in the blood decreases, PTH is secreted and stimulates bone metabolism, which in turn releases calcium from bone in the circulation to restore the appropriate level (Rizzoli and Reginster, 2011).

PTH has been shown to have different effects on bone remodeling depending on the way it is administered. For example, it has been indicated that intermittent administration of PTH causes bone anabolic effects while continuous use favors bone resorption (www.fda.gov). To further support this idea, preclinical and clinical studies have shown that intermittent treatment with PTH stimulated the creation of new bone and improved bone density and bone quality generating strong new bone that was resistant to fracture (Esbrit and Alcaraz, 2013). PTH stimulated also bone formation by increasing the number of osteoblasts (Jilka, 2007).

Nevertheless, serious side effects have been observed after PTH treatment suggesting that careful consideration and further research should be conducted to solve current problems and observed limitations. The way of administration had a significant impact on the resulting outcome. For example, some recent data from animal testing indicated that prolonged PTH administration led to development of osteosarcoma (Subbiah et al., 2010).

1.2.4 Other important growth factors

BMPs and PTH are the most widely used growth factors for bone regeneration. Moreover, platelet-rich plasma isolated from platelets is a source of various growth factors (Marx et al., 1998). It has been shown that many growth factors are released from platelets after activation (Franchini et al., 2005; Frechette et al., 2005) and can support bone formation. Some of these factors are the platelet-derived growth factor (PDFG), the transforming growth factor-β (TGF-β), the insulin-like growth factor-1 (IGF-1), the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF) (Lieberman et al., 2002).

1.2.5 Problems resulting from the employment of growth factors

The only currently approved osteoinductive molecules for bone anabolic treatment are the recombinant proteins BMP2, BMP7 and PTH. One major drawback for all of these proteins is their high cost due to the recombinant technology used for their production and purification. Moreover, a problem for the patients is the fact that current recombinant protein therapies must be administered through either repeated injections or surgical implantation leading to painful and invasive procedures (Bessa et al., 2008; Deal and Gideon, 2003). There is also the issue of immunogenicity of these molecules, which have been reported to cause undesirable immune reactions in patients (Dingermann, 2008; Hwang et al., 2009a; Schellekens, 2010).

One major issue with the therapeutic use of drugs is that they are administered in very high, super physiological concentrations of milligrams in assays, which are a million times higher as compared to the nanogram range *in vivo* (Bessa et al., 2008; Gamradt and Lieberman, 2004) and can often exhibit serious side effects and complications (Mroz et al., 2010).

The last years, problems of increased swelling and significant ectopic bone formation leading to significant pain and limit limb function as well as a high risk of adverse side effects (Lo et al., 2012b) have been observed and associated with the use of recombinant BMP2 (Smoljanovic et al., 2009; Smucker et al., 2006).

Research has indicated that effective doses of BMP in humans are very high compared to smaller animals and a possible reason for this could be related to a less responsive BMP signaling pathway in humans (Bishop and Einhorn, 2007). Finally, one major concern is the widely off-label use of BMPs for various applications leading to undesirable side effects (Woo, 2011).

As mentioned previously, PTH is widely applied for treatment of bone defects. However, apart from the above-mentioned general problems of all growth factors, this hormone exhibits some additional important aspects. To be more specific, parathyroid hormone-related protein (PTHrP) has been shown to regulate tumor-relevant genes and to play a role in tumorigenesis, modulation of tumor progression and response to treatment in breast cancer and bone metastases (Liao and McCauley, 2006; Luparello et al., 2001). In particular, PTHrP has been indicated to interfere with multiple cell survival and apoptosis signaling pathways by inhibiting both death receptor and cell cycle–mediated apoptosis signaling (Mak et al., 2012).

1.3 Necessity for novel approaches and alternative solutions for clinical treatment

The discovery of recombinant human BMPs for clinical treatment of bone defects in combination with the methods of TE created a new era in scientific research and opened up the field for more effective therapies. FDA approval of the use of growth factors led to several successful therapeutic applications and decreased the need for postoperative surgical interventions after complications.

Results of various *in vitro* and *in vivo* applications were really encouraging on the beginning. There appeared, however, significant issues and side effects related with the use of the growth factors. The major problem was the high cost of recombinant technology and the high concentrations of these molecules needed for effective treatment of bone diseases. There appeared also incidents of carcinogenesis and immunogenicity related with the employment of increased levels of growth factors.

All the above-mentioned findings plus protein instability created concerns among scientists and made urgent the need towards the pursuit of an alternative and more effective way of treatment of bone disorders. The idea was to identify a molecule that could induce the secretion of these osteoinductive factors and activate the required signaling pathways without the need to be provided in high concentrations.

Regenerative medicine (RM) applications and tissue engineering (TE) strategies emerged the last years as a new promising solution to try and effectively treat and heal all the potential bone related problems that could appear.

1.3.1 Regenerative medicine (RM)

RM is considered an interdisciplinary field of research aiming at repairing, replacing or regenerating compromised tissues and impaired functions resulting from injuries, diseases, congenital defects or aging. It employs different approaches including the use of soluble molecules, gene therapy, stem and progenitor cell therapy, TE and the reprogramming of cell and tissue types. These methods often stimulate and support the body's own self-healing capacity going beyond traditional transplantation therapies (Daar and Greenwood, 2007).

1.3.2 Tissue Engineering (TE)

TE is one of the major components of RM. Nowadays, TE and RM are considered as the same field of research and the same definition applies often to both of them. TE employs principles from engineering and life sciences and tries to better understand tissue formation and regeneration in order to create biological substitutes to improve tissue function (Kneser et al., 2006; Langer and Vacanti, 1993; Lyons et al., 2008).

In 2002, in a paper by Godbey and Atala, they distinguished *in vitro* and *in vivo* TE indicating the cellular and acellular approach of TE. *In vivo* TE was referring to the use of scaffolds with no cells and was relying solely on the body's regenerative capacity to heal the defect (Godbey and Atala, 2002). Acellular approaches employing different scaffolds are still used but for treatment of small-scale defects. Nowadays, the important contribution and the significant role cells play for tissue repair has been recognized and the majority of TE applications employ the cellular pathway.

To be more specific, TE is utilizing three main components to deliver its target, known as "the tissue engineering triad" (Figure 1.4) (Murphy et al., 2013).

The triad consists of an appropriate cell source, a suitable scaffold and the necessary regulatory signals (Murphy et al., 2013). TE aims at replacing damaged and non-functioning tissues with 3D cellular constructs obtained from the seeding of cells within a natural or synthetic scaffold (Baptista et al., 2009). These constructs should be functionally, structurally and mechanically equal to, if not better than, that which they have been designed to replace (Logeart-Avramoglou et al., 2005; Stock and Vacanti, 2001).

For successful outcome of TE applications, it is crucial not only to really understand the role of each component of the TE triad individually but also to realize the interplay between the cells, the scaffolds and the regulatory signals (Murphy et al., 2013).

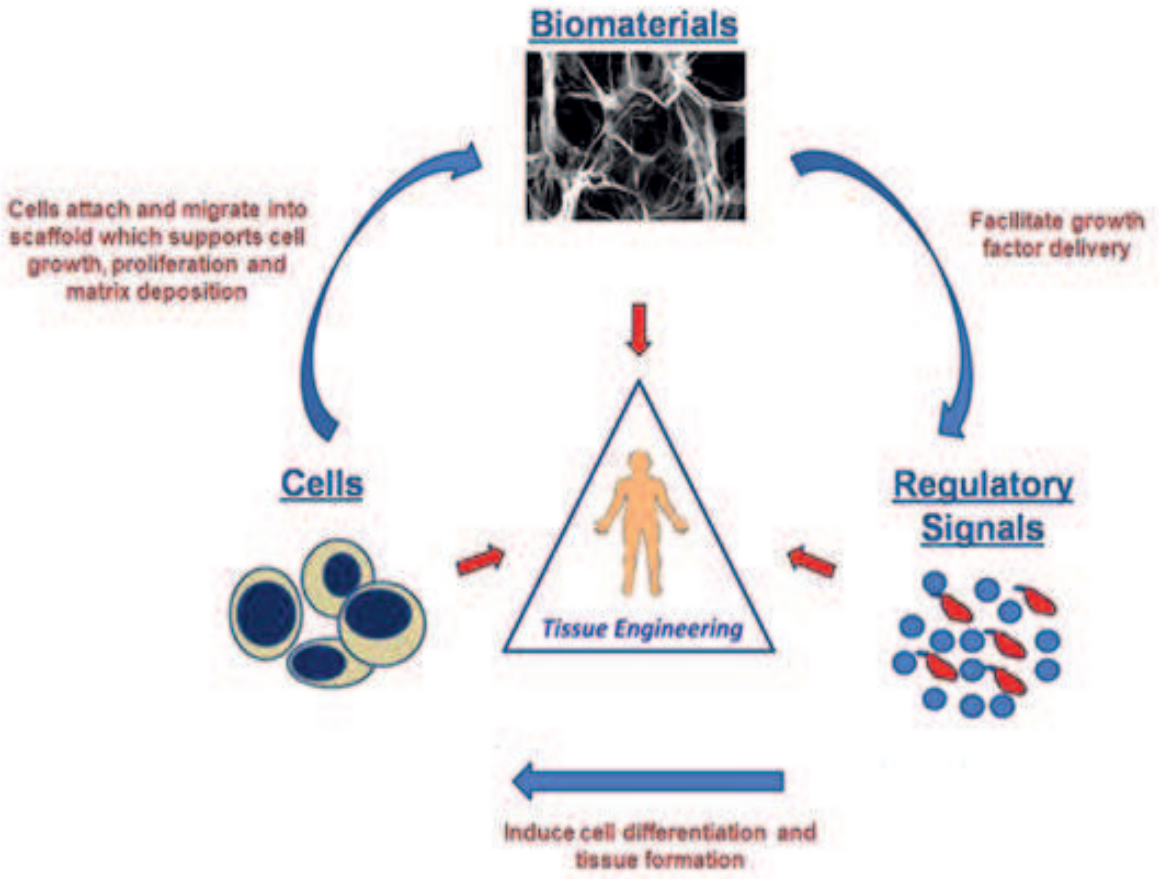


Figure 1.4: The concept of TE and the essential components of the TE triad. Adapted from (Murphy et al., 2013)

The choice of the cells is crucial in order to facilitate the formation of the desired tissue. Scaffold has an essential role in providing structure and substrate for the growth and development of the tissue. The scaffold is responsible for supporting 3D tissue formation within the construct and for mimicking the function of the natural ECM until the cells start producing their own matrix (Baptista et al., 2009; Stock and Vacanti, 2001). Finally, the use of appropriate growth factors or biophysical stimuli is essential in order to create the environment needed for properly directing the growth and differentiation of cells within the construct (Murphy et al., 2013).

1.3.3 3D bioprinting

Recent years, the use of microscale technologies has emerged as a novel approach for manipulating biological materials in the context of TE. The reason for this change is related with the advantages offered by this technology, which allows the reduction of the platform scale guiding to the need for low sample and reagent volumes as well as short experimentation times and cost reduction. The small experimental scale allows a better control over cell manipulation and permits testing of several different experimental parameters. Cell encapsulation and bioprinting strategies are some of the widely used microfabrication techniques to create 3D cell-containing materials (Selimovic et al., 2012).

Three-dimensional printing (3DP) otherwise known as additive manufacturing is a rapid prototyping technique that enables the creation of biocompatible materials, cells and supporting components into complex 3D functional living tissues (Murphy and Atala, 2014; Oryan et al., 2014). The main limitation of that method is the restricted options of materials to be used (Castilho et al., 2013).

3D bioprinting has already been applied for the production of tissues like skin, heart, bone and for research purposes like drug discovery and toxicology (Murphy and Atala, 2014). Moreover, it has been used to generate 2D and 3D structures for various purposes, including fabrication of scaffolds and tissue constructs for tissue regeneration. One major problem of the constructs created using printing technology is the lack of mechanical strength and integrity due to the innate properties of hydrogels (Seol et al., 2014). An alternative and recently discovered method for fabricating scaffolds with microscale and nanoscale resolution constituted the layer-by-layer microfluidic patterning, where cells and matrix materials flew through specific channels with controlled flow rates. 3D structures were generated by the sequential deposition of cells and matrix within the microchannels (Khademhosseini et al., 2006).

1.3.4 Gene therapy

A novel approach used for growth-factor delivery in the field of BTE is gene therapy (Caplan, 2000; Chen, 2001). This method includes the transfer of genetic material into the genome of the cell of interest, thus allowing the cell to express important bioactive factors inducing different functions for long time.

Gene transfer can be performed using a viral (transfection) or a non-viral (transduction) vector. Gene therapy poses issues related with high cost, efficacy and biological safety, which need further research before the method is applied to humans. There are, however, promising results, especially with the use of BMPs, from various performed animal experiments (Calori et al., 2009; Dimitriou et al., 2011; Tang et al., 2008b).

1.3.5 MicroRNAs

The recent discovery of microRNAs (miRNAs) and their ability to control global gene expression patterns gives new potential to the more effective treatment of bone defects and the more efficient stem cell differentiation.

MiRNAs are short, non-coding RNA molecules involved in transcriptional regulation that function to inhibit the expression of target mRNAs and regulate significant developmental processes (Erson and Petty, 2008; Lee et al., 2006). These small RNAs shorten the half-life of the mRNAs by binding to their 3' untranslated region and prevent their translation into functional proteins (Mariner et al., 2012).

MiRNAs have been associated with the control of bone formation and the regulation of osteoblast differentiation. Reports suggested that miRNAs were implicated in various levels of gene regulation during bone development and in several functions, such as the initial response of progenitor cells and the structural and metabolic activity of the mature tissue (Lian et al., 2012). Future application of miRNAs as gene therapy targets for the clinical treatment of metabolic bone diseases and bone injuries has been proposed (Dong et al., 2012)

1.4 Stem cell bioprocessing and TE for bone regeneration

The significant role of TE strategies and the contribution of stem cell bioprocessing protocols for the efficient cell differentiation have been recognized. One of the widely applied field of research and application of these methods is bone regeneration.

Bone tissue engineering (BTE) strategies, by combining cells with the right biomaterial and the use of necessary osteogenic factors, can generate a 3D construct that mimics natural bone tissue and can be used for bone formation offering an alternative solution to current existing limitations. Necessary component is the employment of the appropriate bioreactor in order to automate and scale up cell production (Figure 1.5) (Seong et al., 2010).

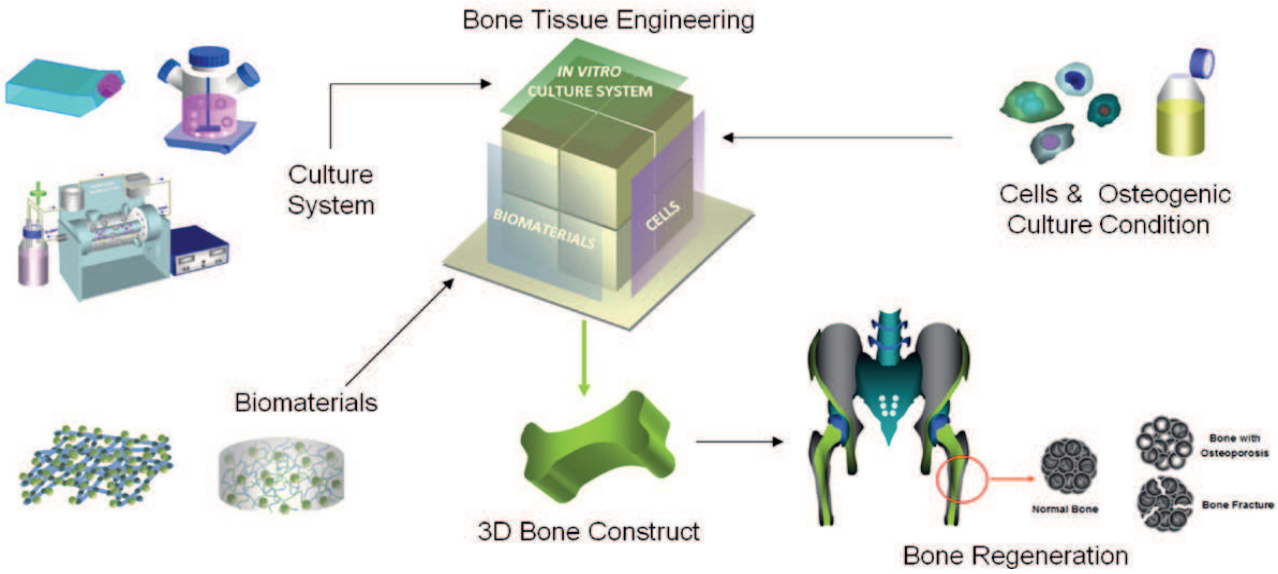


Figure 1.5: Components of BTE. Modified from (Seong et al., 2010)

The aim of BTE is try to eliminate many of the pitfalls of current treatments and create a more effective healing process. To further support this function, bioprocess engineering can contribute to alleviate possible limitations and support the efficient transition to 3D culture, which is closer to the original environment. The development of bioprocess technology can enhance the successful transfer of laboratory-based practice of stem cell and tissue culture to the clinic as therapeutics through the application of engineering principles (Placzek et al., 2009).

Each of the main three components of BTE will be examined and analyzed separately in the following sections where appropriate examples will also be provided and thoroughly described. It is important to mention that later, the initial trial idea has been reconsidered and the importance of the mechanical environment as the fourth necessary element for proper support and completion of bone regeneration and fracture healing has been introduced (Guilak et al., 2001; Virk and Lieberman, 2012).

1.4.1 Cell types

The first component of BTE is considered the appropriate cell type to be employed. Two main cell groups are widely employed: primary cells and stem cells. Primary cells mainly used include osteoblasts and chondrocytes whereas stem cells are divided in two main groups based on their origin, the somatic stem cells (SSCs) and the embryonic stem cells (ESCs). SSCs are subdivided to adult stem cells (ASCs) and foetal stem cells (FSCs) based on the age of the person they are derived from (Figure 1.6).

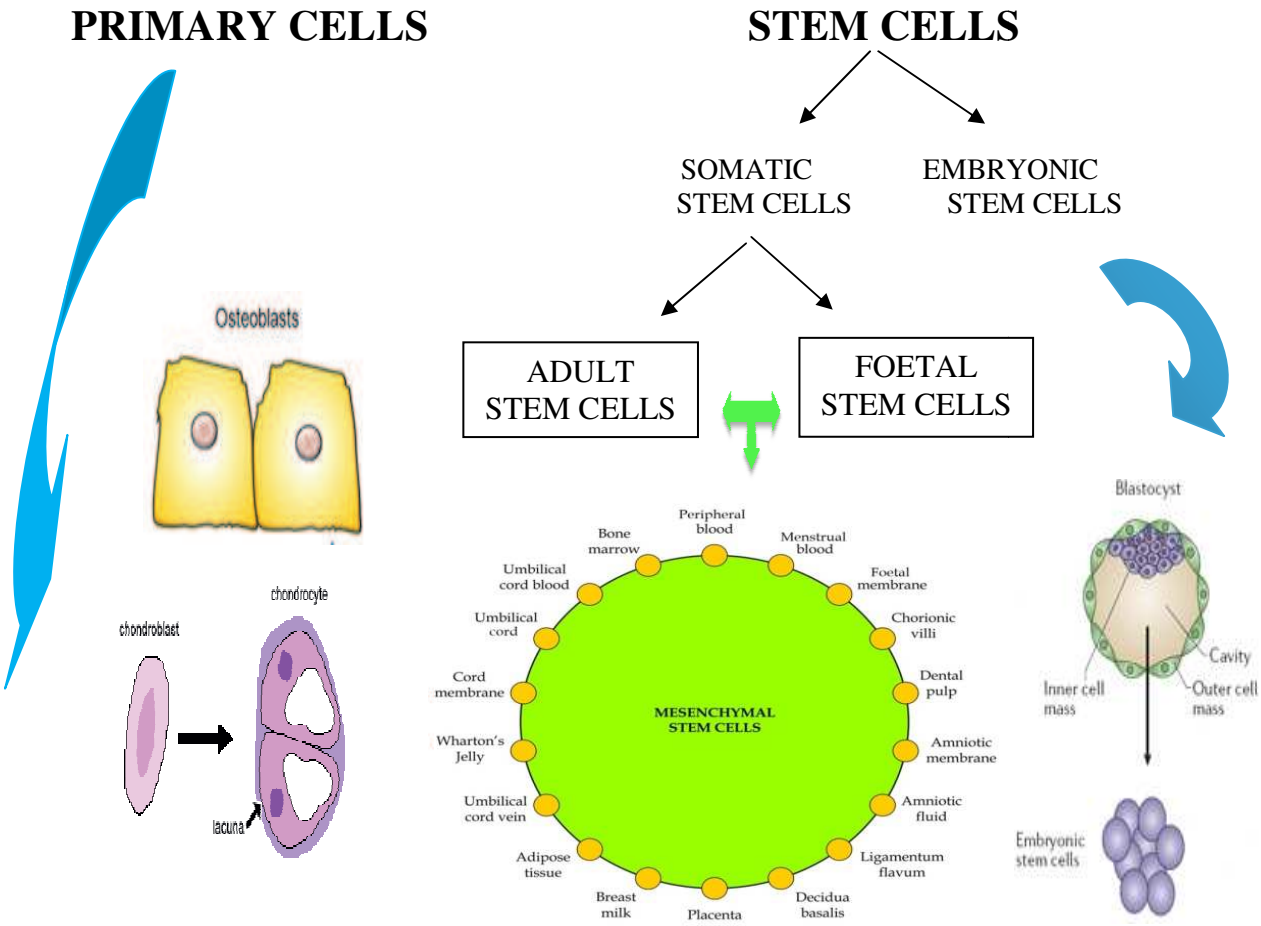


Figure 1.6: Cell types available for regenerative medicine applications. Obtained from (O'Connor and Crystal, 2006; Van, 2011)

1.4.2 Primary cells

A primary cell would be defined as a cell or cell line taken directly from a living organism, which is not immortalized. It refers to an end product with the ability to only regenerate the specific tissue. In the case of bone, this definition includes the various osteogenic cells that could be isolated from several adult tissues, including bone, bone marrow, periosteum and adipose

tissue (Hutmacher and Sittinger, 2003; Mailhot and Borke, 1998; Zuk et al., 2001). Previous studies have indicated the existence of considerable variation (Bouet et al., 2014) and differences in cell yield, proliferation and osteogenic potential between these sources (Jaquiery et al., 2005; Sakaguchi et al., 2005). Primary cells are also sensitive to the batch of serum used (Gong et al., 2009; Zheng et al., 2006).

Widely used candidates in various applications are osteoblasts. There are also several examples with the use of patient's own bone marrow cells and committed stromal cells of osteoblastic lineage. Chondrocytes have also been employed as a primary source for subsequent bone formation. The main advantageous characteristic of using primary cells directly isolated from the bone of the patient is the fact that these bone-forming cells lack immunogenicity and can be directly implanted on the defect site and start producing bone matrix immediately without risk of rejection (Meijer et al., 2007). However, these cells exhibit restricted proliferation capacity *in vitro* and for this reason, their application is limited (Jayakumar and Di Silvio, 2010; Liu et al., 2007; Meyer et al., 2004).

There are few studies reporting the direct use of primary cells and most of them employed osteoblasts. In one of these studies, researchers used periosteal derived osteoblasts and indicated ECM production and mineralisation (Wiesmann et al., 2003). In another study, researchers seeded osteoblasts on titanium and analyzed the observed interactions (Muller et al., 2006). Siggelkow ang his collegues studied the expression pattern of bone-related genes during osteoblast differentiation in primary human osteoblasts (Siggelkow et al., 1999) and compared it with the already established and known panel of rat calvarial osteoblasts (Owen et al., 1990; Stein et al., 1990). Neonatal calvarial osteoblasts were also employed in a recent study and compared the quality of the generated final product with that of ESCs and MSCs (Gentleman et al., 2009).

Primary bone cells can be obtained from various anatomical locations within the humanbody (Marolt et al., 2014). One recently discovered source includes primary humanalveolar bone cells that could be potentially used for periodontal and maxillofacial TE-based bone repair. Constructs made of these cells have been shown to enhance de novo bone formation in critical-size skull defects in immunodeficient mice (Pradel et al., 2008; Xiao et al., 2004; Xiao et al., 2003) and were used to treat jaw bone defects in several clinical case studies (Pradel et al., 2006; Pradel and Lauer, 2012; Springer et al., 2006). Prior work indicated that osteogenic cells originating from the jaw bone exhibit distinct differentiation properties both *in vitro* and *in vivo* (Akintoye et al., 2006) and also diverse drug responses compared to osteogenic cells originating from the long/iliac bones (Marolt et al., 2012a; Marolt et al., 2012b; Stefanik et al., 2008).

1.4.3 Stem Cells

Stem cells can be defined as clonal precursors, which have two significant characteristics: the ability to self-renew producing identical daughter cells or to differentiate into various specialized cell types (Placzek et al., 2009). Differentiation potency or plasticity varies between different stem cell types (Figure 1.7). Based on that, there are totipotent stem cells, which can give rise to a whole organism. Zygote, produced from the fusion of an egg and sperm cell, is the only totipotent cell. Next group are the pluripotent stem cells, which can differentiate into cells derived from any of the three germ layers (endoderm, mesoderm and ectoderm) of the embryo or germ cells but not cells of the placenta. Finally, there are multipotent stem cells, which can generate all the cells only of their tissue of origin. For example, HSCs can develop into several types of blood cells, but cannot develop into brain cells or other types of cells (Wobus and Boheler, 2005).

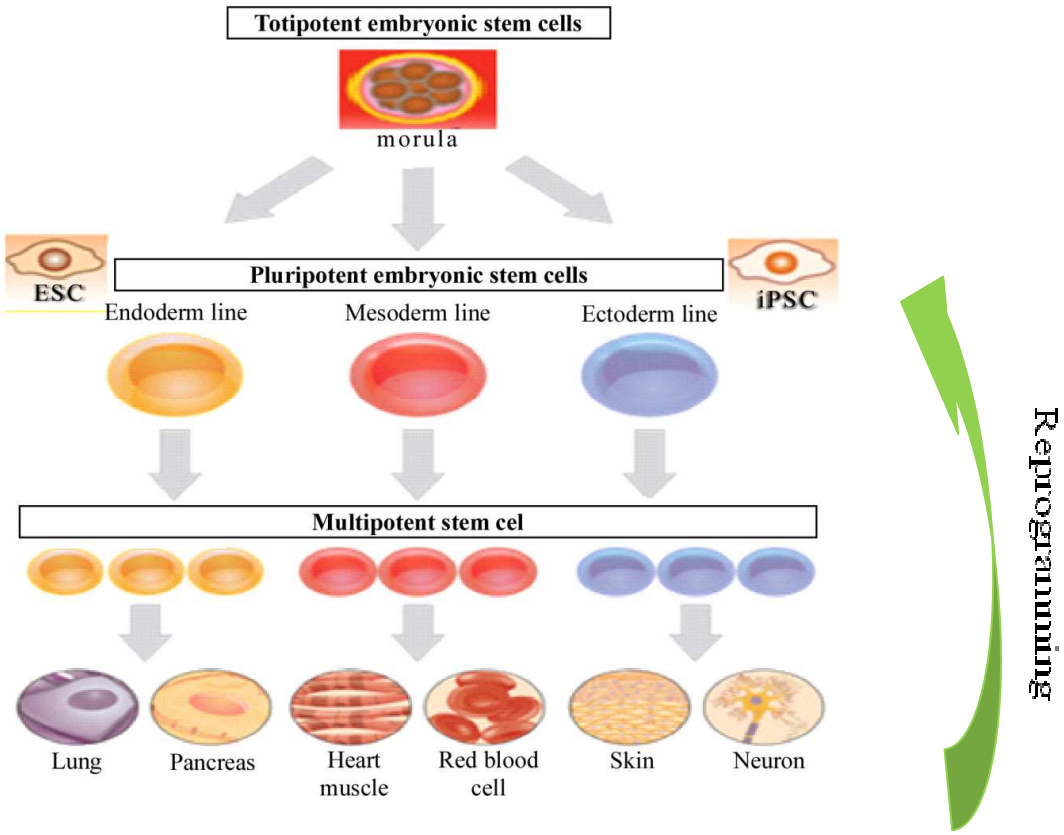


Figure 1.7: The hierarchy of stem cells. Adapted from (Kolomvounis, 2013)

Stem cells can be isolated at different developmental stages from embryo to adulthood and from various sources. There are two main groups: SSCs, which include ASCs and FSCs and ESCs from the blastocyst of the embryo (Guillot et al., 2006). Due to their advantageous characteristics over primary cells, different types of stem cells are widely employed in the majority of current applications aiming to achieve bone formation.

1.4.4 Somatic stem cells (SSCs)

1.4.5 Adult stem cells (ASCs)

SSCs have been isolated from various foetal, neonatal and adult tissues such as bone marrow, skin, liver, eyes, gastrointestinal tract and heart (Brittan and Wright, 2002; Fausto, 2004; Lavker et al., 2004; Leri et al., 2005; Modlin et al., 2003; Tumbar et al., 2004). Bone marrow contains the best characterised populations of stem cells including two types of stem cells, with notably different properties: MSCs and HSCs. MSCs can differentiate into mesodermal tissues such as bone, fat and cartilage, while HSCs can differentiate into cells present in the hematopoietic system (Friedenstein et al., 1974). The frequency of SSCs declines with age, with only 1 in 100.000 cells being a stem cell in teens and 1 in 400.000 cells at the age of 50 (Caplan, 2007).

SSCs are believed to reside in specific regions called niches within a tissue (Walker et al., 2009). Stem cell niche is the microenvironment composed of cells, which supports the development and nourishes the SSCs. It also contributes to the maintenance of tissue homeostasis (Moore and Lemischka, 2006). Stem cell niche has a pivotal role in regulating the balance between the quiescent and the activating state of SSCs. This is achieved through a complex network of interactions between somatic and supporting stem cells and also through the activation of various signaling pathways (Mimeault et al., 2007).

Niche cells provide the appropriate environment to protect stem cells from differentiation and apoptotic stimuli that would change their state (Moore and Lemischka, 2006). Because of the significant role of stem cell niche, SSCs have limited ability to expand *in vitro*. Nevertheless, they have the advantage of not forming tumors when transplanted *in vivo* and they are not rejected because they originate from the same tissue and they have similar immunological features. Another advantage is that ASC populations are more accessible than ESCs and for this reason they have already been used in cell replacement therapies within the tissue of their origin under pathological conditions. For example, they stimulate endogenous regenerative process after tissue injury (Brittan and Wright, 2002; Bryder et al., 2006; Mimeault and Batra, 2006).

Adult MSCs are multipotent and they can produce all cells of a particular germ layer or more restrictedly of a specific tissue. There is also strong evidence that ASCs may stimulate to self-renew or differentiate, for example into gastrointestinal cell lineages (Brittan and Wright, 2002; Moore and Lemischka, 2006) after activation by exogenous application of specific growth factors or cytokines *in vivo* (Mimeault et al., 2007). MSCs are the first cells that have been

widely used for BTE applications and bone replacement therapies. MSCs can differentiate relatively easy *in vitro* into osteoblasts by treatment with Dex that stimulates proliferation and promotes osteogenic differentiation. Other supplements such as ascorbic acid (AA) phosphate and 1,25-dihydroxyvitamin D3 (vitD3) and members of the BMP family of growth factors are also commonly used for osteogenic induction of MSCs (Muller et al., 2008).

MSCs have been widely tested *in vivo* in various animal models to evaluate their capacity in repairing large segmental bone defects. Initial experiments were conducted in mice or rat models (Bruder et al., 1998a; Bruder et al., 1998b; Bruder et al., 1998c; Pereira et al., 1998) and performed using allogeneic MSCs in order to assess their ability to facilitate bone healing. Results indicated the ability of allogeneic MSCs to efficient repair bone defects in the same degree as autologous MSCs. These positive results are related with the most advantageous property of MSCs, which is their immunosuppressive ability and regenerative capacity (Tsuchida et al., 2003). The absence of immunogenicity of allogeneic MSCs is the critical feature that renders these cells suitable for clinical applications in orthopaedics. For this reason, there are many experiments and applications using these cells, which can be guided towards osteoblast differentiation relatively easy (Guo et al., 2009).

The rat/mice model was an important demonstration of the principle of MSC-mediated therapy and results were successful but these animal models do not really represent human conditions. Prior to transferring this technology to the clinic, it should be first translated to a larger animal model such as the canine, which is biomechanically relevant to humans. Following previously applied protocols, experiments with large animal models were initiated and obtained results were considered more accurate and representative to be tested in humans. One of the first large animals to be tested was canine. Researchers isolated MSCs from canine bone marrow, they seeded them on a porous ceramic scaffold and demonstrated that they can form both bone and cartilage upon implantation in subcutaneous and intramuscular sites of canine animal model (Kadiyala et al., 1997). Further experiments demonstrated that new-bone formation could be elicited in critical-sized segmental defects in dogs by the implantation of autologous MSCs (Bruder et al., 1998b).

To take it one step further, a few years later the same group used allogeneic MSCs loaded on a ceramic scaffold and implanted in a critical-sized segmental defect in the canine femur. Results indicated repair of the defect and although there was no use of any immunosuppressive therapy, there was no observation of any immune reaction (Arinzeh et al., 2003).

The next animal model to be evaluated was sheep. In one study, researchers used bone marrow stromal cells seeded on coral scaffolds and they implanted the construct in a bone defect of clinically relevant volume in a sheep model. It has been shown that bone remodeling in these animals is comparable to that in humans and coral material has been previously employed in several clinical applications (Yukna, 1994; Yukna and Yukna, 1998). Obtained results indicated acceleration of the repair process and successful reconstruction of the large bone defects (Petite et al., 2000). A few years later, people from the same group repeated this experiment by implanting bone constructs made of MSCs and coral granules of a standard size in a sheep model and they observed repair of the large segmental defects. They also noticed that the osteogenic ability of the employed bone constructs approached significantly that of bone autografts (Viateau et al., 2007).

Kon and his collegues used a sheep model to evaluate the osteogenic ability of marrow-derived osteoprogenitor cells. Autologous cells were seeded on hydroxyapatite ceramic (HAC) scaffolds and implanted in critical-size tibial gaps. HAC scaffolds without cells were also implanted. Obtained results indicated a faster bone repair and regeneration of the defect in the cell-seeded scaffolds compared to the scaffolds alone (Kon et al., 2000).

MSCs were also tested in children with **Osteogenesis Imperfecta,** a congenital disease characterized by defective bone formation. Report showed that three months after infusion of cells into children, there was a general and significant improvement of mineralisation together with new bone formation and reduction of fractures (Horwitz et al., 1999).

Nowadays, MSCs are usually combined with different porous biomaterials and the generated constructs are then implanted in the defect site. This approach has better outcome and seems to enhance bone regeneration more efficiently (Bruder et al., 1998a; Bruder et al., 1998b; Bruder et al., 1998c; Cancedda et al., 2003; Richards et al., 1999; Rose and Oreffo, 2002; Vats et al., 2003). It is generally accepted that local implantation of porous biomaterials covered with autologous bone marrow MSCs represents the most effective approach for repairing bone defects (Quarto et al., 2001).

1.4.6 Foetal stem cells (FSCs)

FSCs may be harvested from most tissues and organs of the fetus (Gerrard et al., 2005) and mainly from bone marrow, blood, liver, kidney or from cord blood and placenta and tend to be more primitive the earlier in gestation they are derived. FSC collection causes ethical issues

because they are mainly isolated from abortal tissue after first or second trimester termination of pregnancy (Guillot et al., 2006). These cells do not pose the risks of ESCs such as the immune rejection and at the same time they are in a more progenitor stage in comparison to ASCs.

Amniotic fluid-derived stem cells (AFS) have shown the capacity to differentiate into various cell types of the three germs layers such as osteoblasts, adipocytes, chondrocytes, muscles, endothelial cells, neurons and hepatic cells (De Coppi et al., 2007; Delo et al., 2006; Kolambkar et al., 2007). Specifically, human AFS are the first foetal cells to be tested for their ability for osteogenic differentiation. These cells express a combination of ESC and ASC markers and exhibit some of the features of both. They have unlimited expansion potential but unlike ESCs they do not seem to be tumorigenic. They can be expanded for more than 250 population doublings retaining long telomeres and a normal chromosomal karyotype unlike ASCs (De Coppi et al., 2007).

Human amniotic fluid mesenchymal stem cells (hAMSCs) were seeded on microcarriers and cultured in a perfusion culture system to proliferate and achieve high cell numbers before induced to undergo osteogenic differentiation. Osteogenic differentiation was performed with the classic DAG protocol described previously for ESCs and MSCs. Mineral deposition and calcium content were confirmed with increased ARS. There was also increased gene expression of bone markers ALP, COL 1 and BGLAP (Chen et al., 2011).

In another study, aMSCs were seeded on scaffolds and the generated constructs were implanted in animals for sternal repair. Two months later, obtained results indicated defect closure in all animals and also increase in ALP activity. This finding demonstrated the promising role of these cells as candidates for alternative and potentially more efficient treatment of bone defects (Steigman et al., 2009). Researchers from another group seeded AFS on porous medical-grade poly-e-caprolactone (mPCL) scaffolds and they observed significant production of mineralised matrix. Bone formation was also confirmed *in vivo* in a rat subcutaneous model. This outcome suggested that AFS could be a potentially effective cell source for the production of large, mineralised constructs to be used for functional repair of large bone defects in various clinical cases (Peister et al., 2009).

Apart from the amniotic fluid, cells isolated from the placenta have been indicated to stimulate bone formation (Li et al., 2011). A relatively recent and novel cell source for bone regeneration is tooth-derived stem cells isolated from very young donors so they belong to the group of FSCs (Bluteau et al., 2008; Gronthos et al., 2002; Gronthos et al., 2000; Park, 2013; Yen and Sharpe, 2008).

1.4.7 Embryonic Stem Cells (ESCs)

ESCs are isolated from the ICM of the blastocyst of the developing embryo. Human ESCs (hESCs) were isolated for the first time by Thomson and his group at the University of Wisconsin (Thomson et al., 1998). ESCs have also been isolated from rodents (Graves and Moreadith, 1993; Martin, 1981) and primates (Thomson et al., 1995). In particular, the first pluripotent mESC line from mouse embryo was derived in 1981 (Evans and Kaufman, 1981).

Since their discovery, mESCs initially and hESCs the last years are widely used by many scientists all over the world because they possess two significant characteristics, which make them an ideal experimental tool. Firstly, ESCs can be propagated indefinitely *in vitro* as a stable self-renewing cell population without losing their undifferentiated status and secondly, they are pluripotent cells meaning that they can differentiate into any cell type in the embryo apart from the placenta (Handschel et al., 2010; Wobus et al., 2001).

Several authors have reported the differentiation of ESCs into cardiomyocytes (Kehat et al., 2002; Xu et al., 2002; Yang et al., 2008), hematopoietic cells (Kaufman et al., 2001; Wiles and Keller, 1991), neurons (Bain et al., 1995; Carpenter et al., 2001; Lee et al., 2007a; Schuldiner et al., 2001), chondrocytes (Hwang et al., 2008; Kramer et al., 2000; Oldershaw et al., 2010), adipocytes (Dani et al., 1997), muscle cells (Rohwedel et al., 1994), osteoblasts (Ahn et al., 2006; Kuznetsov et al., 2011; Mateizel et al., 2008), pancreatic islets (Assady et al., 2001) and endothelial cells (James et al., 2010).

There are also many studies demonstrating the capacity of hESC to differentiate into mesenchymal cells as a first step and subsequently used to regenerate a specific defected area (Barberi et al., 2005; de Peppo et al., 2010; Lian et al., 2007; Olivier et al., 2006).

ESCs are also characterized by long telomeres and high telomerase activity. Telomerase is an enzyme, which protects the ends of chromosomes, the so-called telomeres and expands them after each cell division in order to maintain their length. Telomere shortening is associated with replicative senescence (Mimeault and Batra, 2006; Thomson et al., 1998).

These properties make ESCs an ideal cell source for cell therapies and TE applications. One major demand in various therapies is the need for high cell numbers. ESCs with their unlimited self-renewal capacity and maintenance of undifferentiated status can provide the high number of cells needed for cell replacement therapies and other clinical applications. They can also be differentiated to all cell types of human body and applied for treatment of various diseases (Howard et al., 2008; Jukes et al., 2008).

In their undifferentiated state, ESCs express various markers that have been associated with pluripotency, the most important of which are the transcription factor octamer binding protein-4 (Oct-4) of the POU family, which prevents stem cells from differentiating and helps them maintain self-renewal and pluripotency (Scholer et al., 1990a; Scholer et al., 1990b), the homeodomain protein Nanog, which directs pluripotency and differentiation of undifferentiated ESCs and works together with the transcription factor SOX2, which regulates transcriptionally active genes involved in pluripotency maintenance (Chambers et al., 2003). Pluripotent ESCs in humans also express stage specific embryonic antigens SSEA-3 and SSEA-4, the keratan sulfate-associated antigens Tra-1-60 and Tra-1-81 and the zinc-finger containing gene Rex-1 at high levels (Chen and Daley, 2008; Guillot et al., 2007). Moreover, ESCs are characterized by high ALP activity (Wobus et al., 2001).

ESCs seem a very promising candidate for cell therapies and TE applications based on their advantageous performance *in vitro*. There are, however, a couple of important considerations regarding their transfer to *in vivo* applications in human beings.

It is not very easy to efficient direct ESCs to fully differentiate towards a specific lineage. Because of that, it has been shown that when injected *in vivo* into severe combined immunodeficient (SCID) mice, undifferentiated ESC form teratomas (Mimeault and Batra, 2006; Thomson et al., 1998). Tumor formation is a very serious risk and it indicates the importance to create more efficient differentiation protocols and reassure all cells are fully/terminally differentiated before *in vivo* implantation.

Another important hurdle for *in vivo* therapy is the immunological incompatibility and the challenge to identify a strategic solution to circumvent immune rejection (Grinnemo et al., 2008). Experiments using both human and mouse ESCs revealed that these cells were rejected after transplantation into immunocompetent mice in contrast to immunodeficient mice. Prior sensitization of the host by repeated transplantation resulted in accelerated ESC death. These results suggested that transplanted ESCs triggered cellular and humoral immune responses. The use of immunosuppressive drug regimens may be a solution (Swijnenburg et al., 2008).

1.4.8 Induced pluripotent stem cells (iPSCs)

In 2006, Takahashi and Yamanaka generated a new cell line that aimed to combine the advantages of embryonic and adult stem cells and eliminate their drawbacks. iPSCs were created from adult fibroblasts with the addition of four defined factors that resulted to their reprogramming back to their pluripotent state (Takahashi and Yamanaka, 2006). iPSCs have similar properties to ESCs without the ethical consideration of destroying embryos and the risk of immune rejection due to the fact that they come from the patient himself and not from a donor (Jiang et al., 2014).

There are already examples of successful derivation of osteoblasts from iPSCs. One of the first *in vitro* experiments directed the differentiation of mouse iPSCs (miPSCs) to mesenchymal cells as a first step and subsequently to osteoblasts. Cells were then seeded on scaffolds and were implanted *in vivo* in mice. Both *in vitro* and *in vivo*, cells demonstrated the capacity to generate a stable bone phenotype and an osteogenic matrix (Bilousova et al., 2011).

In another study, miPSCs were employed and exposed to osteogenic growth factor TGF-beta 1 or 3 in order to differentiate to osteoblastic cells. The cells were then seeded on ceramic scaffolds and implanted in SCID mice. Results showed the generation of progenitors from miPSCs that have the potential to make bone and they also demonstrated bone deposition on the scaffolds. These findings however should be tested in human iPSCs (hiPSCs) to evaluate if they are transferable (Li and Niyibizi, 2012).

A very recent study utilizes a new strategy trying to establish a stepwise differentiation protocol of miPSCs and hiPSCs into osteoblasts using four small molecules under serum-free and feeder-free conditions. The steps include an initial mesoderm induction, followed by osteoblast induction and finishing with ostoblast maturation. They noticed increased expression of osteoblast-related genes and proteins in both miPSCs and hiPSCs (Kanke et al., 2014).

In Table 1.3, the main advantages and disadvantages of various cell types employed in TE applications are presented.

Table 1.3: Characteristics of cell types employed in TE applications: Advantages and Disadvantages

CELL TYPES	ADVANTAGES	DISADVANTAGES	REFERENCES
Primary cells	- Lack immunogenicity - No risk of rejection	- Restricted proliferation capacity *in vitro* - Sensitive to serum batch - Different sources exhibit variations in: + Cell yield + Proliferation capacity + Osteogenic potential	(Bouet et al., 2014) (Jaquiery et al., 2005) (Sakaguchi et al., 2005) (Gong et al., 2009) (Zheng et al., 2006)
ASCs	- No tumor formation - Not immune rejection - Accessible	- Limited ability for *in vitro* expansion	(Brittan and Wright, 2002) (Bryder et al., 2006) (Mimeault and Batra, 2006) (Moore and Lemischka, 2006)
FSCs	- Not risk for immune rejection - More primitive stage in comparison to adult	- Ethical issues, use of abortal tissue	(Gerrard et al., 2005) (Guillot et al., 2006) (De Coppi et al., 2007)
ESCs	- Unlimited self-renewal capacity without losing undifferentiated status - Differentiation to any cell type from the three germ layers - Long telomeres and high telomerase activity	- Difficult to fully and efficiently differentiate - Tumor formation - Risk for immune rejection	(Handschel et al., 2010) (Wobus et al., 2001) (Mimeault and Batra, 2006) (Thomson et al., 1998) (Howard et al., 2008) (Jukes et al., 2008)
iPSCs	- No ethical considerations - No risk of immune rejection - Similar advantageous properties of ESCs	- Not efficient reprogramming protocol - Gene therapy strategies using virus are employed	(Jiang et al., 2014) (Warren et al., 2010) (Mikkers et al., 2012) (Maherali and Hochedlinger, 2008) (Soldner et al., 2009)

1.5 Culture systems: 2D versus 3D

Traditionally, stem cell culture and expansion is performed in 2D flat surfaces such as well plates and tissue culture flasks. The extended use of these conventional monolayer platforms is due to their simple structure, which makes them easy to handle and convenient for cell culturing purposes. An important advantage is also their low cost (Figure 1.8).

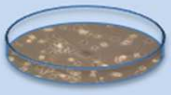
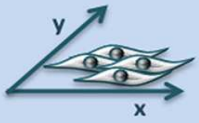
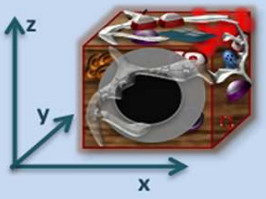
	STATIC condition	STATIC condition
Hydrodynamic forces		
Dimensionality	 **2D**	 **3D**
Characteristics of tissue-specific micro-environment (compared to the *in vivo* situation)	**a) Absence (loss) of:** • tissue architecture/ dimensionality (3D) • complex heterotypic cell-cell/ cell-matrix interactions • stroma/parenchyma interplays **b) Air-liquid interface** **c) Low mass transfer**: passive diffusion of gas and nutrients **short-term preservation of differentiated cell functions**	**a) Preservation of the integrity of:** • tissue architecture/dimensionality (3D) • complex heterotypic cell-cell/cell-matrix interactions • stroma/parenchyma interplays **b) Air-liquid interface** **c) Low mass transfer**: passive diffusion of gas and nutrients → • decreasing gradient of oxygen and metabolites from outer to inner parts of the explant: development of central core necrosis ( ) **rapid cell suffering/death**

Figure 1.8: Major characteristics and comparison between 2D and 3D static culture. Adapted from (Ferrarini et al., 2013)

However, 2D culture presents with a number of drawbacks, which render it not an optimal cell culture configuration. The main disadvantage of 2D plastic substrates is the limited capacity to reproduce the complex *in vivo* environment. It cannot support high cell numbers needed for cellular therapies due to the limited surface area. It cannot also mimic and recreate the *in vivo* environment where cells normally reside and cannot provide the appropriate signaling for cell differentiation. Consequently, it is not possible to support complex structures. Transport of important molecules is different between 2D and 3D cultures. Diffusion of molecules is the

mechanism employed in 2D cultures whereas convection is the advantageous mechanism in 3D. Another important issue is oxygen consumption. Cells in 3D consume more in comparison to 2D. This is due to the fact that cells in 2D are more space restricted and they reach confluence faster and stop proliferating due to contact inhibition whereas 3D cultures provide a larger surface area for cells to grow and increase their numbers. Sufficient oxygen supply is critical in the early stages of proliferation where cells need and consume more oxygen compared to late stages (Malda et al., 2004; Radisic et al., 2006).

Table 1.4: Advantages and Disadvantages between 2D and 3D culture configuration

Culture configuration	Advantages	Disadvantages	References
2D Cultures	- Easier environmental control, cell observation, measurement and manipulation than 3D - A rich body of literature exists, easier to perform comparisons with obtained results	-Altered gene expression - Different growth characteristics - Deficiency in cell-cell and cell-matrix interactions - Less compatibility with *in vivo* systems and different cell behavior - Elongated, unnatural cell shape - Unable to depict traits exhibited by *in vivo* systems. - Unnatural interactions between cells. - Cell monolayer. - Poorer stem cell expansion.	(Ferrarini et al., 2013) (Malda et al., 2004) (Radisic et al., 2006) (Abbott, 2003) (Cukierman et al., 2001) (Zhang, 2004) (Serra et al., 2012)
3D Cultures	- Cells contact with other cells - Cells behave more like those *in vivo* - More natural cell shape - More natural interactions between cells - Cell multilayer - Improved stem cell expansion.	- Diffusional transport limitations - Accumulation of toxic waste products - Culture-dependent alterations in gene expression, cell proliferation, viability, productivity and product quality due to nutrient deprivation	

Cells cultured on 2D culture flasks are forced to adapt to a flat and rigid surface, which is completely different from the normal *in vivo* microenvironment within humanbody and this results in significantly altered metabolism and declined functionality. Moreover, these cells exhibit major differences regarding morphology, proliferation and differentiation compared to 3D cultures. Gene expression patterns also indicate differences between 2D and 3D culture configurations (Abbott, 2003; Cukierman et al., 2001; Zhang, 2004).

Important drawbacks of 2D culture platforms are also the lack of proper control, cell heterogeneity and generation of low production yield making them unsuitable to be used for clinical applications. On the other hand, 3D culture strategies mimic more what actually occurs in the native microenvironment and by this way they can significantly improve cell viability and function and offer better results regarding efficiency, robustness, consistency and predictability of the cultured product. This fact indicated the promising role of 3D configuration in preclinical research (Serra et al., 2012).

1.5.1 3D culture

Scaffolds are considered the second essential component of TE, which provides the 3D configuration. There are various types of scaffolds for different tissues and applications.

1.5.2 Scaffolds

Scaffolds are considered the second essential component of TE. A scaffold is mainly designed to provide the appropriate stimulatory 3D environment needed for tissue formation (Frohlich et al., 2010). It should also be able to mimic the function of the natural ECM (Baptista et al., 2009). Their choice is important and they need to possess several features in order to support their role.
A scaffold must be biocompatible, which means to be able to integrate in the host's tissue without eliciting an immune response. It should also have the appropriate structural and mechanical properties and thus, to be highly porous and permeable for cell seeding, infiltration, nutrient transport, tissue ingrowth and vascularisation. Porosity and interconnectivity are particularly significant for the accurate diffusion of nutrients and the removal of metabolic waste products resulting from the activity of the cells. Pore size is another important feature that needs to be taken into consideration. Small pores will be occluded by cells and this in turn will cause problems in matrix production, vascularisation and cellular penetration (Salgado et al., 2004).
In the case of bone engineering, scaffolds with pore sizes in the range of 200 – 900 μm (Abukawa et al., 2004; Ishaug-Riley et al., 1998; Logeart-Avramoglou et al., 2005) that mimic the structure of bone, have been shown to enable cellular penetration, ECM production and eventual blood vessel ingrowth (Frohlich et al., 2010). A scaffold is also vital to have mechanical strength in order to withstand hydrostatic pressure and maintain the spaces required for cell growth and matrix production. Ideally, it should have properties matching those of the native bone (Leong et al., 2003). Furthermore, it should gradually be degraded in non-toxic derivatives and replaced by engineered tissue deposited by the cells. Finally, a scaffold should be

osteoconductive, osteoinductive and osteointegrative (Frohlich et al., 2008).

Osteoconduction refers to the ability of the scaffold to allow bone growth on its surface (Albrektsson and Johansson, 2001). It also characterizes the recruitment of osteoblasts to the defect area by the surrounding tissues (Yuan et al., 2010).

Osteoinduction refers to the process where undifferentiated progenitor cells are stimulated to differentiate towards the osteogenic lineage resulting in de novo bone formation (Yuan et al., 2010). In particular, it has been suggested that a material is considered osteoinductive if it has the capacity to induce bone formation heterotopically (Barradas et al., 2011).

Osseointegration is referring to the area of bone/implant interface (Dohan Ehrenfest et al., 2010) and characterizes the bone anchorage to the implant material (Albrektsson and Johansson, 2001) and the stability of the implant itself (Albrektsson and Zarb, 1993). Initial definition was targeting only metal implants but nowadays the term is also applied on non-metal surfaces (Wenz et al., 2008), although the followed biochemical mechanism underlining the two processes is different. The choice of the appropriate material for the production of a scaffold is important for BTE. Various materials, from both natural and synthetic sources, have been utilized up to now.

Natural biomaterials usually come from an *in vivo* source and this means they are constantly available in large quantities and reasonable prices. Their biggest advantage is their biocompatibility due to the fact that they contain binding sites for cells and adhesion molecules. However, they have some disadvantages like their limited mechanical properties. There is also the risk for impurities, which may cause unwanted immune reactions. Lot-to-lot variability is another concern of *in vivo* sources. Some widely use natural biomaterials for bone applications are collagen, silk, alginate and chitosan (Table 1.5) (Bartis and Pongrácz, 2011).

In TE applications nowadays, a number of synthetic biomaterials are widely employed. Their main advantage is the high reproducibility. It is also very useful that their properties can be tailored and adjusted to current needs. Mechanical properties, degradation rate, shape and composition are some of the features that can be easily controlled. Synthetic materials however often lack sites for cell adhesion and many times they do not possess biocompatibility and they are not able to support stem cell differentiation. Some widely applied synthetic biomaterials are ceramics and metals (Table 1.5) (Bartis and Pongrácz, 2011). Metals and most of the ceramics are not biodegradable. Some materials like bioceramics have been shown to induce bone formation without prior cell implantation. This strategy could be used for small defects where low amount of cells is needed but it would not be effective for critical size bone defects due to the large number of cells required to repair the damage (Perka et al., 2000).

From the synthetic biomaterials, calcium phosphate ceramics are widely used as bone graft substitutes due to their similar properties with the mineral part of natural bone tissue. These ceramics have indicated great biocompatibility and osteoconductive capacity without exhibiting any adverse immune reactions (Gosain et al., 2002). However, at the same time, ceramics, such as HA or HA-tricalcium phosphate do not provide good mechanical properties and they are fragile (Grundel et al., 1991; Moore et al., 1987).

Nowadays, scientists examine and focus on the use of biodegradable materials, such as hydrogels, which seem to be better candidates for BTE. These can be either natural or synthetic (Hutmacher, 2000; Yang et al., 2001).

Table 1.5: Materials used to form scaffolds for the regeneration of orthopaedic tissues. Adapted from (Evans, 2013)

Class	Examples
Natural polymers	Collagen Gelatin Silk Fibrin Alginate Chitosan Hyaluronan Coral (hydroxyapatite)
Ceramics	Hydroxyapatite β-Tricalcium phosphate (TCP) Biphasic calcium phosphate (BCP) Calcium sulfate (plaster of Paris) Octacalcium phosphate Bioglass
Synthetic, biodegradable polymers	Polylactic acid Polyglycolic acid (vicryl) Poly (lactic-co-glycolic acid) Polycaprolactone Polyhydroxyalkanoate Polyurethane
Tissue extracellular matrix	Demineralised bone matrix (DBM) Small intestine sub-mucosa (SIS) Skin Dermis Fascia Pericardium
Other	Self-assembling peptides Hybrid scaffolds Collagen-glycosaminoglycan Collagen-hydroxyapatite Gelatin - hyaluronan Hyaluronan – polycaprolactone Polycaprolactone - polyurethane

1.5.3 Hydrogels

Hydrogels are considered an attractive option and have been extensively examined for their capacity to be employed as scaffolds for various TE applications due to their advantageous features, which resemble natural tissues (Peppas et al., 2000a; Peppas et al., 2000b; Slaughter et al., 2009). Hydrogels are highly hydrated polymer materials, which possess a number of beneficial characteristics for BTE, for example biocompatibility and controllable biodegradability. Moreover, due to their soft and pliable nature, they exercise minimized mechanical or frictional irritation to the surrounding tissue and it is convenient to change their physical and chemical properties in order to be applied in any surgery. Furthermore, they allow rapid diffusion of nutrients and metabolites (Vinatier et al., 2006) and provide homogenous cell distribution in a 3D structure (Bienaime et al., 2003; Chia et al., 2000; Uludag et al., 2000).

Nowadays, cell-laden hydrogels are considered ideal candidates for minimally invasive applications. It is suggested that gel immobilization increases transplantation efficiency and protects the cells during implantation. Furthermore, hydrogels mimic the physiologic microenvironment of osteoblastic cells until they start to synthesize their own supportive ECM (Kneser et al., 2006). They also provide a temporal mechanical support for the cells (Gomez-Barrena et al., 2011).

As mentioned previously, the problem with the development of robust 3D structures is the limited mass transport through diffusion. Perfusion culture configuration holds the promise of eradicating these limitations and promoting the development of complex and clinically relevant tissues. The use of hydrogel scaffolds allows diffusive permeability to oxygen and mass transport of nutrients to encapsulated cells (Li et al., 2012).

1.5.4 Alginate

The alginate gel is considered as one of the most applied biomaterials for the differentiation of stem cells in a 3D system (Bosnakovski et al., 2006; Diduch et al., 2000; Ma et al., 2003; Mehlhorn et al., 2006). Extracted from seaweed, alginate is a polysaccharide that is composed of D-mannuronic acid residues, which contribute to its elasticity and L-guluronic acid residues, which contribute to mechanical strength, stability, and porosity (Figure 1.9).

Cells are suspended in the liquid alginate and in the presence of calcium ions; the semisolid gel can be formed by way of the crosslinking of alginate chains under mild conditions. When the

cells are encapsulated in alginate gel, interconnected pores can promote cell attachment, proliferation and differentiation and thereby provide pathways for nutrient and protein diffusion (Fragonas et al., 2000; Murtas et al., 2005).

One of the main advantages alginate gel has is that it is injectable and can be used in a minimally invasive manner to transplant cells and fill lesions with irregular shapes (Stevens et al., 2004). High porosity has also been reported, along with an ideal porous structure, biocompatibility, and high affinity to water (El-Sherbiny and Yacoub, 2013), which is necessary for engineering scaffolds.

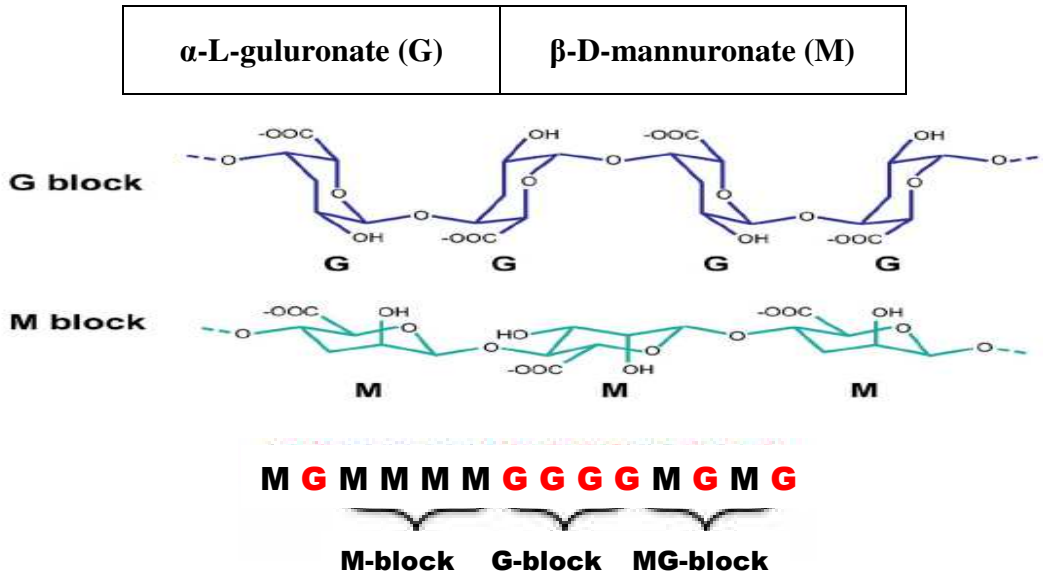


Figure 1.9: Chemical structure of alginate polysaccharide composed of **β**-D-mannuronic acid (M) and **α**-L-guluronic acid (G) with a variation in composition and sequential arrangements. Adapted from (Paredes Juarez et al., 2014; Wilson and McDevitt, 2013).

Cai and his group suggested that the application of hydrophilic and porous materials, such as alginate, might enhance the communication of seeded cells and the refreshing of nutrition. The plasticity of alginate/cell constructs makes them fit easily in the adjacent tissues surrounding the defects (Cai et al., 2007). Alginate hydrogels have been widely used for engineering various tissues in human body. There are examples of encapsulation and delivery of Langerhans islets (Johnson et al., 2011), ovarian follicles (Tagler et al., 2012) and stem cells for neural (Banerjee et al., 2009) and skeletal muscle (Liu et al., 2013) regeneration. Alginate has also been tested for engineering cartilage (Wan et al., 2011) and intervertebral discs (Renani et al., 2012).

There are several examples of alginate hydrogels applied for BTE applications and regeneration.

Zhou and his colleagues encapsulated human umbilical cord mesenchymal stem cells (hUCMSCs) in alginate-fibrin microbeads and they noticed excellent proliferation, osteodifferentiation and bone mineral synthesis indicating a good delivery system to promote bone regeneration (Zhou and Xu, 2011).

In another study, researchers used rabbit-derived bone marrow cells (BMCs) and encapsulated them in alginate microbeads. They concluded that this system could support the proliferation of BMCs and also their osteogenic differentiation (Abbah et al., 2008). The same group also experimented with murine-derived adipose-tissue stromal cells (ATSCs) encapsulated in alginate microcapsules and cultured in the presence of osteoinductive factors. Their results indicated enhanced proliferation and osteogenic differentiation. Osteogenic activity was also confirmed from high expression of ALP and OCN mRNA. The alginate microenvironment supported cell viability and allowed cell differentiation towards osteoblastic lineage (Abbah et al., 2006).

In a more recent study, adipose-derived stem cells (ADSCs) were encapsulated in alginate microbeads and they were also combined with platelet-rich plasma (PRP), which has been shown to contain a number of growth factors. *In vitro* results showed increased osteogenic differentiation of the encapsulated cells while *in vivo* experiment in nude mice indicated vascularization and mineralisation in heterotopic site (Man et al., 2012).

Hydrogels possess a hydrophilic nature, which does not allow protein adsorption and for this reason, mammalian cells cannot interact with them. Cell anchorage is crucial for cell viability. It has been observed that ionically crosslinked alginates lose mechanical properties over time *in vitro*, presumably due to an outward flux of crosslinking ions into the surrounding medium (Shoichet et al., 1996). For these reasons, many groups use covalently modified alginate by incorporating cell adhesion ligands, usually the RGD peptide (Rowley et al., 1999).

For example, alginate modification with RGD-containing peptide showed to promote osteoblast adhesion and spreading. Researchers used a murine pre-osteoblast cell line (MC3T3-E1) for *in vitro* encapsulation and experimental analysis. They observed upregulation of important bone differentiation markers indicating increased osteoblast differentiation of the murine osteoblastic cells. To get one step further, they performed animal experiments where they transplanted primary rat calvarial osteoblasts encapsulated in RGD-modified alginate hydrogels and observed statistically significant *in vivo* bone formation (Alsberg et al., 2001).

Another study used the preosteoblastic cell line MC3T3-E1 and encapsulated them in RGD modified alginate gels. Researchers suggested that RGD peptides promoted adhesion and differentiation of MC3T3-E1 preosteoblasts while there was increased collagen production and secretion to the ECM (Evangelista et al., 2007).

More recently, mESCs were encapsulated in alginate and cultured in a RWV bioreactor. The generated 3D mineralised constructs displayed morphological, phenotypical and molecular features of osteogenic lineage while presenting with the characteristic mineralised calcium/phosphate deposition (Hwang et al., 2009b).

1.6 Culture systems: 3D Static versus Dynamic

In the previous chapter, the limitations of conventional 2D culture configurations were reported and the necessity to change for 3D culture protocols which better mimic the *in vivo* environment where cells normally grow. In this chapter, comparison between 3D static and 3D dynamic configurations takes place (Figure 1.10). 3D static culture seems to possess important limitations, which do not accurately represent the *in vivo* microenvironment. The most significant limiting factor is oxygen. Oxygen has low diffusion capacity and solubility coefficient in aqueous solutions leading to the creation of significant oxygen gradients in static culture. Oxygen concentration with its turn, affects cell viability/ survival.

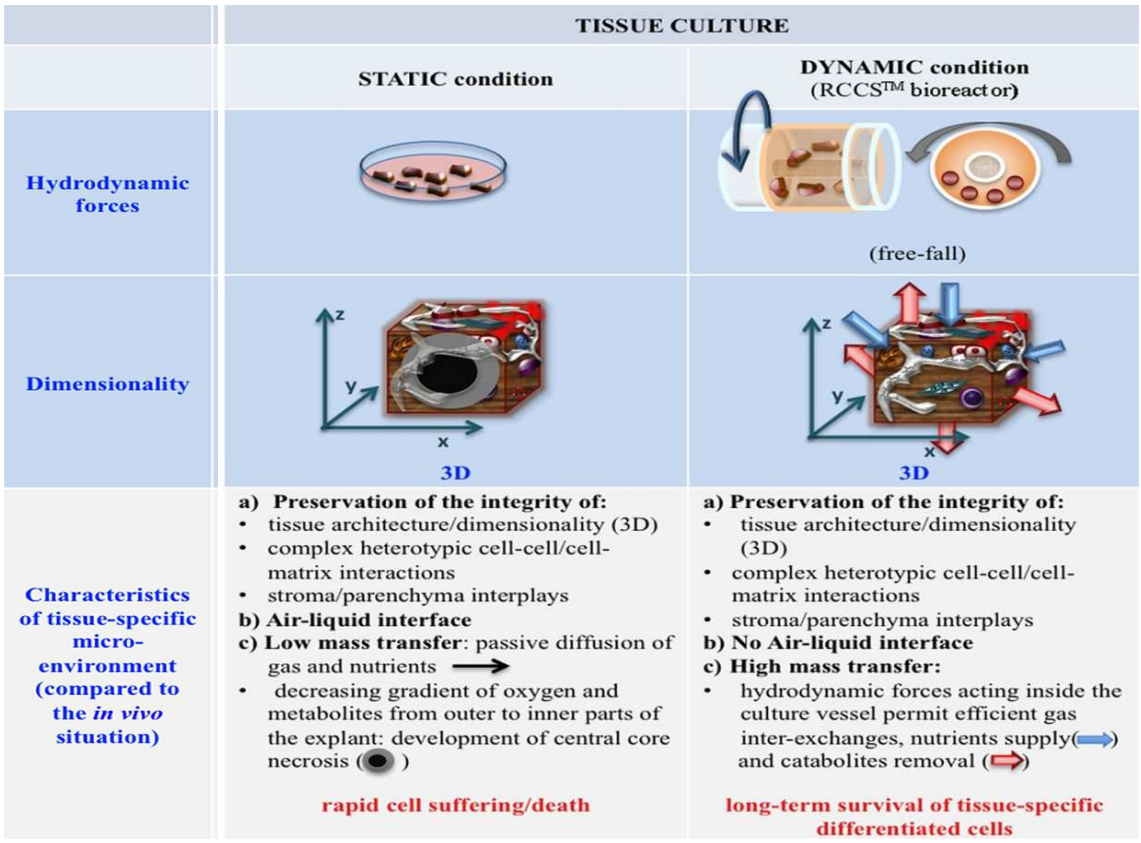


Figure 1.10: Major characteristics and comparison of 3D static versus dynamic culture in a bioreactor Adapted from (Ferrarini et al., 2013)

Static culture also indicates uneven levels of nutrient supply and waste removal. These gradients in the level of oxygen and nutrient configuration have a direct effect on tissue quality (Arkudas et al., 2007; Malda et al., 2007). Cells in 3D culture exhibit high proliferative capacity, which is severely disrupted by the oxygen gradients in static culture, which may possible cause cell death (Volkmer et al., 2008). Dynamic culture systems were developed to overcome many of the previously mentioned limitations. Flow perfusion bioreactors can improve nutrient and oxygen delivery and waste removal from 3D tissue cultures (Abousleiman and Sikavitsas, 2006; Martin and Vermette, 2005).

The use of bioreactors is considered nowadays an essential and integral part of any TE applications. Bioreactors provide the essential environment required to support 3D culture configuration and sustain the culture of clinically relevant high cell numbers needed for therapy of bone disorders. They were also introduced to improve the quality of different types of engineered tissues, including bone and to overcome diffusional limitations of static culture. They provide a better recapitulation of *in vivo* environment. The third essential compound of TE is the use of the appropriate culture platform, a bioreactor that would control the culture conditions and create an environment ideal for the cells to proliferate and reach high numbers and subsequently differentiate to the desired cell type.

1.6.1 Bioreactors

Bioreactors are devices in which biological and biochemical processes develop under controlled and regulated environmental (temperature, pH and oxygen) and operating conditions. Recent years, there is increasing interest on the use of bioreactors for various bioprocessing applications. The automatisation, the product quality and the reproducibility they offer in the expansion and differentiation of cells is one of their major advantages (Martin et al., 2004). Traditional *in vitro* static culture methods of 3D scaffolds cannot maintain 3D tissue-engineered constructs due to the inherent limitation in chemo transportation of nutrients (Sailon et al., 2009). Diffusion is not sufficient to supply nutrients in the centre of large scaffolds and this leads to different cell densities either because cells die creating a necrotic center in the core of the scaffold or cells move from the core and grow at the periphery (Goldstein et al., 2001). Bioreactor systems are used to reduce these limitations by continuously mixing media, thus supplying nutrients to cells and removing metabolites throughout cultivation (Suck et al., 2007).

In the case of BTE, there are three main dynamic culture bioreactor systems that have been extensively used in order to improve *in vitro* culture: spinner flask (Goldstein et al., 2001), rotating wall (Botchwey et al., 2003a; Botchwey et al., 2003b; Yu et al., 2004) and flow perfusion bioreactors (Zhao and Ma, 2005) (Figure 1.11).

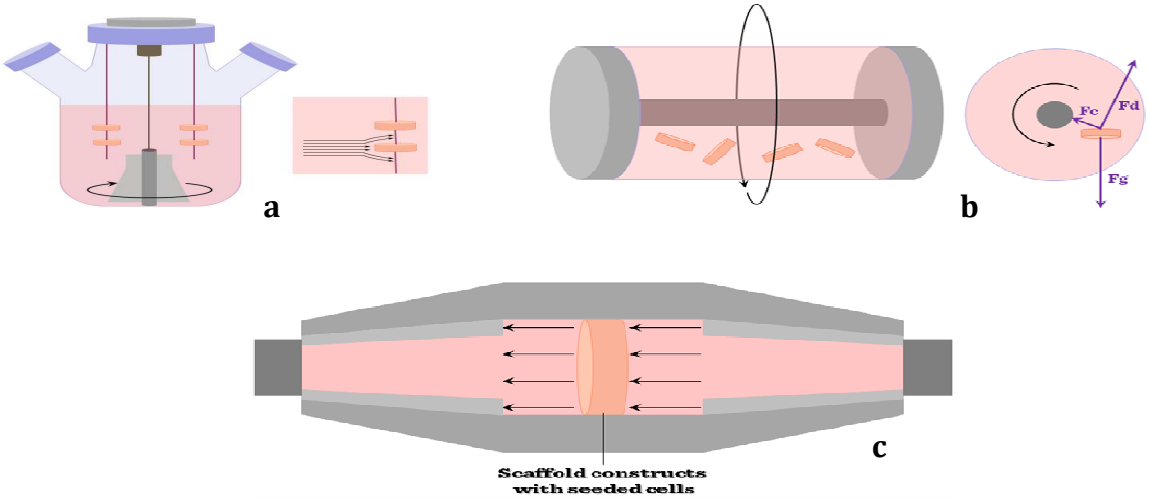


Figure 1.11: Spinner Flask (a), Rotating Wall Vessel Bioreactor (RWV) (b), Flow Perfusion Bioreactor (c). Adapted from (Bartis and Pongrácz, 2011)

There are several examples demonstrating the effects of these bioreactors on osteoblastic differentiation. Dynamic flow culture bioreactor systems have been shown to enhance *in vitro* bone tissue formation by facilitating mass transfer and providing mechanical stimulation.

Spinner flasks were designed to improve mass transport and provided enhanced fluid flow by continuous stirring of the culture medium. They have been shown to promote cell proliferation and osteogenic differentiation. However, the turbulent environment generated by the stirring has proved to be injurious to the cells and the newly formed ECM. Spinner flask couldn't support nutrient transfer through the pores; cell growth and matrix development only occured on the surface of the scaffold (David et al., 2011; Zhang et al., 2010b).

RWV bioreactors provide a dynamic culture environment by rotating at a speed that allows free suspension of cellular constructs. They support high mass transfer and low shear stress environment. Shear stress is defined as the force exerted over the cells due to the flow of the

media (Chen and Hu, 2006). However, they face problems with random collisions between scaffolds and the culture vessel wall, which result in non-homogenous cell growth and matrix development (David et al., 2011; Zhang et al., 2010b).

Spinner flasks and rotating wall bioreactors use convection to provide a well-mixed environment and increase homogeneity of nutrients in the media around the scaffold but they don't perfuse media effectively into the scaffold. They don't also show a significant increase in nutrient transfer in comparison with static culture (Yeatts and Fisher, 2011).

Widely applied and well-know bioreactors are the NASA-designed RWVs available in two different geometries; the High Aspect Ratio Vessel (HARV) and the Slow Turning Lateral Vessel (STLV). The advantageous characteristic of both configurations is the creation of a low shear stress environment (Begley and Kleis, 2000), which has been shown to support various cell culture applications (Goodwin et al., 1993). The simulated microgravity environment created by these bioreactors has been used to study bone and cartilage differentiation in space (Freed and Vunjak-Novakovic, 1997; Freed and Vunjak-Novakovic, 2002).

A RWV bioreactor consists of a cylindrical chamber in which the outer wall, inner wall, or both are capable of rotating at a constant angular speed. Cell constructs cultured within a RWV bioreactor remain suspended in the culture media due to three main forces exerted on the particles. The vessel walls are rotated at a speed that enables a balance between the downward gravitational force (Fg) and the upward hydrodynamic drag force (Fd) resulting in a state of free-fall of the particles induced by the action of the centrifugal force (Fc) (Figure 1.12)(Martin et al., 2004; Partap et al., 2010b).

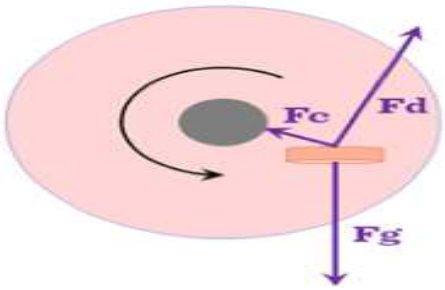


Figure 1.12: Vector Velocity Diagram showing the forces acting on a particle rotating in a fluid. The wall of the vessel rotates, providing an upward **hydrodynamic drag force (Fd)** that balances with the downward **gravitational force (Fg)**, resulting in the scaffold or cells remaining suspended in the media. **Fc** indicates the **centrifugal force** exerted on a particle. Adapted from (Bartis and Pongrácz, 2011).

RWVs keep microcarriers/cells suspended and provide an environment that alleviates large shear stress while ensuring adequate mass transfer. Rotation replenishes the media around the cells. This low turbulence culture environment promotes the formation of large, 3D cell clusters, which more closely resemble the original tissue found in the body due to their high level of cellular organization and specialization (Schwarz et al., 1992).

Cell constructs grown in a rotating bioreactor on Earth eventually become too large to stay suspended in the nutrient media. However, in the microgravity environment of the RWV, cell-seeded scaffolds are placed inside the vessel and are kept in a state of constant free-fall by adjusting the speed of rotation so that the centrifugal force balances the force of gravity and the fluid drag on the objects inside (Jessup et al., 1993; Unsworth and Lelkes, 1998).

The size of the particles, however, changes during culture due to cell growth and proliferation causing an increase in sedimentation velocity, which is proportional to the square of a particle's radius. In order to prevent the particles from hitting the vessel wall, it is important to increase the speed of rotation. Sedimentation of the particles offers also benefits in matters of mass transfer. The constant movement of the particles in the media is advantageous because nutrient, oxygen and waste are not limited by diffusion, as it is the case in static cultures (Hammond and Hammond, 2001; Klaus, 2001).

Because of these properties, the RWV bioreactors are used to simulate some aspects of a microgravity environment in the laboratory. They are usually combined with the employment of encapsulation technology of cells to hydrogels, such as alginate, which has been shown that it does not interfere with cell function. The system employing the above-mentioned two components can minimize significantly shear stress experienced by surface seeded cells (Bucaro et al., 2004).

The main advantages and disadvantages between the various types of bioreactors, described above, are summarized in Table 1.6.

Table 1.6: Advantages and disadvantages between various types of bioreactors. Adapted from (Panoskaltsis et al., 2005; Serra et al., 2010)

BIOREACTORS		
TYPES	**ADVANTAGES**	**DISADVANTAGES**
STATIC	- Simplicity - Ease of handling - Low cost	- Mass transport only with diffusion - Inhomogeneous environment/ Concentration gradients - Support low cell densities - Low total cell output - Difficult to scale up - Increased handling time - Increased risk of contamination - Increased cost
SPINNER FLASK /STIRRED TANK	- Enhanced mass transport - Increased cell density - Eliminate the concentration gradients of nutrients - Create a homogeneous physicochemical environment	- Damage cells/tissues - High Shear stress generation from the impeller
ROTATING WALL VESSEL: HARV & STLV	- Low shear stress environment - High mass transport - Efficient mixing - Efficient gas transfer	- Collision/damage of scaffolds with the bioreactor wall - Mineralisation effects and culturing benefits are limited to the outside of the scaffolds - Internal nutrient transport diffusional limitations
FLOW PERFUSION	- Enhanced mass transport - Less metabolic stress-removal of metabolites - Higher cell expansion - Maintain high cell density - Homogeneous cell distribution throughout scaffolds	- High Flow rate, Washing out of cells, Excessive shear stress - More compex system

The HARV bioreactor has been widely used for BTE applications because of the features of low shear and high mass transfer that provides. In one study, Yu and his collegues used rat primary

calvarial cells and they created 3D microsphere polymeric scaffolds made from mixed poly (lactic acid glycolic acid) (PLAGA). The microspheres were cultured in the HARV bioreactor for 7 days and the dynamic flow environment affected bone cell distribution within the 3D constructs. The created environment improved significantly transport of nutrients and metabolites resulting in increased cell viability within the scaffolds. Dynamic flow also promoted osteoblast differentiation and mineralisation of the constructs. High expression of the bone markers OCN and OPN confirmed the positive effects on bone formation (Yu et al., 2004).

Lv and his collegues developed novel 3D PLAGA/ nano-hydroxyapatite (n-HA) mixed scaffolds composed of lighter-than-water (LTW) and heavier-than-water (HTW) composite microspheres in order to improve mechanical properties and bioactivity. For *in vitro* evaluation, they used human MSCs seeded on the composite scaffolds and cultured in the HARV bioreactor for 21 days. HMSCs exhibited increased proliferation, differentiation and mineralisation when compared with pure PLAGA scaffolds probably due to surface roughness and ability of n-HA to absorb multiple proteins and initiate mineralisation (Lv et al., 2009).

STLV bioreactor has been also employed in various experiments from different groups and results indicated successful osteogenic differentiation. Inanc and his collegues seeded human periodontal ligament fibroblasts on poly (lactide-*co*-glycolide) (PLGA) scaffolds and the generated constructs were cultured in an STLV microgravity bioreactor. Results indicated that the employed culture system was able to support osteogenic differentiation and contributed to the regeneration of the defected area (Inanc et al., 2006). The following year, the same group repeated the experiment using the same cell population but seeded on a different scaffold, on Chitosan-HA Microspheres. Osteogenic differentiation was successful (Inanc et al., 2007).

Marolt and his collegues used bone marrow stromal cells seeded on porous silk scaffolds and the constructs cultured within an STLV microgravity bioreactor. Calcium deposition and mineralisation were observed in the end of the experiment. The positive outcome was partially attributed to the improved mass transfer and better oxygen supply provided by the rotating bioreactor to the cultured cells (Marolt et al., 2006). The important observation from the previous experiments, apart from the desired osteogenic differentiation, was the fact that culture within the RWV bioreactors improved the initial seeding density and provided more uniform cell distribution within the scaffolds. It also supported better media supply to the cells and reduced nutrient and metabolite transport limitations (Sikavitsas et al., 2002).

Flow perfusion bioreactors will be described extensively and separately on a following chapter.

1.6.2 Bioreactor feed operation: Batch, Fed-batch and Perfusion

Cell culture nowadays can be performed via three possible operational modes: batch, fed-batch or continuous perfusion mode.

In a batch culture, all necessary fresh media components and the employed cell population are added in the bioreactor on the beginning of the experiment and the obtained products are only collected in the end of the culture. As cells grow, they consume media and waste products are accumulated. Medium components concentration is not controlled and varies throughout the experimental procedure while pH, temperature and dissolved oxygen are held constant (Lim and Shin, 2013).

In a fed-batch process, the characteristic is the replacement of media and gradual provision of fresh nutrients at certain times during the growth process by manipulating feed rates in order not only to sustain but also to improve productivity and prevent nutrient depletion although temporal variation in nutrient supply has been observed, characterized as “feast and famine” periods (Patterson, 1975). Culture volume increases until reaching maximum amount. Here as well, the obtained products are only collected in the end of the run (Lim et al., 1977; Lu et al., 2013; Shin and Lim, 2006).

Finally, widely used mode nowadays, which is the employed mode in this research project, is perfusion culture. In perfusion operation mode, there is continuous media exchange and feeding supply with fresh nutrients and at the same time, constant removal of waste byproducts and dilution of metabolites. In this way, the culture system achieves and maintains a steady-state condition. Cellular metabolism is properly regulated and allows the support and generation of higher cell concentrations. Bioreactors with perfusion feeding minimize the accumulation of toxic metabolic by-products and are suitable candidates for the efficient support of long-term cultures with high cell densities. Such cultures pose reduced contamination risks and require less labor and fewer material costs (Liu et al., 2014; Sart et al., 2014). Another important feature of perfusion configuration is the exposure of cells to controlled fluid mechanical forces, which has been indicated to enhance cell differentiation in many cases (Li et al., 2009).

To summarize, different feeding operation modes exert different effects on stem cell cultures. The unregulated culture environments experienced in fed-batch cultures do not seem to be optimal for culture of the sensitive population of pluripotent stem cells. On the other hand, perfusion system has been indicated to properly and efficiently regulate significant metabolic and signaling factors influencing pluripotent stem cell fate.

The main advantages and disadvantages of the three-operation bioreactor modes described above are summarized in Table 1.7:

Table 1.7: Advantages and Disadvantages of Batch, Fed-batch and Perfusion culture

BIOREACTORS			
MODES OF OPERATION	**ADVANTAGES**	**DISADVANTAGES**	**REFERENCES**
BATCH	- Relative simple to terminate and start a new experiment	- Constantly changing conditions - Batch-to-batch variability	(Lim and Shin, 2013)
FED-BATCH	- Operational simplicity and flexibility - Increased production - Robustness	- Unregulated cellular metabolism - Generation of complex data patterns difficult to interpret - Arbitrarily determined feeding schedules - Accumulation of toxic levels of endogenous secretions - Detrimental consequences to the cells - High start-up costs	(Patterson, 1975) (Lim et al., 1977) (Lu et al., 2013) (Shin and Lim, 2006)
PERFUSION/ CONTINUOUS	- Greater degree of homeostasis - Sustain longer culture periods, higher productivity - Reduced risk of contamination - Improved product quality - Achieve steady-state condition	- More difficult to operate - Need for greater process control - Flow disturbances of the soluble cellular microenvironment - Convective mass transport eliminate local concentration gradients - Limit diffusive transport of solutes to and from cells - Large volumes of medium required	(Liu et al., 2014) (Sart et al., 2014) (Li et al., 2009) (Gomez, 2008) (Paguirigan and Beebe, 2008) (King et al., 2008)

The bioreactor operation mode in this project was perfusion configuration and it will be analyzed in more details in the following section where appropriate examples will also be presented.

1.6.3 Perfusion

Flow Perfusion bioreactors use a pump to perfuse media through the interconnected pores of a scaffold in order to improve mass transport inside the 3D cellular constructs by utilizing

interstitial flow (Bancroft et al., 2003; Goldstein et al., 2001; Yeatts and Fisher, 2011). Perfusion bioreactors are broadly classified into indirect or direct systems, depending on whether the culture medium is perfused around or throughout/within the tissue-engineered constructs (Cartmell et al., 2003; Meinel et al., 2004; Sladkova and de Peppo, 2014). It is important to reassure that flow goes through the scaffolds, minimizing the nonperfusing flow which is the path of least resistance that goes around the scaffold (Yan et al., 2011).

Nowadays, perfusion bioreactors are widely used for TE of different organs and their use is combined with specific scaffolds on which cells are seeded at different concentrations thus, creating a 3D environment that mimics the *in vivo* conditions. The most important features for creating a 3D environment is the existence of a balance between the mass transfer of nutrients and waste products, the maintenance of matrix components within the construct and the control of fluid shear stress within the scaffold (Martin et al., 2004). Perfusion enhances culture performance by replacing exhausted nutrients and removing inhibitory metabolic by-products (King and Miller, 2007). Perfusion systems, which expose cells to shear stress, have been shown to enhance more efficiently nutrient transfer by effectively perfusing media throughout the scaffold. It has been also indicated that they enhance mineralised matrix deposition, they increase calcium deposition and they upregulate osteoblastic markers in response to culture (Yeatts and Fisher, 2011).

Perfusion system offers several advantages in comparison to static culture system including enhanced transfer of materials by fluid flow, the ability to provide mechanical forces influencing tissue development and better control of the culture conditions (Martin et al., 2004). It is well known that *in vivo* bone remodels in response to mechanical forces and it has been shown that *in vitro*, mechanical stimulation through fluid shear stress influences bone differentiation and mineralisation (Bancroft et al., 2003; Bilodeau and Mantovani, 2006).

In static culture, there are mass transport limitations to the interior of the scaffolds such that only cells around the perimeter of the scaffold have access to nutrients in the medium whereas in the bioreactor, cells can receive a constant supply of food and oxygen, while harmful metabolic products can be excluded from the inside of the 3D scaffold, which is now able to support greater cell growth, as evidenced by higher cell numbers achieved (Holtorf et al., 2005).

Evidence shows that the bioreactor enhances matrix formation by bone cells and it induces cell viability. It is significant the fact that perfusion reduces problems of mass transfer limitations, which exist in all kinds of cultures within a bioreactor. Moreover, it enhances cell survival and achieves higher seeding efficiencies by supplying sufficient transport of nutrients, especially oxygen, for cells within constructs, facilitating uniform distribution of the cells and enhancing bone tissue formation (Grayson et al., 2008).

To summarize, experiments in perfusion bioreactors demonstrated that culture medium flows through the interstitial pores of the construct rather than around its periphery (Grayson et al., 2008), which enables local supply of oxygen and nutrients and removal of metabolites, thus providing better control of the cell microenvironment. The drawback of using this system is its complex and difficult mechanism to assemble and operate it (Goldstein et al., 2001). Perfusion systems also provide biophysical stimulation of the cells in large constructs (Grayson et al., 2010) and are commonly used for various BTE applications.

It is strongly believed that these conditions better mimic the native bone environment and it has been shown that mechanical conditioning alone in the absence of Dex, the standard osteogenic supplement, can induce osteogenic differentiation of BMSC in perfusion culture (Frohlich et al., 2008). Holtorf and his collegues suggested that flow perfusion culture induces osteogenic differentiation of rat MSCs and that there is a synergistic effect of enhanced osteogenic differentiation when both flow perfusion culture and Dex are used, resulting in very high mineral content. It is believed that initial exposure to Dex may offer these cells a boost down the osteogenic pathway, which flow perfusion culture helps to maintain (Holtorf et al., 2005).

Nowadays, the importance of perfusion culture to properly control nutrient transport has been recognized and is widely applied for BTE applications. Many examples show perfusion's positive effect on osteogenic differentiation and bone formation. Datta and his collegues used a different approach to indicate the important role of perfusion cultures in bone generation. They employed titanium (Ti) fiber mesh discs with incorporated pre-generated bone-like ECM. They seeded them with MSCs and cultured them in a flow perfusion bioreactor with or without the osteogenic culture supplement Dex. They observed that the bone-like ECM together with the fluid shear stress from the bioreactor synergistically enhanced the osteodifferentiation of MSCs. There was also increased mineralisation even in the absence of Dex (Datta et al., 2006).

In a following study, Gomes and his collegues used highly porous starch-based fiber mesh scaffolds seeded with marrow stromal cells and cultured under flow perfusion conditions. They identified that the combination of these scaffolds with the fluid flow bioreactor improved nutrient transport and provided mechanical stimulation to the seeded cells, which demonstrated increased osteogenic differentiation and mineralisation (Gomes et al., 2006). This finding confirmed previous results, which suggested the important role of fluid flow not only in mitigating nutrient transport limitations in 3D perfusion cultures of MSCs but also in providing mechanical stimulation to seeded cells in the form of fluid shear stress, resulting in increased deposition of mineralised matrix (Bancroft et al., 2002; Sikavitsas et al., 2003).

The same year, Hosseinkhani and his collegues performed similar experiments to identify the important role of perfusion cultures. They seeded MSCs in a custom made hybrid scaffold made of a hydrogel formed through self-assembly of peptide–amphiphile (PA) with cell suspensions in media and a collagen sponge reinforced with poly (glycolic acid) (PGA) fiber incorporation and the whole system cultured in a flow perfusion bioreactor. Researchers confirmed *in vitro* results of osteogenic differentiation indicated from previous groups. They took it however one-step further and implanted the constructs *in vivo* in rats where they observed ectopic bone formation (Hosseinkhani et al., 2006a).

Most of the initial experiments with perfusion bioreactors tested the use of MSCs. More recently, a number of research groups started to experiment with the promising but controversial cell source of ESCs. It has been shown previously that the hESC-derived mesenchymal progenitors behave similarly to the adult bone marrow-derived MSCs (de Peppo et al., 2010). Based on these findings, Marolt and her collegues decided to use hESC-derived mesenchymal progenitors seeded on osteoconductive scaffolds and cultured in a bioreactor with interstitial flow of culture medium. *In vitro* osteogenic differentiation and formation of compact bone constructs was observed. Engineered bone constructs were subsequently implanted in immunodeficient mice for eight weeks. Maintenance and maturation of bone matrix with continuous bone development was observed. There were no signs of teratoma formation compared to implantation of undifferentiated hESCs. These results suggested the potential transfer and use of protocols developed for osteogenic differentiation of ASCs to ESCs (Marolt et al., 2012a).

The same group, one year later, performed the same experiments using this time hiPSC cells. Based on their previous idea, they cultured hiPSC-derived mesenchymal progenitors on osteoconductive scaffolds in a flow perfusion bioreactor. Molecular analysis indicated repression of proliferation and increased expression of bone genes. Constructs were then subcutaneously implanted in an animal model and kept a stable bone phenotype for twelve weeks. More research is needed but initial indications show promising results from the use of iPSCs (de Peppo et al., 2013a). These findings confirmed the capacity of flow perfusion cultures to augment bone formation and improve osteogenic differentiation.

1.7 Examples and culture protocols of *in vitro* bone differentiation from ESCs

When kept in culture, murine ESCs remain undifferentiated in the presence of leukaemia inhibitory factor (LIF). When LIF is removed, mESCs differentiate by spontaneously forming EBs when cultured in suspension *in vitro* (Mimeault and Batra, 2006). EBs consist of spheres

containing a variety of more differentiated progenitor cell types and contain cells from all three germ layers. The use of specific growth factors or cytokines during the outgrowth of EBs in culture *in vitro* might induce their differentiation into the specific cell lineages (D'Amour et al., 2005; Trounson, 2006; Yao et al., 2006).

During *in vivo* gastrulation, the visceral endoderm plays an important role in inducing mesoderm formation. Hepatic cells are known to be very similar with visceral endoderm in their biological function. It has been reported that culturing mESCs in human hepatocarcinoma cell line-derived conditioned media (HepG2-CM) enhances mesoderm formation, thus resulting in increased osteogenic differentiation in the presence of osteogenic supplements such as b-GP, AA and Dex (Hwang et al., 2006).

EB formation has the problem of spontaneous and uncontrolled differentiation. Recent approaches tried to find a way to bypass the step of EB formation and it has been reported that ESCs were differentiated into osteogenic lineage by treatment with HepG2-CM without EB formation and by culturing them in the presence of b-GP, AA and Dex (Hwang et al., 2008; Karp et al., 2006).

B-GP provides the phosphate ions needed while AA facilitates osteogenic differentiation by acting as a cofactor in Col I synthesis and by inducing ECM production (Both et al., 2007; Choi et al., 2008; Langenbach and Handschel, 2013).

In cell cultures, nowadays, ascorbic acid-2-phosphate is used as a more stable analogue of AA. Jaiswal and his group observed that better osteogenic differentiation of human BMSCs is achieved by using 50μM ascorbic acid-2-phosphate (Jaiswal et al., 1997).

Dex is a synthetic member of the glucocorticoid class of steroid hormones, which exerts a strong effect on the osteodifferentiation of MSCs. In particular, it has been shown that Dex is such a potent induction agent that when MSCs are exposed to it immediately after harvest, they may start differentiating before completing proliferation. There have been observed higher cell numbers and slower differentiation when cell are cultured without Dex. However, cells retain a greater osteogenic capacity when Dex is present in the medium immediately after harvest (Holtorf et al., 2005).

Apart from the above-mentioned protocols used for osteogenic differentiation, the incorporation of various growth factors is widely employed and has been suggested as a method to enhance bone formation. There are several examples from different cell lines used for the experiments.

BMPs are characterized as the strongest osteoinductive factors. For this reason, they were employed in various *in vitro* osteogenic differentiation protocols. In particular, BMP-2, -4, -6, -7 and -9 have been shown to promote osteogenic commitment and terminal osteogenic differentiation in MSC (Dorman et al., 2012; Kang et al., 2009a). BMP-2, the most commonly studied BMP ligand has been

shown to induce MSC osteogenic differentiation both *in vitro* and *in vivo* (Varkey et al., 2006). Its application is mainly through gene transfection studies (Kempen et al., 2008; Park et al., 2009; Tang et al., 2008b; Wegman et al., 2011). BMP4 was used for osteogenic differentiation of mESCs and results indicated an induction in the expression of the tested bone markers. However, mineralisation was only observed when both BMP4 and DAG protocol were combined indicating that BMP4 is insufficient to promote osteogenic differentiation when acting alone (Kawaguchi et al., 2005). To further support the previous study, Camargos and his collegues recently performed osteogenic differentiation of mESCs using BMP4 and they indicated an upregulation of Activin A, a growth factor released by mature osteoblasts and of the bone markers OCN and ALP (Camargos et al., 2014). In another study, Trikkonen and his collegues tried the efficiency of BMP2, BMP7 and VEGF growth factors alone or in combination with the classic osteogenic media employing the DAG factors to perform differentiation of adipose stem cells. Obtained results indicated that application of classic osteogenic media alone significantly enhanced osteogenic differentiation and there was no extra benefit from the addition of the growth factors (Tirkkonen et al., 2013).

Table 1.8: Examples of protocols for osteogenic differentiation and the resulting outcome. Osteogenic supplements (DAG) include b-glycerophosphate, ascorbic acid and dexamethasone.

STEM CELLS	DIFFERENTIATION PROTOCOL	OBSERVED OUTCOME
ESCs	EB formation	Osteogenic supplements (DAG)
ESCs	HepG2-CM	Osteogenic supplements (DAG)
	GROWTH FACTORS	
MSCs	BMP2	Both *in vitro* and *in vivo*
mESCs	BMP4	- Transfection - Insufficient when acting alone - BMP4 + DAG efficient combination
Adipose stem cells	BMP2, BMP7, VEGF	- DAG media supported efficient osteogenic differentiation -No additional benefit from growth factors
mESCs	FGF7	Enhanced calcium deposition induced by the DAG protocol
Bone marrow MSCs	IGF-1	Enhanced osteogenic differentiation
Human dental pulp stem cells	IGF-1	Enhanced osteogenic differentiation

Another growth factor that was employed for osteogenic differentiation was Fibroblast growth factor-7 (FGF7). FGF7 was used on mESCs and it enhanced the calcium deposition supported by

the DAG protocol alone. It also indicated increased osteogenic gene expression of Runx2, OSX, BSP and OCN. The use of the growth factor was again combined with the DAG protocol (Jeon et al., 2013).

IGF-1 is another candidate that has been shown to enhance osteogenic differentiation of bone marrow MSCs and more recently of human dental pulp stem cells (DPSCs) (Feng et al., 2014). IGF-1 has been indicated to have an important function in osteogenic differentiation and bone remodeling by stimulating the proliferation and differentiation of osteoprogenitor cells (Kostenuik et al., 1999) and the formation of osteoclasts (Wang et al., 2006b).

ESCs have the potential to generate any cell type of the three germ layers. This is the challenge that research needs to overcome and try to properly and efficiently control the differentiation of these cells to the desired lineages. Differentiation of ESCs to bone cells has been achieved using protocols from bone marrow MSCs. Initial experiments were performed for many years in mESCs. It has been shown that after induction to EB formation, mESCs could be guided to differentiate to osteoblasts using Dex in combination with b-GP and AA. Bone nodule formation and collagen matrix deposition were used to confirm osteoblastic differentiation (Buttery et al., 2001).

In the next level, researchers started to experiment with human ESCs. Using the same protocols for osteogenic differentiation as they employed with mESCs, they observed mineralisation in two independently isolated hESC lines (Thomson et al., 1998). Molecular analysis was also performed and revealed up regulation in the expression level of various osteogenic markers like Cbfa1, COL 1, PTH receptor, OPN, BSP and OCN, which are related with osteogenic commitment (Aubin et al., 1995; Karsenty, 2000; Malaval et al., 1999). Another significant indication was also the fact that mineralised nodules were composed of HA, demonstrating the directed commitment of human ESCs to the osteogenic lineage (Sottile et al., 2003).

Bielby and his collegues demonstrated the derivation of osteoblasts from hESCs using the previously mentioned protocol. They indicated the possibility of making functional osteoblasts able to make mineralised tissue both *in vitro* and *in vivo*. They implanted cells *in vivo* and noticed the formation of areas of mineralised tissue whereas there were no signs of any tumor formation. This group proved that the culture methodology established for differentiation of mESCs was entirely transferable to hESCs (Bielby et al., 2004). Several other studies followed, where they examined and confirmed the potential of hESCs to efficiently differentiate into osteoprogenitor cells and to support *in vivo* bone formation (Ahn et al., 2006; Kuznetsov et al., 2011; Mateizel et al., 2008).

In a more recent study, de Peppo and his collegues followed a different pathway. They decided to first differentiate hESCs into mesenchymal progenitors and then seeded these cells on 3D osteoconductive scaffolds and induced their osteogenic differentiation while culturing them in a bioreactor with interstitial flow of culture medium. It has been shown previously that hESC-derived mesenchymal progenitors have similar properties with adult bone marrow-derived MSCs (de Peppo et al., 2010). Following the observation of the previous paper, they decided to follow this alternative way of differentiation in order to enhance osteoblast generation and reduce the risk for teratoma formation. This approach resulted in augmented osteoblast differentiation and *in vivo* formation of bone tissue, which when subsequently implanted *in vivo* didn't exhibit any signs of tumors (Marolt et al., 2012a).

1.7.1 Drawbacks of currently used differentiation protocols

Currently used differentiation protocols possess significant limitations. The DAG protocol contains compounds, which are not osteoblast specific and could lead to the generation of other cell types as well. Moreover, Dex as a steroid can have some uncontrolled effects over osteogenic differentiation. On the other hand, use of BMPs and other growth factors has been shown to have several problems related mainly to their high cost, their short half-lives and their use in very high concentrations due to their ineffectiveness (Garrison et al., 2007; Garrison et al., 2010; Zuk et al., 2011; Zuk et al., 2001). Clinical use of growth factors has been restricted because of the high doses needed in humans, which may cause unexpected physiological effects ranging from bone resorption (Giannoudis et al., 2007) to heterotopic ossification (Axelrad et al., 2008; Wysocki and Cohen, 2007).

Alternative methods for effective osteoinduction are under investigation and they mainly focus on the use of mechanical stimulation (McCullen et al., 2010; Tirkkonen et al., 2011). These findings made urgent the need to discover a new and safer molecule able to induce osteogenic differentiation without the need for exogenous supplementation of growth factors.

One of the first experiments to be performed towards that direction identified the small molecule compactin as a potential factor to induce osteogenesis in mESCs and upregulate BMP2 expression (Phillips et al., 2001).

1.7.2 Small molecules

Small molecules seem ideal candidates possessing a number of advantages over the use of growth factors (peptides or proteins). Small molecule is defined as a non-peptide biologically active organic compound with a molecular size usually less than 1000 Da. These molecules can be designed to have desired features such as being selective, potent, water-soluble and cell permeable. Their strongest point is the fact that as small molecule drugs, they are very unlikely to create any immune reaction and also they possess very low cost of synthesis in comparison to recombinant proteins (Lo et al., 2012a; Lo et al., 2014; Wieghaus et al., 2006). Apart from the fact that they are more affordable, small-molecule based drugs are also more stable and require lower dosage to achieve bone regeneration and to stimulate bone formation in comparison to recombinant proteins (Carbone et al., 2014). (Table 1.9)

Table 1.9 Comparison of therapeutic proteins and small molecules. Adapted from (Lo et al., 2012a)

	Advantages	**Disadvantages**
Protein	Specific	Unstable
		Impurities
		High cost
		Immunogenic
		Given by injection
Small molecule	Ease of manufacturing	Non-specific
	Low cost	Off target side-effects
	Stable	
	Non-immunogenic	
	Orally available	
	Ease of delivery	

Small molecules have their drawbacks as well. The most significant concern is their nonspecific side effects (Lo et al., 2012a; Weiss et al., 2007). Due to their small size, they can enter other cells apart from the target ones and elicit unwanted physiological reactions (Brouwers et al., 2011). The solution to that problem is the employment of a drug delivery method to direct the drug directly to the desired target. The design of appropriate materials can coordinate the release kinetics of the small molecule drug. More research is also needed to define the optimal dosage requirements (Tayalia and Mooney, 2009).

1.7.3 Statins

Statins are widely used small molecule drugs to lower cholesterol. Their mechanism of action involves the inhibition of the enzyme HMG-CoA reductase, which participates actively in the pathway of cholesterol production.

The mevalonate pathway is responsible for the production of cholesterol from acetyl-CoA. One of the key steps in this pathway is the conversion of HMG-CoA into mevalonate by the enzyme HMG-CoA reductase. Statins are blocking the enzyme by competitive inhibition with the normal substrate in the enzyme's active site (Schachter, 2005). They alter the conformation of the enzyme when they bind to its active site and prevent the enzyme from attaining a functional structure. This action leads gradually to the reduction in the intracellular content of cholesterol. Cells react by increasing the number of low-density lipoprotein (LDL) receptors in the cell membrane and they remove higher levels of circulating LDL resulting in reduced cholesterol levels in the bloodstream (Corsini et al., 1999). Due to their properties, statins are also employed for the treatment of hyperlipidemia, arteriosclerosis and cardiovascular diseases (Davignon, 2004; Frostegard, 2013; Gotto, 2011; Kobayashi et al., 2011).

There are hydrophobic and hydrophilic statins. Hydrophobic/lipophilic statins are lovastatin, Sim, compactin, fluvastatin, cerivastatin and atorvastatin (Fong, 2014) and have the major advantage of being able to get in the cell with passive diffusion. Hydrophilic statins are pravastatin and rosuvastatin and can only get in the cell with active transport (McTaggart et al., 2001; McTavish and Sorkin, 1991). Lovastatin, Sim and pravastatin are naturally derived from fungal fermentation (Henwood and Heel, 1988; Kishida et al., 1991; Todd and Goa, 1990) while atorvastatin, fluvastatin, cerivastatin, pravastatin, pitavastatin and rosuvastatin are fully synthetic compounds, which are chemically synthesized (Davidson, 2002; Lennernas and Fager, 1997). (Table 1.10)

Table 1.10: Members of statin family are distinguished as hydrophophic or hydrophilic and as natural or synthetic compounds

STATINS				
Hydrophobic/ Lipophilic	**Hydrophilic/ Lipophobic**		**Natural Fungal fermentation**	**Synthetic Chemically derived**
Lovastatin	Pravastatin		Lovastatin	Atorvastatin
Simvastatin	Rosuvastatin		Simvastatin	Fluvastatin
Cerivastatin			Pravastatin	Cerivastatin
Fluvastatin				Rosuvastatin
Atorvastatin				Pitavastatin
Pitavastatin				

1.7.4 Pleiotropic effects of statins and the mevalonate pathway

Statins are well known drugs for lowering cholesterol and they are used for treatment of cardiovascular diseases, atherosclerosis and hyperlipidemia. They perform that function through inhibition of the enzyme HMG-CoA reductase in the mevalonate pathway, which is responsible for cholesterol production. Apart from their main role in lowering cholesterol levels, statins exert a number of **cholesterol-independent** functions. The mevalonate pathway leads also to the production of a number of important molecules called isoprenoids, which are involved, in various important cellular functions (Wang et al., 2008). The inhibition of isoprenoid synthesis is the reason behind the various actions of statins on cells and tissues within the body, which are referred to as pleiotropic effects (Ray and Cannon, 2005).

Statins are involved in immunomodulation (Greenwood et al., 2006), neuroprotection (Kivipelto et al., 2005) and cellular senescence (Brouilette et al., 2007). More recently, there are data suggesting the implication of statins in improvement of endothelial dysfunction, increased nitric oxide bioavailability, antioxidant properties, inhibition of inflammatory responses and stabilization of atherosclerotic plaques (Davignon, 2004; Endres, 2005; Gazzerro et al., 2012; Giurgea et al., 2006; Zhou and Liao, 2010). These findings also indicate the application of statins in various diseases affecting vascular tissue such as arterial hypertension (Deschaseaux et al., 2007), alzheimer's dementia, rheumatoid arthritis, multiple sclerosis (Holmberg et al., 2006; Jung et al., 2004), fibroproliferative disorders (Abe et al., 2012) and atherosclerosis (Kurata et al., 2012).

One of the significant pleiotropic functions of statins is the anabolic effect on bone metabolism suggested from various *in vitro* and *in vivo* results. Statins are under investigation as a safe and effective drug for managing skeletal injuries by stimulating the healing of fresh fractures, non-unions and spinal fusions (Park et al., 2009; Wang et al., 2007; Zou et al., 2012).

1.7.5 Effects of statins on skeletal system

In 1999, Mundy and his group looked for agents that could enhance bone formation and osteoblast differentiation. They were interested in small molecules that could activate the promoter region of BMP2 gene. After examining more that 30.000 compounds from natural products, they identified the statin lovastatin as the only natural product that strongly activated the promoter of BMP2 (Mundy et al., 1999).

After their discovery, statins started to be widely used and it has been proved that they stimulate bone formation both *in vitro* and *in vivo*. Initially, experiments were largely performed *in vivo*, by

injecting statins in animal models. Mundy and his group were the first who did animal experiments using statins which were orally provided to rats or injected to mice and they noticed increase on bone volumeand in bone formation. They also tested statins *in vitro* and they noticed that these molecules stimulated bone formation in cultured osteoblasts. The same group also observed a decrease in osteoclast cell numbers but the effect was minor compared to the anabolic action on bone formation (Mundy et al., 1999).

Following this initial study, several research groups suggested that statins also inhibit bone resorption. In particular, another research group tested 40 different statin analogs and they observed that they acted as potent inhibitors of osteoclastic bone resorption *in vitro* (Staal et al., 2003). To further support this idea, another group suggested that statins ability to reduce fracture risk is more a result of inhibition of resorption rather than a stimulation of bone anabolism (Grasser et al., 2003). (Figure 1.13) A very recent paper indicated that statins reduced alveolar bone resorption observed during periodontal disease and after tooth extraction (de Mones et al., 2015). It has been proposed that statins may increase bone formation through inhibition of osteoblast apoptosis (Ruan et al., 2012). Sim in particular has been shown to inhibit osteoclast differentiation (Moon et al., 2011; Yamashita et al., 2010).

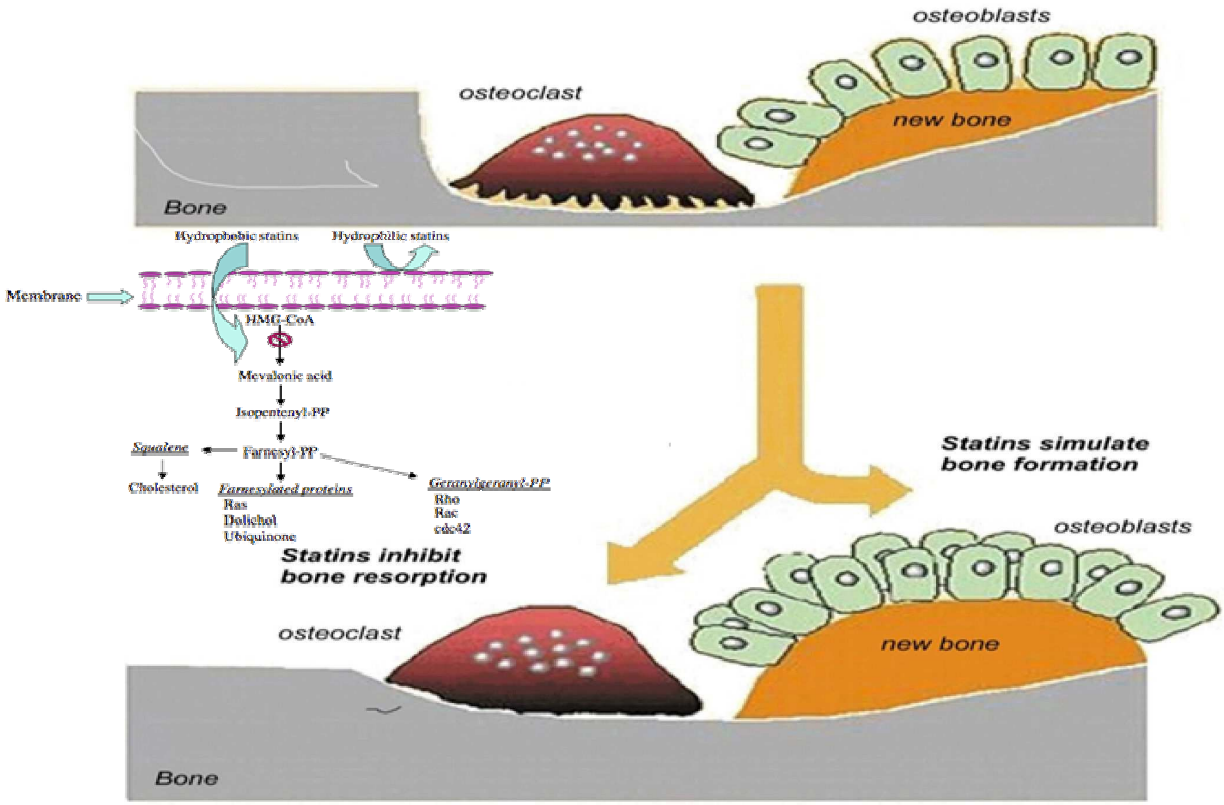


Figure 1.13: Mechanism of action of statins and bone formation. Adapted from (Danesh et al., 2003; Rogers, 2000)

As mentioned previously, statins are widely used drugs to lower cholesterol levels. For this reason, the majority of the experiments and clinical studies performed from the beginning on human patients and on animal models. The aim of these trials was to evaluate the effect of statins on bone formation. Targeting population for the experimental procedures were post-menopausal women or patients with hypercholesterolaemia. After treatment with statins for specific time duration, researchers observed anabolic effects on various parameters of bone metabolism of the tested patients (Chan et al., 2001; Mostaza et al., 2001; Stein et al., 2001). They also noticed a beneficial influence of statins in bone mineral density (BMD) (Chung et al., 2000; Edwards et al., 2000; Lupattelli et al., 2004; Montagnani et al., 2003; Sirola et al., 2002; Uzzan et al., 2007) and a decrease in the risk of fractures (Bauer et al., 2004; Meier et al., 2000; Pasco et al., 2002; Rejnmark et al., 2004; Schoofs et al., 2004; Wang et al., 2000).

Similar experiments were also performed in animal models, in rats in particular and results confirmed previous findings indicating an increase in BMD (Oxlund et al., 2001; Serin-Kilicoglu and Erdemli, 2007). However, there were some controversial results published on the effect of statins on the skeletal system between the previously research groups and more experiments should be performed to confirm the above findings.

A recent study examined the effect of statins on two skeletal dysplasias, the thanatophoric dysplasia (TD) and achondroplasia (ACH) which are both, caused by mutations on the fibroblast growth factor receptor 3 gene (FGFR3). Fibroblasts obtained from patients with one of these skeletal dysplasias and converted to iPSCs for *in vitro* testing. There was also a mouse model of FGFR3 skeletal dysplasia created for *in vivo* studies. Chondrogenic differentiation of TD iPSCs and ACH iPSCs resulted in the formation of degraded cartilage. Application of statins indicated the ability to correct the degraded cartilage in both chondrogenically differentiated TD and ACH iPSCs. ACH is causing disproportionate short-limb dwarfism. Treatment of ACH mice with statin resulted in significant recovery of bone growth and bone elongation. These results showed that statin treatment rescued both human iPSC disease models and mouse disease models, indicating the possibility to be used for treatment of patients with these diseases (Yamashita et al., 2014).

On the other side, there are few studies, which did not observe any positive effects on bone fracture (LaCroix et al., 2003; Pedersen and Kjekshus, 2000; Reid et al., 2001; Yue et al., 2010). These findings indicate the need for further research in order to elucidate the statin influence on bone skeleton. Research is also needed in order to define the potential side effects of statin therapy and identify ways to avoid them. Several studies have reported that statin use is associated with myopathy, which rarely can progress to rhabdomyolysis (Baker and

Tarnopolsky, 2001; Ballantyne et al., 2003; Omar et al., 2001; Thompson et al., 2003).
Rhabdomyolysis occurs when extensive muscle damage results in the release of cellular contents into systemic circulation. Statins can cause serious muscle toxicity resulting in muscle pain and weakness. According to a recent study, the risk is increased with enhanced system exposure to statins and also in people with renal disease (Hedenmalm et al., 2010).

To summarize, it is generally accepted and has been proved that statins exert their skeletal anabolic effect mainly due to increased gene expression of BMP2, the strongest osteoinductive factor. Statins stimulate the transcription of BMP-2 and also increase the endogenous expression of BMP-2 mRNA and protein in human osteoblastic cells (Ohnaka et al., 2001; Sugiyama et al., 2000).

1.7.6 Simvastatin

The lipophilic statin, Sim, seems to be the most attractive candidate as it is readily available, inexpensive and has shown the most consistent positive effects on bone formation (Tang et al., 2008a). Moreover, due to the better potency, it started to be commonly used in several experiments. A number of animal experiments using ovariectomized mice confirmed previous results from Mundy and his group, showing the anabolic effects of Sim on bone formation and an improvement in fracture healing (Skoglund et al., 2002). Similar experiments from another group indicated increased bone formation and decreased bone resorption in the mice after oral administration of Sim (Pytlik et al., 2003).
In another study, when Sim was provided locally to the fracture area, exhibited a strong positive effect on biomechanical parameters and the strength of fracture healing (Skoglund and Aspenberg, 2007). In a study performed in 2007, Sim was injected subcutaneously into tissue in close proximity of the fracture in ovariectomized rats and it promoted the process of fracture healing. Local application had better and more controllable outcome compared to oral administration of the drug (Wang et al., 2007).
Further research was conducted to evaluate the interaction of statin with implants. Titanium implants are widely used for the treatment of many bone defects. Sim has been shown to enhance osteogenesis around titanium implants in one experiment (Ayukawa et al., 2004) and to improve osseointegration of these implants in osteoporotic rats (Du et al., 2009). Sim induced bone healing and enhanced bone formation around the implants. This result may be associated with the way Sim acts by increasing BMP2 expression, which stimulates osteoblast differentiation (Mundy et al., 1999).

Sim has also a significant influence on osteogenic gene expression. It has been shown initially that Sim enhances the expression of BMP-2 and VEGF (Maeda et al., 2003). In a following study by the same group, Sim has been shown to stimulate the expression of the differentiation markers ALP, Col-1, BSP and OCN leading to increased formation of mineralised nodules indicating mineralisation (Maeda et al., 2004). Several *in vitro* studies have documented increased osteoblast differentiation and mineralisation induced by application of Sim on different cells. One of the early studies, performed by Maeda and his collegues, tested the effects of Sim in non-transformed osteoblastic cells (MC3T3-E1) and rat bone marrow cells and they observed that relatively low doses of Sim (significant at 10^{-8} M and maximal at 10^{-7} M) stimulated osteoblast differentiation and enhanced ALP activity and mineralisation (Maeda et al., 2001).

Baek and his collegues used humanbone marrow stromal cells (hBMSCs) in their study and they observed that Sim (10^{-6}) had anabolic effects on bone by promoting osteoblast differentiation of the tested cells. There was also increased expression of ALP, OCN and mineral deposition. In this study, however, inhibition on the proliferation of hBMSCs was noticed (Baek et al., 2005). Sim has been also tested on mouse bone marrow stromal cells and indicated that it could promote osteoblastic differentiation and inhibit adipocytic differentiation of these cells. It also increased significantly BMP2 expression levels (Song et al., 2003). Periodontal ligament (PDL) cells were also tested with Sim. Results showed that low concentrations enhanced cell proliferation and osteoblastic differentiation. Sim also stimulated ALP activity, OPN content and increased calcium deposition after three weeks of culture (Yazawa et al., 2005).

More recently, Sim has been indicated to induce the osteogenic differentiation of mESCs in the absence of osteoinductive supplements. In the same study, there was also an increase in the expression of the significant bone markers OSX and OCN (Pagkalos et al., 2010). To summarize, Sim has been reported to promote osteoblastic activity and inhibit osteoclastic activity and thereby, increase cancellous bone volume, bone formation rate and cancellous bone compressive strength *in vivo* (Montagnani et al., 2003).

In vivo animal research strongly supported the idea that Sim exerted beneficial effects on bone metabolism and indicated anabolic effects on bone formation and fracture healing (Tang et al., 2008a). These findings showed the potential use of statins for bone regeneration and the majority of these studies demonstrated these effects via systemic, transdermal or local subcutaneous administration of the statins.

However, *in vitro* results are conflicting about the effects of Sim. This is due to the fact that different parameters are employed in the experiments and probably influence the outcome. These important influential features are the method of administration, duration of exposure,

experimental animal model and bioavailability. Further research is needed to determine the optimal therapeutic threshold, mode of application and the effectiveness for humans for bone regeneration (Park, 2009).

One more superior element supporting strongly the use of statins in bone industry is the fact that they possess a long history of clinical systemic usage with very acceptable good toxicity profiles (Garrett, 2007). This indicates faster approval times to be used clinically for orthopaedic applications once their properties are established in a large animal model (Carbone et al., 2014). These features render them an appealing choice to be used for the treatment of bone diseases. It is generally accepted and required from people all over the world the discovery of affordable, safer and more effective therapies for the future. In the case of bone diseases, TE can offer a promising solution with less invasive and more effective treatments. Further research should be conducted in order to achieve the desired outcome.

1.8 THE NOVELTY OF THIS PROJECT

This PhD thesis recommends and describes extensively the application of an alternative solution trying to solve currently existing limitations in the field of BTE, presented before in the literature review section.

The novelty of this project lied on two elements. The first one was the use of a custom made RWV perfusion bioreactor for the culture of cellular constructs. The design of this bioreactor was based on NASA's STLV bioreactor idea with incorporated improvements. The main change was the use of a permeable to oxygen membrane, made from Teflon and silicon, around the vessels. There was also an oxygenator system to provide appropriate oxygenation level to the media before getting into the vessel. This biorector has been designed and used for osteogenic differentiation and bone formation by Dr Jae Min Cha (Cha, 2010). Dr Cha performed osteogenic differentiation using the widely employed DAG protocol and compared the custom-made RWV perfusion bioreactor with NASA's designed HARV biorector. Following and continuing his work, the RWV perfusion bioreactor was used for my experiments and comparison was performed with static configuration. To take it one step further and get more insight information, the system was operating under 24-hour continuous perfusion mode, something that was different from the previous work conducted by Dr Cha. Continuous perfusion combined with rotating movement provided significant and novel information regarding osteogenic differentiation.

The RWV perfusion bioreactor has been also employed by other PhD students for different purposes. In particular, Dr David C. Yeo used this platform to support and maintain pluripotency of mESCS while Dr Ailing Teo employed the same configuration and indicated successful cardiomyogenesis of ESCs. Both researchers have published their work on the RWV perfusion bioreactor (Teo et al., 2014; Yeo et al., 2013).

The second novel element was the employment of the small molecule Sim. Sim was tested in a very low concentration, in the range of nanomolar, as an agent for performing chemical induction and achieve osteogenic differentiation without the use of expensive growth factors, such as BMPs. It was supplied in the osteogenic differentiation media at specific and appropriate time point during the experiment. The aim was to get some more insight information on the possible role of statins in BTE applications. Sim has been tested previously by Dr Joseph Pagkalos (Pagkalos et al., 2010), who defined an optimal concentration and designed a differentiation protocol based on his experiments, which were all performed in 2D configuration. He ended up using a very low concentration of Sim. Based on these previous findings, I tried as a first step to replicate his results and test the efficacy of his protocol. Initial outcome was not encouraging and a short optimization was performed to define the appropriate time point for supplementation of Sim in the culture media. Next step involved the transition and experimentation in 3D culture configuration. It was the first time to employ such a low concentration of Sim as part of the differentiation media in order to perform osteogenic differentiation of mESCs in the dynamic environment of the RWV perfusion bioreactor. Moreover, Qiao and his collegues performed osteogenic differentiation of mESCs using Sim and obtained important information regarding the followed signaling pathway (Qiao et al., 2011). My contribution on this part was the extended gene expression analysis performed and the significant information provided regarding the osteogenic differentiation using this protocol.

The use of mESCs and their encapsulation in alginate hydrogels have been already tested and optimized. A single step bioprocess design for the efficient expansion and osteogenic differentiation of the encapsulated in alginate mESCs within the custom-made RWV perfusion bioreactor has been previously created (Cha, 2010).

2ND CHAPTER

2. AIMS AND OBJECTIVES

The overall aim of the proposed PhD study is to explore an alternative paradigm in BTE that could be potentially employed for future orthopaedic clinical applications. The major target of this project is to generate 3D mineralised cellular constructs made from mESCs encapsulated in alginate hydrogels, chemically induced by Sim and cultured within an automatable and scalable custom-made RWV perfusion bioreactor. In particular, the main objective is to assess and evaluate the ability of Sim to induce osteogenic differentiation of mESCS in a 3D configuration.

The specific objectives of this research project are:

- To analyse and evaluate the osteoinductive capacity of Sim.
- To determine the effects of dynamic rotary culture on cell proliferation.
- To utilize extensive gene expression analysis in order to gain more insight information regarding the whole differentiation pathway of mESCs, from the initial undifferentiated status to the final osteogenic phenotype.
- To compare bioprocess parameters between static and dynamic 3D culture platform configurations.
- To compare the Sim employed chemical induction protocol with the previously used protocol with Dex; distinguish similarities and differences among them.

3RD CHAPTER

3. MATERIALS & METHODS

In this chapter, the employed cell culture techniques are initially described followed by the various methods of experimental analysis used during this project.

3.1 Cell culture in 2D configuration

3.1.1 mESCS expansion and maintenance media formulation

All procedures associated with cell culture were carried out in a cell culture hood. The E14 thioguanine resistance 2 mESC (E14Tg2a) line (cat. No. CRL-1821, purchased from American Type Culture Collection – ATCC) was routinely cultured on tissue culture flasks coated with 0.1% gelatin (Sigma-Aldrich, Poole, UK) in a 95% humidified cell culture incubator (Nuaire, Nu – 5510E, Triple Red Ltd) set at 37ºC and 5% carbon dioxide (CO_2) level.

Undifferentiated mESCs (<passage 20) were subcultured every 3 days at a seeding density of (2 – 4)×10^4 cells/cm^2 and fed every day with fresh maintenance medium based on high glucose Dulbecco's Modified Eagle Medium (HG-DMEM) without sodium pyruvate (Invitrogen, Paisley, UK) supplemented with 10% (v/v) of foetal bovine serum (FBS) batch-tested/heat inactivated (Invitrogen, Paisley, UK), 100 units/mL penicillin and 100 μg/mL streptomycin (LGC Standards, Middlesex, UK), 2 mM of L-glutamine (LGC Standards, Middlesex, UK), 0.1 mM of 2-mercaptoethanol (Sigma-Aldrich, Poole, UK) and 1000 Units/ml of LIF (Millipore (U.K.) Limited, Watford, UK).

Prior to sub-culture, culture media was aspirated and tissue culture flasks were washed with 1× phosphate buffered saline (PBS), without calcium and magnesium (Gibco) before dissociation using 0.05% (v/v) trypsin ethylenediaminetetraacetic acid (Trypsin-EDTA; Gibco). Trypsin was applied for 5 minutes at 37^{o}C, 5% CO_2, resuspended using gentle pipetting for 1 minute to ensure complete dissociation, before fresh maintenance media was applied to inactivate trypsin and stop the reaction. Centrifuging at 360g/1280rpm for 5 minutes was used at all steps to separate the cell pellet from its supernatant before fresh maintenance medium was added for further subculture.

3.1.2 Embryoid body (EB) formation and osteogenic differentiation in 2D configuration

EB formation involved careful preparation of mESCs prior to suspension culture and was well documented (Desbaillets et al., 2000; Hopfl et al., 2004; Martin and Evans, 1975). Nonetheless, empirical determination of the correct conditions before suspension was established with the E14Tg2a cell line. Briefly, cells in monolayer culture should be around 70% confluent on either day 2 or 3 of culture and morphologically undifferentiated. Cells were shortly trypsinised for 1-2 minutes ensuring cell clumps. The clumps were then centrifuged at 300g for 3 minutes at room temperature and then re-suspended in EB formation medium based on Minimum Essential Medium Alpha (a-MEM) (Invitrogen) containing 15% (v/v) of FBS batch-tested (Invitrogen), 1% (v/v) of penicillin and streptomycin 100 units/ml (Invitrogen) and distributed evenly into two 90 mm diameter bacteriological grade petri dishes.

On day 3 of EB formation the medium was exchanged with fresh one and on day 5 the EBs were harvested, transferred to 50 ml centrifuge tubes and then settled down for 30 minutes in a CO_2 incubator. The medium was aspirated and replaced with pre-warmed PBS to wash away traces of serum. The EBs were settled down again and the PBS was aspirated. After PBS washing, 2 ml of Trypsin-EDTA was added to the EBs for 3-5 minutes in a CO_2 incubator to make them a single cell suspension. 8 ml of pre-warmed medium was then added to halt trypsinization. An Erythrosin B cell count (Sigma-Aldrich) was performed together with centrifuging the remaining 10 ml of TE/media/cell suspension at 1280 rpm for 5 minutes. The cell pellets were re-suspended in medium and seeded at 3 x 10^4cells/cm^2 density to induce osteogenic differentiation. The following day, the media was changed to osteogenic media based on the EB media containing 50 µg/ml L-ascorbate-2-phosphate (Sigma-Aldrich) and 10 mM b-GP (Sigma-Aldrich) from day 8 to 29 and 1 µM Dex (Sigma-Aldrich) from day 21 to 29.

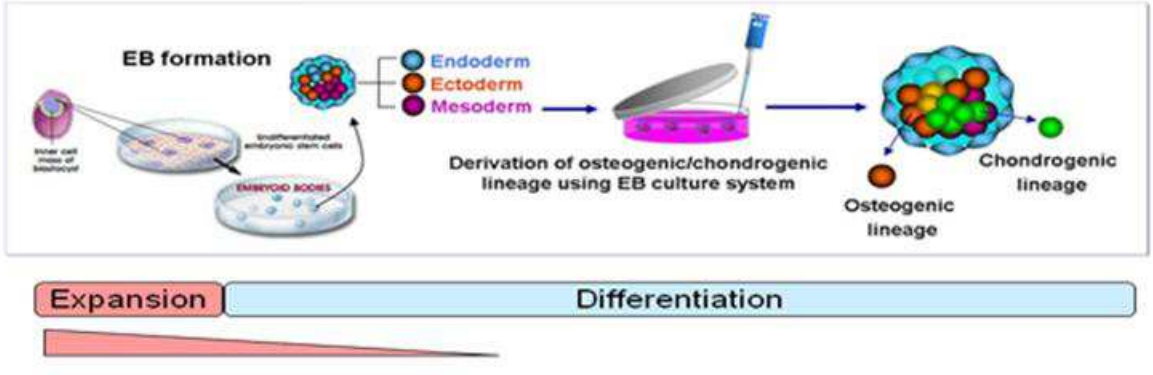


Figure 3.1: Conventional osteogenic/chondrogenic differentiation of mESCs via EB formation. Adapted from (Hwang et al., 2007).

3.1.3 Erythrosin B cell counting

Cell counting was performed using Erythrosin B staining for dead cells in order to quantify the total cell number as well as the percentage of live cells. Cells were dissociated into single cells and seeded into a haemocytometer and observed using a Leica DM-IL inverted phase microscope (Leica). Individual cells having compromised membranes were stained red (non viable) whereas viable cells were unstained.

3.2 3D cell culture for BTE

Initial step included cell expansion in 2D cell culture flasks following the previously mentioned protocol in order to obtain the required cell numbers to proceed for alginate encapsulation and culture within the bioreactor. The next steps included collection of HepG2-CM, alginate cell encapsulation, set-up and operation of the bioreactor and finally, 3D cell culture and osteogenic differentiation within the cell culture platform. Detailed description of each step follows.

3.2.1 HepG2-CM formulation

HepG2 cells (ATCC HB-8605) were cultured in the same way with our previous work (Hwang et al., 2006). Briefly, cells were initially cultured in a density of 5.0×10^4 cells/cm^2 with DMEM containing 10% (v/v) FBS and 1% (v/v) of penicillin and streptomycin. The culturing medium was not changed for the following 4 days and then collected to the 0.22-μm filter-unit (VWR International, Poole, UK) for filter-sterilization followed by supplementing 0.1 μM of 2-mercaptoethanol and 2 mM of L-glutamine. HepG2-CM was formulated with 50% (v/v) of the fresh mESC growth medium and 50% (v/v) of the medium from the culture above and subsequently 1000 units/ml of LIF was added in the mixture (Rathjen et al., 1999).

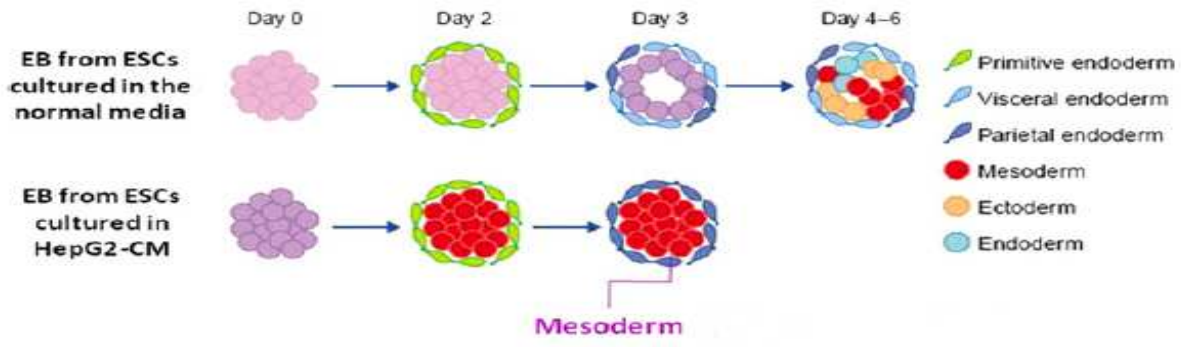


Figure 3.2: Enhanced mesoderm derivation from ESCs using HepG2-CM. Adapted from (Lake et al., 2000)

3.2.2 Alginate encapsulation and characterization of the generated beads

Undifferentiated mESCs were trypsinized from T-flasks, dissociated into a single cell suspension, counted and resuspended at a density of 2.5 x 10^6 cells/ml (20.000 cells/bead) in 0.22-μm sterile filtered solution composed of 1.1% (w/v) alginic acid (Sigma-Aldrich) and 0.1% (v/v) porcine gelatin (Sigma-Aldrich) (both dissolved in PBS, pH 7.4) as described in previous study (Randle et al., 2007).

Before the beginning of encapsulation, tubes were autoclaved and washed initially with ethanol and then with sterile PBS. After that, the mESC–alginate solution was passed through a peristaltic pump (Model P-1, Amersham Biosciences, Buckinghamshire, UK) and dropped in the solution for cross-linking the alginate beads, composed of 100 mM calcium chloride ($CaCl_2$; Sigma-Aldrich), 10 mM N-(s-hydroxyethyl) piperazine-N-(2-ethane sulfonic acid) (HEPES; Sigma-Aldrich) and 0.01% (v/v) Tween 20 at pH 7.4.

HEPES is a buffering agent and Tween 20 is a non-ionic surfactant. Tween 20 was employed in order to improve the resolution of the microgel assembly patterns and in particular to reduce the surface tension (Du et al., 2010). Nonionic detergents are considered mild surfactants; they do not affect protein activity and they are effective in solubilization. They are widely employed as washing agents in immunoblotting and ELISA in order to minimize unspecific binding of antibodies (Kuczynska et al., 2010).

A surfactant can affect polymerization kinetics and the final properties of the conjugated polymers. The incorporation of a surfactant into a conducting polymer can improve the electrical and morphological properties of the polymer as well as its thermal stability because of the introduction of a bulky hydrophobic component into the polymer structure (Yavuz and Gok, 2007). The same action can also improve the biocompatibility and conformation of the polymer and give porous surface morphology at the nanoscale to improve enzyme immobilization (Khan et al., 2009; Uygun et al., 2009).

A flow rate of a peristaltic pump was empirically confirmed in order for 4 ml of mESC/alginate solution to produce approximately 500 beads using a 25G disposable needle (Becton Dickinson, Oxfordshire, UK) and dropped from a height of about 30 mm within 10 minutes. The droplets containing cells were gelled immediately on contact with the Ca^{2+} solution as a shape of bead (approximate diameter size: 2.3mm).

The mESC-hydrogel beads were allowed to set within the cross-linking gently stirred solution for 5 minutes at room temperature. The beads were then washed three times in PBS and placed into HepG2-CM for 3 days. Cells were fed with fresh HepG2-CM every day.

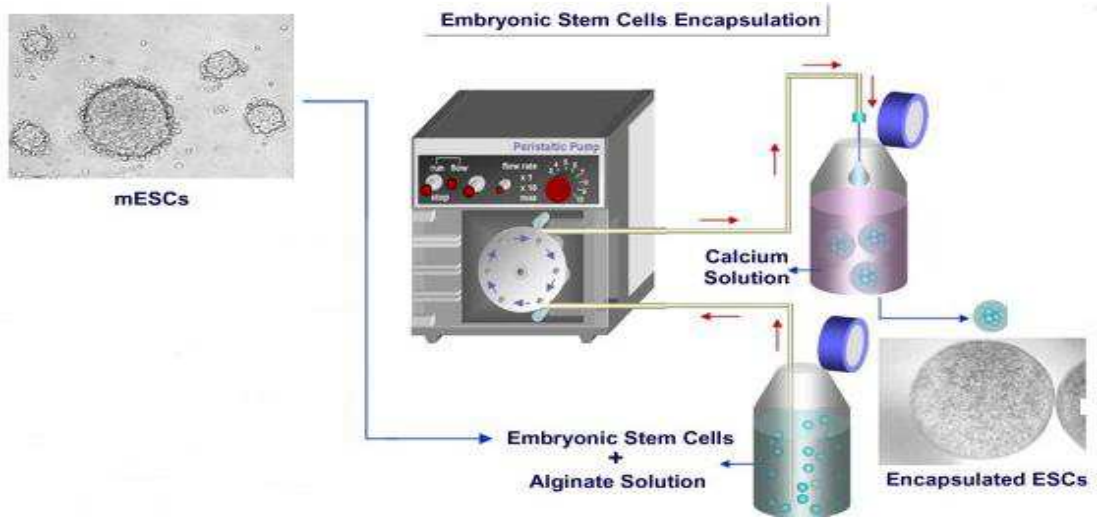


Figure 3.3: Alginate encapsulation as performed in the BSEL laboratory. Adapted from (Hwang et al., 2009b)

In order to carry out experimental analysis, cell release was performed after incubation of the required number of beads in depolymerisation buffer for 10 – 20 minutes at 37^{o}C with gentle agitation. The buffer consisted of 0.22 μm filter sterilized 50 mM tri-sodium citrate dihydrate (Fluka, UK), 77 mM sodium chloride (BDH Laboratory supplies, UK) and 10 mM HEPES (Sigma, UK) in a PBS base at 7.4 pH. Cell pellet was obtained after samples were centrifuged for 5 minutes at 400g and washed twice with PBS.

Alginate bead formation and the chemical interactions upon cell encapsulation in alginate have been described previously in detail by Bienaime and his collegues. They supported that hydrogel beads used to encapsulate cells are well-structured macromolecular constructions possessing an architecture that provides regular geometric structures throughout the gel network. As a result, characteristics such as bead porosity and gel density are closely linked to the gel molecular structures. Knowing the pore size and pore connectivity is neccessary in order to ascertain whether cells encapsulated within the alginate beads have access to all the nutrients in the external medium and also whether toxic metabolites, produced by the cells, are able to perfuse through the bead. They also indicated that bead production is highly reproducible when the same conditions and reagent concentrations are used (Bienaime et al., 2003). The pore size is also a critical factor for cellular activity (Lien et al., 2009). It has been indicated that cross-linking

density determines the final pore structure of the hydrogel, even when the same materials and experimental conditions are employed (Drury and Mooney, 2003).

Dr Wesley Randle evaluated the permeability of the employed alginate beads using SDS-PAGE during his project. His results suggested that in a dynamic environment, such as the one provided by the RWV perfusion bioreactor, the beads allowed proteins of at least 66.2 kDa in size, possibly up to 97.4 kDa to permeate. The difference is related to the inherent bead heterogeneity during manufacture, as different time in calcium chloride solution may affect porosity (Randle, 2006).

The surface topography of the beads used in this study was similar to those produced in the literature (Fundueanu et al., 1999) with pore size on the external surface approximately 0.5 - 1 μm in diameter (McConell et al., 2004). Beads of this size were found to be permeable to molecules ranging between 66.2 kDa and 97.4 kDa, similar to the permeability analysis performed in this thesis and suggesting that it would easily allow the diffusion of LIF (Guo et al., 1989), which ranges in size from 20 to 60 kDa (Gascan et al., 1989; Hilton et al., 1988). This finding also indicates that mESCs (>20 μm in diameter) are not capable of leaving the beads when encapsulated unless they do so through growth and mechanical pressure.

As far as bead morphology is regarding, the 25G needle size was chosen, after varying the gauge and it was employed for encapsulation leading to the generation of beads with almost perfect sphericity. Similar publications (Ma et al., 2003; Murphy and Sambanis, 2001) supported no major differences when varying needle size and the important goal was to allow viable cell growth. He investigated different cell densities per bead that would enable culture for long periods of time, up to 30 days, without causing overcrowding and he defined size of 2.3 mm diameter as an acceptable one to work with. The alginate-gelatin co-polymer beads were uniform in size and sphericity, before and after culture, regardless of the number of cells encapsulated within them. He also conducted scanning electron microscopy (SEM) analysis in an attempt to determine the pore size of the beads. However, the beads almost completely collapsed under the vacuum of the SEM and pore size could not be established.

The total porosity is related to the amount of pore space present in the scaffolds and can be calculated by employing physical properties such as material or bulk density of the scaffolds. Various equipment and computer software can be also used to measure porosity and pore size of scaffolds (Loh and Choong, 2013). Pore size and porosity of hydrogels can only be determined during the fabrication process or using indirect methods of measurements of pure hydrogels with no cells and the assumption is that the material will behave the same way even in the presence of cells in its interior space.

Examples of such indirect methods are fluid intrusion for physical characterization or imaging techniques such as SEM to determine porosity. From SEM and liquid intrusion, only a rough estimate of diameters could be calculated. Obtained values were not accurate and were somewhat arbitrary. Liquid intrusion only gave the largest diameter pores and had high variability (Loh and Choong, 2013).

An alternative approach to SEM for assessing pore size is to use indirect methods such as porometry and porosimetry. In particular, widely used is mercury porosimetry, which allows the determination of the total pore volume fraction, the average pore diameter and pore size distribution of 3D materials (Guarino et al., 2008; Thunemann et al., 2011). Mercury porosimetry is quite well accepted in the determination of the pore size of materials despite the fact that it does not take into account the shape of the pores.

Scaffold permeability method has been also employed to determine the pore size of scaffolds (Sell et al., 2008). To be more specific, scaffold porosity was determined using a liquid displacement method similar to that reported by Zhang and Ma (Zhang and Ma, 1999) and Hsu and his collegues (Hsu et al., 1997). Techniques that measure the movement of probes through soft polymeric foam or hydrogel scaffolds can potentially generate more meaningful information than methods that can distort or disrupt the native scaffold. The identity of the probe may range from a small molecule including dissolved gases to proteins.

One important thing that was also noticed was the clear fact that prolonged culture was possible and the beads were robust enough to support long-term culture. It was also beneficial for cell viability that beads were formed at 37^0C and for this reason, both alginate and calcium chloride solution were pre warmed at this temperature. It has been reported that the incorporation of gelatin with alginate can support the cell/hydrogel structure to keep mechanical stability after prolonged culture (Balakrishnan and Jayakrishnan, 2005).

3.2.3 Mechanical properties of hydrogels

It has been observed previously that ionically crosslinked alginates lose mechanical properties over time *in vitro*, presumably due to an outward flux of crosslinking ions into the surrounding medium (Shoichet et al., 1996). Such hydrogels were also employed in these experiments and obtained results indicated that there was not such an issue. To be more specific and for further validation, Dr Jae Min Cha and Dr Yushik Hwang (Cha, 2010; Hwang, 2007) performed mechanical testing of the alginate hydrogels alone or with undifferentiated mESCs inside on day

0 and also after 29 days of osteogenic differentiation in static configuration using tissue culture flasks and in dynamic configuration using the custom-made RWV perfusion bioreactor or the HARV bioreactor. The obtained results of the calculated Young's modulus not only did not indicate loss in mechanical properties but they actually suggested a significant increase in the experimental group after osteogenic differentiation of 29 days compared to the alginate alone or combined with undifferentiated mESCs (Cha, 2010; Hwang, 2007) (Appendix 3.1, 3.2).
Another significant indication was the fact that on day 29, beads were collected in order to perform several analyses and observation suggested that the hydrogels not only maintained their structure but also, due to the mineralisation, they were white and hardened compared to day 0.
Furthermore, based on the literature, mechanical properties of the hydrogels are affected by changes in the crosslinking or the material properties (Ahmed, 2015; Wan et al., 2008). During all the performed experiments, the exact same protocol, experimental procedure and cross-linking mehod were followed without changes in the alginate or calcium concentration. Based on that, it was not expected to observe changes in the mechanical properties of the generated beads. Finally, it has been reported that the incorporation of gelatin with alginate can help to overcome the inert nature of alginate, increase bead integrity and enhance cell ligand-specific binding properties. In this way, it can support the cell/hydrogel structure to keep mechanical stability after prolonged culture (Balakrishnan and Jayakrishnan, 2005). Normally alginate hydrogels lose Ca^{2+} cations after prolonged culture but it has been indicated that the incorporation of gelatin enables cell-mediated contraction and packing of the scaffold material (Awad et al., 2004). Due to the above-mentioned reasons, the employed hydrogels didn't seem to have any flux of ions and seemed to maintain their mechanical properties during the whole experiment.

3.2.4 RWV perfusion bioreactor set up

A custom-made RWV perfusion bioreactor, consisted of autoclavable parts was used in this project (Figure 3.4). The bioreactor contained two cell culture vessels, which are located on a single base plate and they rotate alongside by a main servo motor system. Each vessel has the "centre rod" through which media is supplied and it is surrounded by a gas permeable membrane which facilitates efficient gas transfer. Each of the vessels has also its own set of peristaltic pumps controlling perfusion rate, oxygenators and fresh/waste medium bottles. Perfusion is performed in a rate of 60 ml/day. The medium follows a specific pathway which starts from its

collection from a fresh medium bottle, through an oxygenator, perfused into the culture vessel and finally discarded in a waste medium bottle. All the electric units are monitored by a computer system using tailor-made software, which controls rotary and feeding parameters.

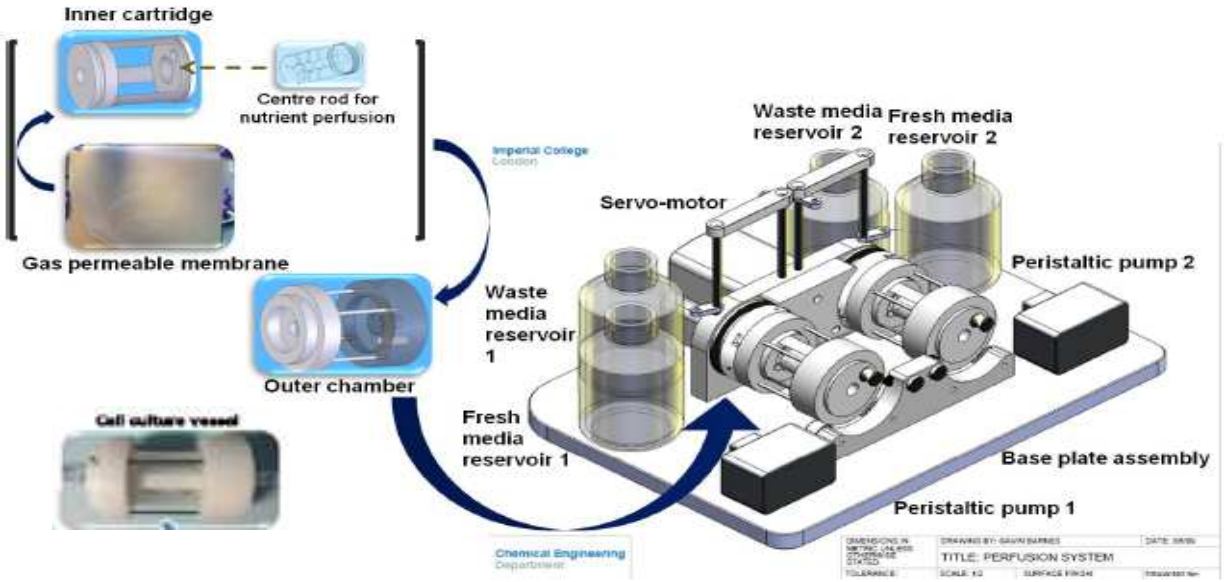


Figure 3.4: A brief schematic of the perfusion bioreactor set-up. Image adapted from (Cha, 2010).

The following images show actual photos of the employed culture platform where the main system components are labelled.

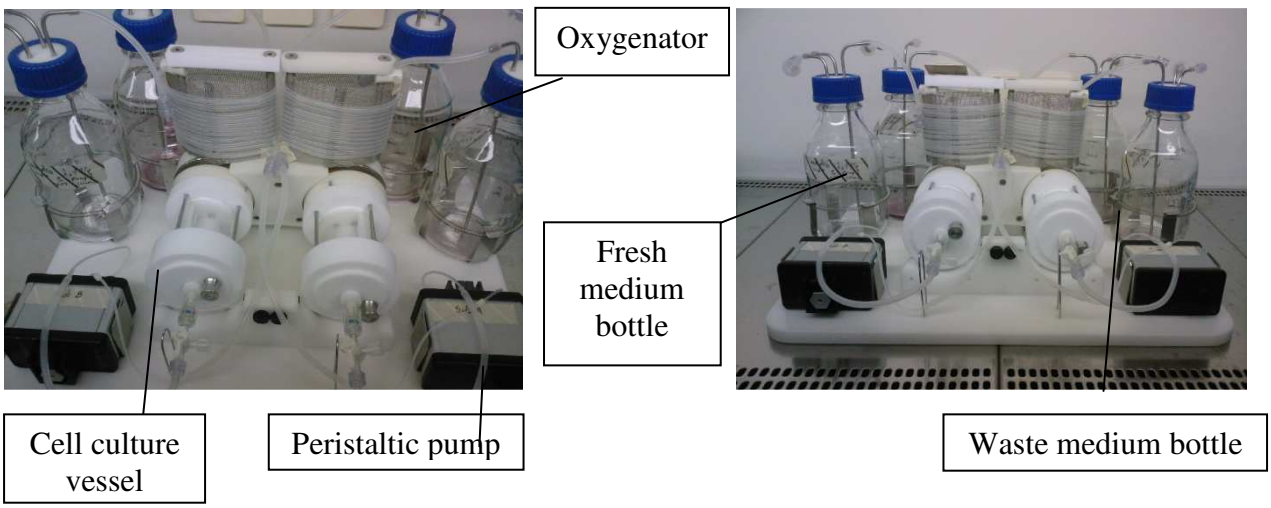


Figure 3.5: Main components of the perfusion bioreactor

3.2.5 Culture in the perfusion bioreactor

For one set of encapsulation, 10 million cells are mixed with 4ml of alginate and the mixture goes through a peristaltic pump and cross links in the end with a $CaCl_2$ solution, thus creating 500 beads each containing 20000 cells. These 500 beads are transferred in one vessel of the custom-made perfusion bioreactor (BSEL). One more set of 500 beads is performed and transferred to the other vessel of the perfusion bioreactor. Each of the vessels could accommodate 60ml of medium. A flow rate of a peristaltic pump was empirically confirmed to produce proper single droplets using a 25-gauge needle (Becton Dickinson, Oxfordshire, UK) and a drop height was 15 mm. The rotation speed was set between 10 rpm and 20 rpm based on the stability of suspended cellular constructs within the vessel. Perfusion was performed in a rate of approximately 60 ml/day. Once per week, a number of beads were collected from each vessel in order to perform various analyses either on the same day or later by the end of the experiment. More detailed information regarding the required number of beads for each analysis is described in the Materials and Methods section.
Photographs of the cells inside the alginate beads were taken using a Leica DM IL microscope (Leica, Wetzlar, Germany) and were analysed using the analysis^D software (Olympus, Munich, Germany).

3.2.6 Differentiation

HepG2-CM was applied in each vessel for the first 3 days to get cell expansion while 'forcing' the cells to go to mesoderm formation. Differentiation to osteogenesis was triggered using alpha Minimal Essential Medium (α-MEM; Invitrogen) containing 15% (v/v) of FBS and 1% (v/v) of penicillin and streptomycin, which has been commonly used for EB formation (Desbaillets et al., 2000; Martin and Evans, 1975) supplemented with 50 μg/ml of L-ascorbate-2-phosphate (Sigma-Aldrich) and 10 mM b-GP (Sigma-Aldrich) from the onset of osteogenic differentiation. Depending on the experimental group, 1 μM Dex (Sigma-Aldrich) was additionally supplemented on day 21 of culture for the last week or 0.1 nM of Sim on day 14 of culture for the last two weeks of the experiment.

Dex is a steroid acting as a strong osteoinductive agent. It is widely employed by many research groups in order to perform osteogenic differentiation of both MSCs and ESCs. Following published protocols by previous people in the lab (Hwang et al., 2006) and by other groups, (Moslem et al., 2015; Qiao et al., 2011; Smith et al., 2010; Zheng et al., 2013) concentration of **1 μM Dex** was supplemented in the osteogenic media without performing any optimization myself. Moreover, Dex was supplemented in the differentiation media on day 21 and for the last week of culture because it has been previously indicated that mineralisation was enhanced and the osteogenic differentiation was more efficient when Dex was supplied in the culture during the late stages of differentiation (Buttery et al., 2001) and specifically, after 14 days of culture. The reason for this effect is the fact that Dex is such a potent osteoinductive agent that when supplied early does not permit the expansion of the cells which stop proliferating and are directed to differentiate (Bielby et al., 2004)

Sim is a small molecule widely used as a drug to lower cholesterol and prevent cardiovascular diseases. Apart from this main role, it has been also demonstrated that it exhibits a number of other pleiotropic effects, one of which is the anabolic effects on bone formation. Based on that, research using various cell types indicated its capacity to enhance osteogenic differentiation through upregulation of BMP2, the strongest osteoinductive factor. Following initially a published paper from previous people working in the lab (Pagkalos et al., 2010), concentration of 0.1 nM Sim was used and the chemical was supplied in the culture media from day 4 of the experiment. Initial results were not satisfactory, no matter the fact that the employed concentration was low and did not have toxic effects. Moreover, literature research indicated the role of Sim as a cell cycle inhibitor. For this reason, a short optimisation was performed regarding the time of supplementation of Sim in the osteogenic media and in the end, day 14 was chosen as a good time point in order to obtain enough cells and increased osteogenic differentiation. The previously suggested concentration was also tested without further optimization.

3.3 Experimental analysis

3.3.1 Qualitative analysis

3.3.1.1 Immunocytochemistry in 2D configuration

Cells were seeded and cultured on chamber slides at a density of 10^4 cells per well. They allowed to attach and then, they were washed with PBS before proceeding and get fixed for 20 minutes at room temperature in 4% (w/v) paraformaldehyde (PFA; BDH Laboratory Supplies). Next step, cells were treated with 0.2% (v/v) Triton-X-100 (BDH Laboratory Supplies) for 15 minutes at room temperature for permeabilization and washed twice with PBS.

The samples were then incubated with 3% (v/v) blocking goat or donkey serum (Vector Laboratories) for 30 minutes at room temperature in primary diluents composed of 0.05% (w/v) bovine serum albumin (BSA; Sigma-Aldrich), 0.01% (w/v) NaN_3 (Sigma-Aldrich) in PBS to block unspecific binding of the antibodies.

The serum solution was removed and the samples were incubated with primary antibodies diluted in primary diluents at 4^oC overnight followed by two washes and incubation with secondary antibodies diluted in secondary diluents consisting of 0.05% (w/v) BSA in PBS for 30 minutes at room temperature in the dark. For dual staining, the same steps after the first treatment of primary antibody were repeated for the second reaction. Finally, the samples were washed twice in PBS and mounted using Vectashield TM with 1.5 μg/ml 4', 6' diamidino-2-phenylindole (DAPI) (Vector Laboratories). Negative controls were also performed. Information on the employed antibodies is shown on Table 3.1.

Table 3.1: Antibody list

Antigens	**Primary antibodies (dilutions)**	**Secondary antibodies (dilutions)**
Oct4	Rabbit polyclonal (Santa cruz Biotech) (1:80)	Goat anti-rabbit IgG FITC (Santa cruz Biotech) (1:200)
SSEA1	Mouse monoclonal (Santa cruz Biotech) (1:100)	Donkey anti-mouse IgG Texas Red (Santa cruz Biotech) (1:200)

FITC: fluorescein isothiocyanate

3.3.1.2 Conventional polymerase chain reaction (PCR)

3.3.1.3 RNA extraction, Reverse transcription, PCR amplification and agarose gel electrophoresis

After dissolving beads, total RNA from the samples was extracted by using Total RNA Purification Kit (Norgen Biotek Corporation, Canada), according to the manufacturer' instructions.

RNA was then reversed transcribed into complementary DNA (cDNA) using the Reverse Transcription System (Promega, Southampton, UK) following the manufacturer's recommendations with some modifications. Briefly, Mastermix for cDNA synthesis was prepared with 1 µg of RNA adding 5 mM $MgCl_2$, 1 mM of 2'-deoxynucleoside 5'-triphosphate (dNTP) mixture, 1 unit/µl recombinant RNasin ribonuclease inhibitor, 15 units/µl AMV reverse transcriptase and 0.5 µg/µl random primers in a total volume of 20 µl per reaction. The RNA samples were heated at 70^{o}C for 10 minutes, briefly centrifuged and cooled on ice for 5 minutes. After the mastermix was added to RNA samples, they were incubated at 25^{o}C for 10min and then reverse transcription was performed at 42^{o}C for 40 minutes followed by denaturation at 90^{o}C for 5 minutes and a cooling step at 4^{o}C for 5 minutes.

50 µl of cDNA samples containing the mastermix, 1x PCR Buffer, 0.2 mM dNTP mixture, 1.5 mM $MgCl_2$, 0.2 µM each primer (sense and anti-sense), 1 unit of Platinum Taq DNA Polymerase, and DNase free water (all supplied by Invitrogen) were amplified with the following PCR conditions: Taq polymerase activation, 94^{o}C for 10 min; thermal cycling, 94^{o}C for 30 sec, annealing temperature (AT) for 30 sec, 72^{o}C for 60 sec; followed by a final elongation step at 72^{o}C for 10 min.

The PCR products were analysed and visualized on a 1.5% (w/v) agarose gel by ethidium bromide staining with ultra-violet light.

Gapdh was used as a housekeeping gene. Pluripotency markers Nanog and Rex 1 were tested. The expression of important bone markers COL 1 and BMP2 was also evaluated. Mesoderm marker Brachyury T, endoderm marker Gata4 and primitive ectoderm marker Fgf5 were also examined.

The information of specific PCR conditions employed for each primer is indicated on Table 3.2

Table 3.2: Primers of genes tested for gene expression analysis

GENES	SEQUENCES	SIZE (bp)	AT (Co)	CYCLES
Nanog	(fwd) CCTGATTCTTCTACCAGTCCCA (rvs) GGCCTGAGAGAACACAGTCC	123	58	34
Rex1	(fwd) TGGAAGCGAGTTCCCTTCTC (rvs) GCCGCCTGCAAGTAATGAG	127	58	32
Fgf5	(fwd) AGTTTCCAGTGGAGCCCTTC (rvs) AATTTGGCACTTGCATGGAG	235	58	32
Gata4	(fwd) CTC CTA CTC CAG CCC CTACC (rvs) GTG GCA TTG CTG GAG TTACC	571	58	30
BrachyuryT	(fwd) GCTTCAAGGAGCTAACTAACGAG (rvs) CCAGCAAGAAAGAGTACATGGC	116	58	32
COL 1	(fwd) CGTGGCGACCAAGGTCCAGT (rvs) AGGGAGACCCAGAATACCGGGAG	165	58	30
BMP2	(fwd) CCTTACCAAAAATGGAGGCTCA (rvs) GGCTCAGTGTCGTCATCATTAAA	201	58	32
GAPDH	(fwd) CATGGCCTTCCGTGTTCCTACCC (rvs) CCTCAGTGTAGCCCAAGATGCCCT	145	58	30

3.3.2 Quantitative analysis

3.3.2.1 ALP activity

ALP activity was measured using r-nitrophenol phosphate (rNPP, Sigma-Aldrich) as a substrate. Cellular ALPase was determined colorimetrically by rNPP contents according to the reaction. Three beads (n=3) were collected from each group and dissolved in the depolymerisation buffer. The cells from beads were incubated in 200 µl of ALPase buffer (Sigma-Aldrich) and 200 µl of rNPP solution at 37^{o}C for 30 minutes with gentle shaking in the dark. 400 µl of 3N sodium hydroxide (NaOH; BDH Laboratory Supplies) solution was added to each sample to stop the reaction and then 100 µl of the final solution was read at 410 nm with ELISA reader (MRX II plate reader; Dynex technologies, West Sussex, UK).

The enzyme activity was calibrated with rNPP standard curve (Appendix 2) and expressed as micromoles of reaction product per minute per total cell number obtained from DNA quantification.

3.3.2.2 DNA quantification

Total DNA content was measured using Quant-iT PicoGreen double-stranded DNA (dsDNA) Reagent and Kits (Invitrogen), as an indirect method of evaluating cell numbers during the experimental procedure (Ng et al., 2005) following a previously used similar protocol (Randle et al., 2007), with minor modifications.

Ten beads were collected from each group, dissolved in the depolymerisation buffer, washed in PBS and cell pellet was stored in -80^{o}C until analysed. For the analysis, cell pellets from the samples were digested in 50 μg/ml proteinase-K solution (Sigma-Aldrich, Poole, UK) based on 100 mM dibasic potassium phosphate, pH 8.0 (K_2HPO_4; Sigma-Aldrich) overnight at 37^{o}C. Proteinase K was inactivated by heating at 90^{o}C for 10 minutes and the samples were stored in the ice.

A 24 well plate was prepared by putting in each well 50 μl of the sample with the digested DNA or 50 μl of 1x Tris-EDTA buffer (TE buffer) for the negative control. Then, 350 μl of 1x TE buffer were added to all of the wells and mixed thoroughly. After that step, the digested cell supernatant was combined with PicoGreen reagent. In particular, a 96 well plate was prepared by putting in each well 50μl of the mix in the 24 well plate along with 50 μl of PicoGreen reagent (Invitrogen, Paisley, UK) which has been diluted in DNase free TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

Finally, the 96-well plate was read in a fluorometer (skanIT Varioskan fluorometer, Thermo scientific) at the excitation wavelength of 480 nm and the emission wavelength of 520 nm. The amount of total DNA from a known cell quantity produced from routinely cultured mESCs was enumerated in order to calibrate the number of cells per sample (DNA quantification standard curve in the Appendix 1).

Taking into consideration the limitations of the various techniques and the results from this study, it is suggested that measuring total DNA is the most reliable strategy for quantifying cell proliferation in high cell density (100.000 cells/cm^2) and 3D cultures (Ng et al., 2005).

3.3.2.3 Alizarin Red S staining (ARS)-based quantification of bone mineralisation

Mineralisation of ESC-based osteogenic constructs was quantified by the method developed by Gregory et al (Gregory et al., 2004), which is based on ARS assay, with some modifications. Twenty beads from each group on days 21 and 29 were collected (n=3), dissolved in the depolymerization buffer and centrifuged at 400g for 10 min. The cell pellets were immerged in 4% paraformaldehyde at room temperature for 20 min and washed with PBS followed through centrifugation in the same way above. 2 ml of 40 mM ARS (pH 4.2, filtered) was added on the PBS-washed cells and incubated with shaking at RT for 20 min, followed by 3 times washing process with double distilled water. 0.8 ml of 10% acetic acid was added to each sample, which was subsequently incubated with shaking at room temperature for 30 min, followed by incubation at 85^{o}C for 10 min and centrifugation at 20,000g for 15 min. The measurement with 150 µl of the supernatant was done in 96 well plates at 405nm using ELISA reader (Dynex technologies) after adding 200 µl of 10% (v/v) ammonium hydroxide.

3.3.2.4 MTS

The number of viable cells was determined with a colorimetric method using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega). The CellTiter 96® AQueous One Solution Reagent contains the MTS tetrazolium compound (Owen's reagent) which is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of living cells in culture. For the analysis, a 48 well plate was used in the first step. One bead was placed to each well (n=5) and media was sucked out. 40 µl of the CellTiter 96 Aqueous One Solution Reagent and 200 µl of basal medium were added per well with lights closed (Dilution 1:5). Negative control wells with no beads were also prepared. The plate was put in a shaker for 20 min and then was transferred in 37^{o}C in an incubator for 2h 40 min. After the 3 hours, the samples were transferred in a 96-well plate to be measured. 100 µl from each well from the 48-well plate were put in a well in the 96-well plate. Two readings were performed for each well. Finally, the absorbance was recorded at 490 nm in an ELISA reader (MRX II plate reader; Dynex technologies, West Sussex, UK).

3.3.2.5 Real-time polymerase chain reaction (RT-PCR)

Extended gene expression analysis was performed during the experimental procedure in order to evaluate the progress of osteogenic differentiation from mESCs. Analysis of bone markers, cartilage and hypertrophic chondrocyte markers and three germ layer markers, was tested. The procedure consisted of three major steps. It started with RNA extraction, followed by reverse transcription of mRNA to cDNA to finish with RT-PCR for amplification of the end product and semi-quantitative gene expression analysis. Each step is analysed separately on the following sections.

3.3.2.6 RNA extraction

Cell pellets from the samples to be analysed were stored in -80^{o}C after each experiment. Before proceeding with RNA extraction, cell pellets were removed from the freezer and allowed to thaw at room temperature. RNA was extracted using Total RNA purification kit (Norgen Biotek, Canada) following the kit instructions. Briefly, cells were dissolved with a lysis solution supplemented with 1% of b-mercaptoethanol and mixed by vortex. 100% ethanol was added to the lysate to precipitate the binding of RNA to the RNeasy membrane and mixed again by vortex. The lysate went through a provided column in order for the RNA to bind on it. Total RNA that bound to the membrane column was washed once with the washing solution provided in the kit. Before use for the first time, the washing solution was supplemented with 100% ethanol. Next step is on-column DNA removal which was performed using DNase treatment as an additional step to digest contaminating DNA followed by washing of the column two more times with the washing solution provided. RNA was then eluted in a 1.7 ml elution tube provided with the kit using 50ul of Elution Solution. Every time a reagent was added in the column, centrifugation was performed twice in 14000g for one minute. RNA quantification was performed on a UV spectrometer and the A260/A280 ratio of greater than 1.70 was required to ensure purity of RNA sample quality. RNA samples then were employed for cDNA synthesis or stored at -80^{o}C until used.

3.3.2.7 cDNA synthesis

RNA was reversed transcribed into cDNA using the Reverse Transcription System (Promega, Southampton, UK) following the manufacturer's recommendations with some modifications. Briefly, samples containing 1 μg of RNA were prepared and were heated at 70^{o}C for 10 minutes;

left them to cool on 4°C until used in the next step. During the incubation, mastermix for cDNA synthesis containing 25 mM $MgCl_2$, RT 10X buffer, 10 mM of 2'-deoxynucleoside 5'-triphosphate (dNTP) mixture, 1 unit/μl recombinant RNasin ribonuclease inhibitor, 15 units/μl AMV reverse transcriptase and 0.5 mg/μl random primers in a total volume of 10.1 μl per sample, was prepared. The mastermix was added to RNA samples giving a total volumeof 20ul per reaction. The samples were incubated at 25°C for 10min and then reverse transcription was performed at 42°C for 40 minutes followed by a cooling step at 4°C for 5 minutes. cDNA was stored in -20°C.

3.3.2.8 Quantitative real-time PCR (qRT–PCR)

Gene expression was determined using qRT-PCR in the StepOnePlus Real-time PCR instrument (Applied Biosystems). A number of different genes were tested in order to get information regarding bone formation and how osteogenic gene expression was evolved during the 29 days of differentiation. To get more insight details about which pathway of osteogenesis was used, a number of cartilage markers were also tested. Markers from the three germ layers were tested to show the progress of differentiation and give significant information about bone development (Table 3.5). The usual candidate GAPDH was not giving satisfying results as control gene. For this reason and after looking into the literature, the housekeeping gene RPL27 with more stable expression was employed.

For the reactions, the SensiFAST SYBR Hi-ROX Kit (Bioline, London, UK) was employed. The composition of the reaction mix is shown on Table 3.3. The total volumeof reaction was 20 μl.

Table 3.3 Reaction mix composition

REAGENT	VOLUME	FINAL CONCENTRATION
2X SensiFAST SYBR Hi-ROX Mix	10 μl	1X
10μM Forward primer	0.8 μl	400 nM
10μM Reverse primer	0.8 μl	400 nM
CDNA	2 μl	100 ng
H_2O	up to 20 μl	

A 2-step cycling protocol was used. The thermal cycling conditions consisted of 40 cycles and details are shown in Table 3.4.

Table 3.4 2-step cycling protocol

Cycles	Temperature	Time	Notes
1	95^0C	2 min	Polymerase activation
40	95^0C	5 sec	Denaturation
	60^0C	20 sec	Annealing/extension

After completing the PCR in the end of the 40 cycles, melting curve analysis was performed in order to control for primer–dimer formation. A product melting curve can be obtained during PCR by monitoring the fluorescence of dsDNA dyes as the temperature passes through the product denaturation temperature referred to as the melting temperature, Tm. Melting curve's shape is a function of GC content, length, and sequence. Based on that, primer-dimer products are shorter than the targeted product and they will melt at a lower temperature, something that can be recognized by melting curve analysis (Kubista et al., 2006; Ririe et al., 1997).

Relative gene expression analysis was conducted using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where the amount of target, normalized to an endogenous reference and relative to a calibrator, is given by amount of target = $2^{-\Delta\Delta CT}$ where :

$$\Delta\Delta CT = (C_{T,\,Target} - C_{T,\,Rpl27})_{\,Time\ x} - (C_{T,\,Target} - C_{T,\,Rpl27})_{\,Time\ 0.}$$

The $2^{-\Delta\Delta CT}$ method requires the assignment of a housekeeping gene, which is assumed to be uniformly and constantly expressed in all samples and of a reference sample. The expression of the tested samples is compared with that of the reference sample (Pfaffl, 2001).
In this project, ribosomal protein L27 (**Rpl27**) was used as a housekeeping gene and day 0 cells as a reference sample. The expression of the gene of interest was determined by the difference of its expression normalized with the housekeeping gene expression for the specific time point minus the difference between the mentioned samples for time zero. All the obtained gene expression results were statistically analysed.

Table 3.5 The sequences of primers used for real-time PCR to amplify the region of interest
Abbreviations: (A) antisense/reverse primer, (S) sense/forward primer

Gene	Primer Sequence	Amplicon size (bp)
RPL27	(S) AAAGCCGTCATCGTGAAGAAC (A) GCTGTCACTTTCCGGGGATAG	100
ALP	(S) CCAACTCTTTTGTGCCAGAGA (A) GGCTACATTGGTGTTGAGCTTTT	109
RUNX2	(S) TCGTCAGCATCCTATCAGTTCCCA (A) CCATCAGCGTCAACACCATCATTCTGGTTAG	145
OSTERIX	(S) TTGAGGAAGAAGCTCACTATGGCTCCAG (A) GCTGAAAGGTCAGCGTATGGCT	142
COLLAGEN 1 A1	(S) GCATGGCCAAGAAGACATCC (A) CCTCGGGTTTCCACGTCTC	82
BMP2	(S) TCTTCCGGGAACAGATACAGG (A) TGGTGTCCAATAGTCTGGTCA	125
BGLAP	(S) CAGCGGCCCTGAGTCTGACAAA (A) TCACAAGCAGGGTTAAGCTCACACTG	149
BSP	(S) CAGAGGAGGCAAGCGTCACT (A) CTGTCTGGGTGCCAACACTG	80
OSTEONECTIN	(S) GTGGAAATGGGAGAATTTGAGGA (A) CTCACACACCTTGCCATGTTT	104
OSTEOPONTIN	(S) AGCAAGAAACTCTTCCAAGCAA (A) GTGAGATTCGTCAGATTCATCCG	133
MSX2	(S) TCCGCCAGAAACAGTACCTG (A) TTGCAGTCTTTTCGCCTTAGC	121
DLX5	(S) CACCACCCGTCTCAGGAATC (A) GCTTTGCCATAAGAAGCAGAGG	125
ATF4	(S) CCTGAACAGCGAAGTGTTGG (A) TGGAGAACCCATGAGGTTTCAA	134
PTHR1	(S) CAGGCGCAATGTGACAAGC (A) TTTCCCGGTGCCTTCTCTTTC	125
SOX9	(S) TGGCAGACCAGTACCCGCATCT (A) TCTTTCTTGTGCTGCACGCGC	135
AGGRECAN	(S) AGGTGTCGCTCCCCAACTAT (A) CTTCACAGCGGTAGATCCCAG	96
COLLAGEN 10 A1	(S) TTCTGCTGCTAATGTTCTTGACC (A) GGGATGAAGTATTGTGTCTTGGG	115
GATA 4	(S) TCAACCGGCCCCTCATTAAG (A) GTGGTGGTAGTCTGGCAGT	88
SOX 17	(S) ACCTACACTTACGCTCCAGTC (A) GCCGTAGTACAGGTGCAGAG	141
NESTIN	(S) CCCCTTGCCTAATACCCTTGA (A) GCCTCAGACATAGGTGGGATG	115
PAX 6	(S) TAGCCCAGTATAAACGGGAGTG (A) CCAGGTTGCGAAGAACTCTG	132
DESMIN	(S) GTTTCAGACTTGACTCAGGCAG (A) TCTCGCAGGTGTAGGACTGG	105
HAND 1	(S) CTACCAGTTACATCGCCTACTTG (A) ACCACCATCCGTCTTTTTGAG	101
BMP 4	(S) TTGATACCTGAGACCGGGAAG (A) ACATCTGTAGAAGTGTCGCCTC	117

3.3.2.9 Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) analysis

ATR-FTIR spectroscopic imaging is a highly versatile, label free and non-destructive chemical imaging method, which can be applied to study a wide range of samples and systems. Two of the major advantages of measuring in ATR mode are the opportunity to measure samples that absorb strongly in the IR spectrum, such as aqueous systems, without significant sample preparation and the ability to increase the spatial resolution of the measured image. The chemical specificity of FTIR imaging originates from the interaction (via absorption) of infrared light with the vibrational modes of the molecules being interrogated (Kazarian and Chan, 2013).
ATR-FTIR imaging was conducted using a continuous scan FTIR spectrometer (Varian 7000 FT-IR, Varian Inc. CA, USA) in conjunction to a large sample compartment extension (Varian LS) and a 64 x 64 focal plane array (FPA) detector. Further details about the ATR-FTIR imaging setup can be found in the suggested literature (Chan and Kazarian, 2003). Sample beads were placed onto the measuring surface to be in contact with the ATR crystal (oil analyser, Specac Ltd., Kent, UK). Excess water was removed and the sample was enclosed to prevent further evaporation, thus ensuring that the bead remains hydrated during the measurement. Three to five beads were measured from each sample.

3.3.2.10 Media analysis

Nutrient and metabolite concentrations were analyzed in the Bioprofile 400 Analyzer (Nova Biomedical, Flintshire, UK) using culture supernatant samples (1.0 ml) collected on day 29, the last time point of the experiment. Comparison was performed between samples treated either with Dex or with Sim protocol.

3.3.2.11 Statistical analysis

SigmaStat (Systat Software UK, UK, version 3.5) was used for one-way or two-way analysis of variance (ANOVA). Samples for the quantitative analyses were measured for different protocols and the mean values were calculated. The results were expressed as mean ± standard deviation and were analyzed using ANOVA, comparing all data pairs. Error bars on all graphs represent the standard deviation (SD) of the mean. Statistical significance was considered at $p<0.05$.

RESULTS

4th CHAPTER

4. OSTEOGENIC DIFFERENTIATION OF MESCS WITH THE USE OF CONDITIONED MEDIA AND BY BYPASSING EB FORMATION

4.1 Introduction

ESCs are a unique population of cells with two distinctive properties: unlimited self-renewal capacity and pluripotency, meaning potential to differentiate into all adult cell types, including the germ cells (Bradley et al., 1984; Pera et al., 2000; Smith, 2001). These features make them an attractive option for cell therapies as they can provide a theoretically unlimited source of different cells needed.

The field of research that is the centre of attention in this thesis is osteogenic differentiation. Traditionally, osteogenic differentiation of ESCs begins with their aggregation in suspension culture leading to the formation of EBs. EBs are 3D structures that recapitulate early aspects of mammalian development (Doetschman et al., 1985; Shen and Leder, 1992) and contain cells from all three germ layers (Desbaillets et al., 2000). The problem with EBs is that it is difficult to control spontaneous differentiation and to direct cells to the specific cell lineage needed.

An alternative strategy to bypass this step and to direct more efficiently and enhance osteogenic differentiation of ESCs is with the use of conditioned culture medium. It is known from developmental biology that ESCs generate derivatives of the three germ layers via a primitive ectoderm intermediate (Shen and Leder, 1992). Research on EB formation indicated the existence of important inductive signals involved in primitive ectoderm formation and mesoderm differentiation (Coucouvanis and Martin, 1999; Johansson and Wiles, 1995; van den Eijnden-van Raaij et al., 1991).

Based on that, it has been previously reported that the use of medium conditioned by HepG2 cell line induced the differentiation of ESCs to a homogeneous pluripotent cell population, termed early primitive ectoderm-like (EPL) cells. Characterization of EPL cells revealed that they are close related with primitive ectoderm of the pre-gastrulation embryo (Rathjen et al., 1999). Moreover, it has been suggested that liver cell lines possess characteristics similar to those of visceral endoderm, an early regulator of embryogenesis, which directs cell fate (Rodda et al., 2002). This is the first report of factors affecting ES differentiation to a homogenous cell population without intermediate cellular aggregation constituting EB formation (Rathjen and Rathjen, 2001). Differentiation of mESCs with the use of HepG2-CM resulted in the generation of a restricted repertoire of cell types comprised of mesoderm and parietal endoderm. This method could provide an efficient way to enhance mesoderm formation and eliminate the generation of the other germ layers (Kang et al., 2009b).

Based on the above findings, it has been also shown from previous people in the group that treatment of mESCs with conditioned medium from the HepG2 enhanced mesoderm formation and derivation of osteogenic cells without the need for the step of EB formation (Hwang et al., 2006). Finally, osteogenic differentiation is widely performed in osteogenic media containing b-GP, AA and Dex. In particular, it has been shown that when Dex was supplied in the late stages of the differentiation protocol, mineralisation was enhanced (Buttery et al., 2001).

4.2 Aim and Objectives

The aim of this chapter was to show that successful osteogenic differentiation could be performed from mESCs, by using either traditional protocols with the step of EB formation or newly developed protocols with the employment of conditioned medium. Dex was used as an osteoinductive factor and was supplied in the last week of culture. 2D and 3D culture configurations were tested.

In particular:

1. To evaluate and compare the osteogenic differentiation of mESCS between 3D static and dynamic culture platforms.
2. To estimate the efficiency of the conditioned medium and assess the osteogenic differentiation without the EB formation step.

4.3 Methodology

2D culture was initially performed in tissue culture flasks for cell expansion. Cells were then transferred in Petri dishes for EB formation and finally, in six-well plates for osteogenic differentiation. The differentiation protocol lasted for 29 days.

Immunocytochemistry analysis in 2D culture was performed in order to examine and confirm the initial pluripotent status of the cells before the beginning of the experiment. Photos under the microscope were obtained from each stage, in particular from EB formation and bone nodules, in order to observe the morphology. ARS was also used to evaluate calcium deposition and mineralisation.

For 3D static and dynamic culture, cells were encapsulated in alginate and were cultured in HepG2-CM for 3 days either in flasks or in the perfusion bioreactor. After that period, media was replaced with osteogenic media containing b-GP and AA until day 21, when osteogenic media was further supplemented with Dex for the last week of culture.

In 3D cultures, a number of different analyses were employed in order to examine the osteogenic process. Conventional PCR was performed in samples obtained from the early days of culture where expression of pluripotency and initial germ layer formation markers was evaluated. DNA quantification was employed as an indirect method to calculate cell numbers. Measurement of ALP activity was used as an efficient way to prove osteogenic differentiation. ARS was employed as a method to test for calcium deposition and mineralisation. ATR-FTIR was used to confirm the presence of HA. Photos of the hydrogels under the microscope indicated cell morphology. Finally, gene expression studies using qRT-PCR provided some more insight information and quantitative results on the progress of osteogenic differentiation.

4.4 Results

4.4.1 2D BIOPROCESS OF MESCS TOWARDS OSTEOGENESIS USING EB FORMATION AND DEX

The standard protocol for osteogenic differentiation of ESCs in a 2D configuration started with their expansion, then continued with the step of EB formation and it ended up with bone nodule formation, which proved osteogenic differentiation. It was important to evaluate and indicate the

initial pluripotent status of the cells before beginning any experimental procedures. For this reason, immunocytochemistry for two main pluripotency markers was performed and they both indicated positive expression. Oct4 is the most well known nuclear factor associated with pluripotency. Another important pluripotency factor is the surface marker SSEA1.

Photos under the fluorescence microscope were taken and are presented in this section. Mouse primary antibodies against Oct-4 and SSEA1 were employed. Next step involved the incubation of cells with secondary antibodies raised in different species and labelled with different fluorochromes in each case. In particular, secondary antibody against Oct-4 was FITC-conjugated and was visualised under the green light while secondary antibody against SSEA1 was Texas Red-conjugated and was visualised under the red light in the fluorescence microscope. Samples were subsequently counterstained with DAPI and photos were taken under the blue light. Dual stained photos acquired under the microscope are also presented where FITC-conjugated cells and Texas Red-conjugated cells are combined with DAPI (Figure 4.1).

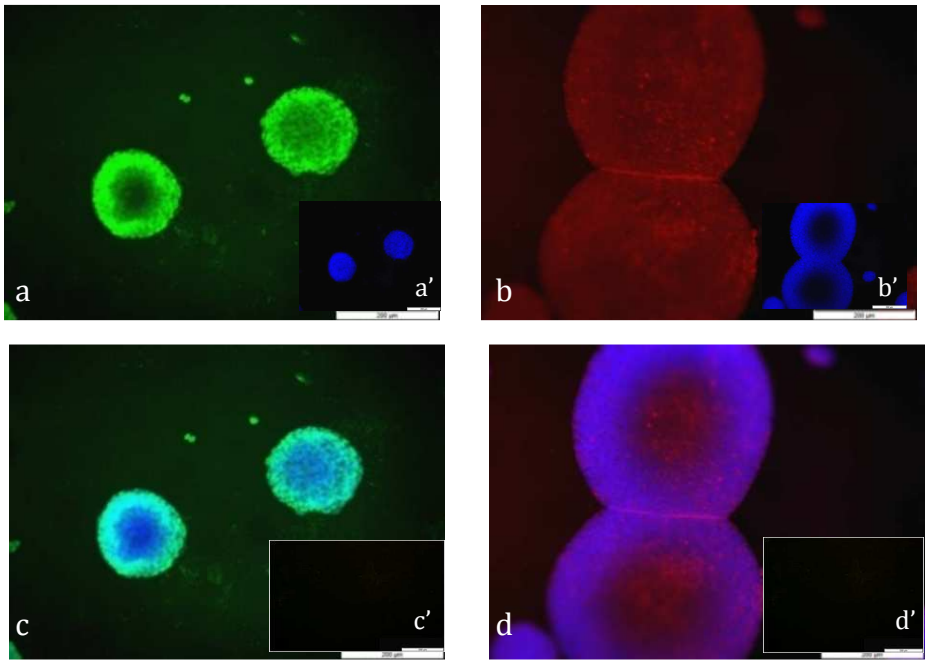


Figure 4.1: Immunocytochemistry photos indicating the expression of pluripotency markers Oct4 (a) and SSEA-1 (b). Nuclear DAPI counterstain was also performed (a', b'). Dual stained photos, combining FITC and DAPI staining for the expression of the nuclear marker Oct4 (c) and Texas Red and DAPI staining for the expression of the surface marker SSEA-1 (d), were analyzed. Negative controls for FITC and Texas Red were also conducted (c', d'). Scale bar is 200μm.

After confirming the pluripotent status of the starting cell population, the 29 days procedure of osteogenic differentiation began. It started with the initial expansion of the cells, followed by EB formation and ended up with bone nodule formation. Photos under the microscope were taken from each stage (Figure 4.2).

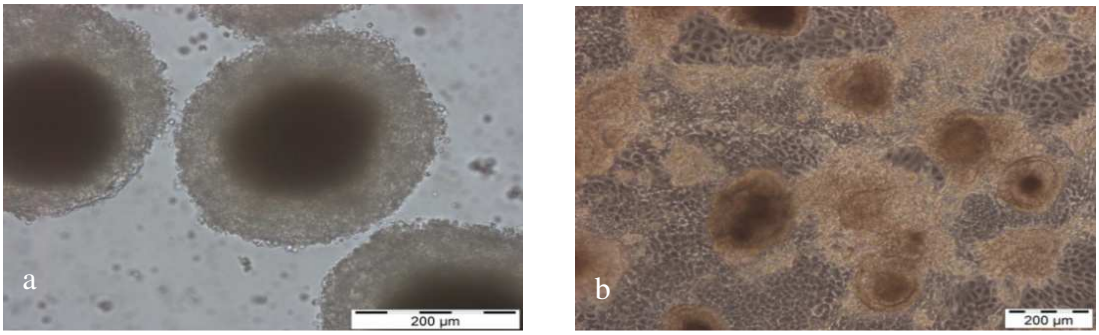


Figure 4.2: Photos obtained under the microscope indicate the formation of EBs (a) and of bone nodules (b). Scale bar is 200µm.

To further validate and confirm osteogenic differentiation and mineralisation, after 21 and 29 days of culture, ARS was used as a method to indicate calcium deposition and mineralisation. Photos using a conventional camera were obtained from the stained six well plates (Figure 4.3).

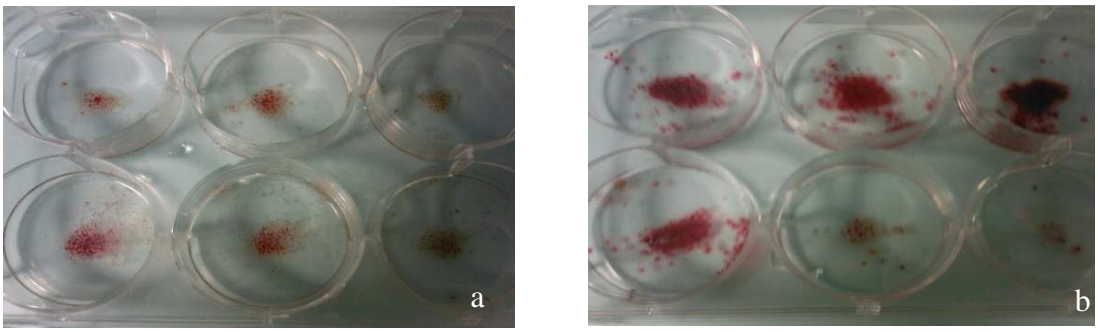


Figure 4.3: ARS was performed in order to evaluate calcium deposition on the six well plates on day 21 (a) and on day 29 (b) of the experiment.

ARS was performed in a six well plate where the same number of cells was initially seeded in each of the six wells. ARS was performed on day 21 and day 29, as it can be observed from the photos of the stained plates. On day 21, all the six wells indicated about the same amount of

staining suggesting similar quantity of calcium deposition. On day 29, however, four of the wells had similar amount of calcium deposition while the other two had less amount of staining. The reason for this is probably related to technical issues that had to do with the procedure followed for quantification of the staining. To be more specific, the first step to quantify ARS involves good washing to remove excess staining. This procedure takes place in the sink under the tap water and there is a chance for some cells to be detached during this process. This is the reason for the fewer stained cells observed in the two wells compared to the rest of the plate.

Subsequent quantification of the ARS was also performed and results are presented in the following graph (Figure 4.4). Statistical analysis indicated that on day 29, there was statistically significant higher calcium deposition compared to day 21, as expected.

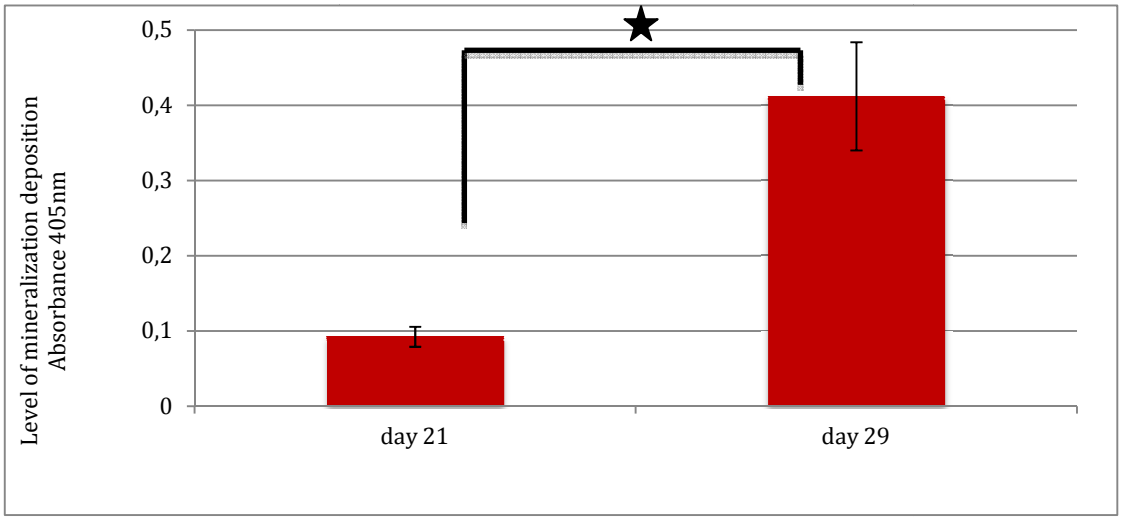


Figure 4.4: Quantification of ARS on day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested time points.

4.4.2 3D BIOPROCESS OF MESCS TOWARDS OSTEOGENESIS USING DEX

As mentioned in the literature review, 2D cultures pose a number of limitations and they are not mimicking the *in vivo* microenvironment. They cannot also provide and support the high cell numbers needed for cellular therapies due to the limited space for the cells to grow in 2D (Xu et al., 2014). 3D culture platforms can overcome these problems and elicit a more physiological state, which can offer high number of better quality cells (Baker and Chen, 2012). One widely

used protocol for the osteogenic differentiation of ESCs comprises of 29 days of differentiation and employs the steroid Dex, which has been shown to enhance mineralisation in various cell types (Blum et al., 2004; Kirton et al., 2006; Mori et al., 1999; Yamanouchi et al., 2001).

In this chapter, cells were encapsulated in alginate and then transferred in tissue-culture flasks with HepG2-CM for three days. After that, media was replaced with osteogenic media containing b-GP and AA. There were two experimental groups. The first group included beads cultured in tissue culture flasks in static configuration and the second group composed of beads cultured in the RWV perfusion bioreactor. On day 21 of the experiment, osteogenic media was further supplemented with Dex for the last week of culture (Figure 4.5). Once per week samples were collected from static and perfusion cultures to perform, either on the same day or later, a number of different analyses and evaluate the differentiation status of the cells.

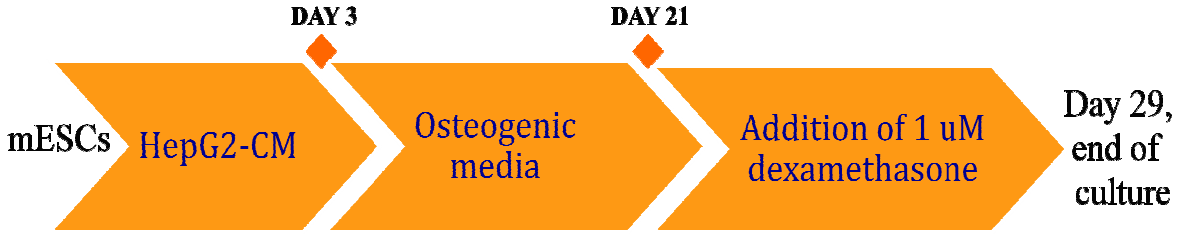


Figure 4.5: The protocol employed for osteogenic differentiation of mESCs

In this PhD project, a custom made RWV perfusion bioreactor was employed for the experimental procedures. This bioreactor combined the advantages and disadvantages of two types of environments: the one generated by the RWV and the one created by the perfusion culture configuration. Following the example of previous people working in the lab and also of other research groups, static cultures, performed in tissue culture flasks, were employed as controls. In order to make the systems comparable, the same number of hydrogels and the same amount of culture media was employed. This static configuration is widely employed by the majority of research groups because of the experimental convenience.

However, it is not an accurate and good control that is really comparable with the dynamic configuration. It would be more appropriate to employ the same culture platform, the bioreactor, for both conditions and culture the hydrogels inside in static configuration. Culturing the cells in the same environment would offer more comparable conditions. Based on that, it would be appropriate for the performed experiments to have two separate controls. One for the RWV would be hydrogels in the bioreactor vessel cultured as a static non-rotating control and another one for the perfusion would be hydrogels cultured in the vessel with rotation but without perfusion.

Simple PCR analysis and gel electrophoresis were performed to evaluate the pluripotent status of the cells in the beginning of the experiment and to follow the initial profile of gene expression and the time line of differentiation during the early culture period. MESCs grew in the presence of LIF to maintain their pluripotent status. As soon as LIF was removed from the culture media, mESCs started rapidly decreasing the expression of pluripotency markers and increasing the expression of lineage markers (Trott and Martinez Arias, 2013).

Nanog and Rex1 are two well-known markers of pluripotency. Results from gel electrophoresis (Figure 4.6) indicated that these two markers were highly expressed in both culture configurations the first six days and they started to decrease by day 12 where the bands were less intense. Starting from day six and increased by day 12, there was expression of the epiblast-affiliated gene Fgf5. Fgf5 has been shown to start its expression when Rex1 expression starts to decrease (Haub and Goldfarb, 1991; Hebert et al., 1991; Rogers et al., 1991; Trott et al., 2012). Rex1 is a marker of naïve population of ESCs while Fgf5 is a marker of primed population of ESCs (Nichols and Smith, 2009; Yeo et al., 2013).

Gata4 is a marker of visceral endoderm and Brachyury T a marker of mesoderm, the first germ layers to be formed. Results obtained from the analysis of the experiment indicated that these markers started to be expressed on day 12. Finally, COL 1 is an early marker of osteogenic differentiation and the most abundant matrix protein. It started to be expressed on day 12 for both culture configurations. In static culture, there was a brighter band compared to the perfusion indicating a higher level of expression at this time point. This result indicated a delayed expression of COL 1 in perfusion culture in comparison to static.

Gapdh was used as a housekeeping gene and its expression was stable at all tested time points. Negative controls were performed for all the tested genes.

In the obtained photos of the results from the gel electrophoresis, it is not easy to observe and distinguish the suggested decrease and increase in the expression of the genes from the acquired bands. Moreover, there are no data obtained from time points after day 12. However, for

confirmation of the above results, there are gene expression data from these markers obtained with qRT-PCR from Dr Jae Min Cha who was working previously in the lab. He tested the expression of these markers on day 6 and day 11 and observed the suggested decrease in the expression of Nanog and Rex1 and increase in the one of Fgf5 (Appendix 5).

GENES	Day 2		Day 6		Day 12		NEG
	Static	Bioreactor	Static	Bioreactor	Static	Bioreactor	
Nanog 123 bp							
Rex 1 127 bp							
Fgf 5 235 bp							
Gata 4 571 bp							
Brachyury T 116 bp							
COL 1 165 bp							
GAPDH 145 bp							

Figure 4.6: Gene expression results after gel electrophoresis

DNA quantification was used as an indirect method to calculate cell numbers (Figure 4.7). Cells cultured in the perfusion bioreactor showed higher proliferation rate resulting in higher cell numbers throughout the whole duration of the experiment. This finding confirmed previous results from other research groups who observed improved cell proliferation in the perfusion culture (de Peppo et al., 2013a; de Peppo et al., 2013b; Zhao and Ma, 2005). This outcome was expected, as the perfusion bioreactor offers a more favorable environment for the expansion of these cells, with continuous supply of fresh media and removal of metabolites. The experiment started with 20.000 cells per bead, which proliferated and reached their maximum number after one week of culture in both tested platforms. Static culture achieved around 100.000 cells/bead while perfusion culture reached a bit more than 200.000 cells/bead. After the first week of the experiment, in static culture cell numbers maintained the same density until the end of the

culture. On the other hand, in the perfusion culture cell numbers were similar until day 21 and then, there was a small increase on day 29 where they reached a bit more than 250.000 cells/bead.

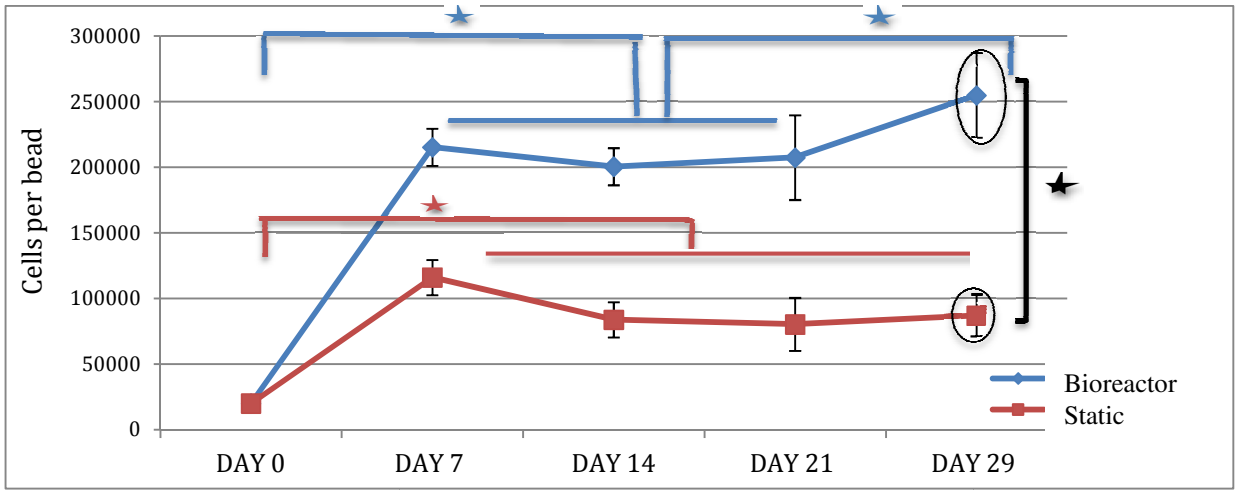


Figure 4.7: DNA quantification throughout the 29 days of the experiment. Comparison was performed between static and dynamic culture configurations. Two-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between these time points within the same platform while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested platforms within the same time point. Values are mean ± SD of N=3.

The important thing to consider was that on day 21, Dex was supplemented in the culture media. This finding suggested that there was a heterogeneous cell population, which reacted differently to Dex. This population consisted of progenitor cells and of more mature cells. The majority of the cells completed the osteogenic process and proceeded towards mineralisation while there were probably still some not fully differentiated cells, which continued to proliferate. This could explain the observed increase in cell numbers on day 29 in the perfusion bioreactor.

The above results confirmed previous findings showing that the favorable environment of the perfusion culture could support much cell numbers compared to static configuration (Fong et al., 2005; Gomes et al., 2003; Marolt et al., 2012a; Oh et al., 2005). It was also crucial to consider that in static culture, there was high chance of cell death occurring in the beads and also creation of necrotic core centre due to the lack of media effectively perfused inside the bead (Carpenedo et al., 2007; Sailon et al., 2009), thus explaining the low cell numbers calculated.

The next important analysis that was performed was the measurement of ALP activity. ALP is one of the most frequently used biochemical markers of osteoblast activity (Balcerzak et al., 2003). ALP is an enzyme secreted from osteoblasts. It is required for osteoid formation and

matrix mineralisation. It is expressed early during the development and it is soon observed on the cell surface and in matrix vesicles. It functions in the initial phases of the process while later in the developmental program its expression declines (Golub, 2009; Golub, 2011).

To be more specific, it has been shown that ALP activity is high in cells undergoing early osteodifferentiation and is maintained until maturation into osteocytes. ALP is thought to facilitate mineralisation of organic matrix through calcium binding, generation of free phosphates or degrading mineralisation inhibitors (He et al., 2010). In this analysis, the same two groups were compared, static versus perfusion. ALP activity was initially presented with the obtained raw absorbance values. Subsequently, ALP activity was normalised with cell numbers obtained from DNA quantification and the results were expressed as μM/cells/min.

ALP activity, before normalisation with cell numbers (Figure 4.8), was reaching a peak after one week of culture, for both tested platforms. After that point, the expression profile was different between the two culture configurations. In perfusion culture, ALP was maintained at the same high level throughout the whole duration of the experiment. In static culture, ALP started to gradually decrease from day 7 until day 21. After day 21 and during mineralisation, ALP activity increased again by the end of the experiment on day 29. ALP in perfusion was higher than static. Similar finding suggesting that the favorable environment of the RWV perfusion bioreactor enhanced ALP activity has been indicated previously by other groups (Bancroft et al., 2002; Goldstein et al., 2001).

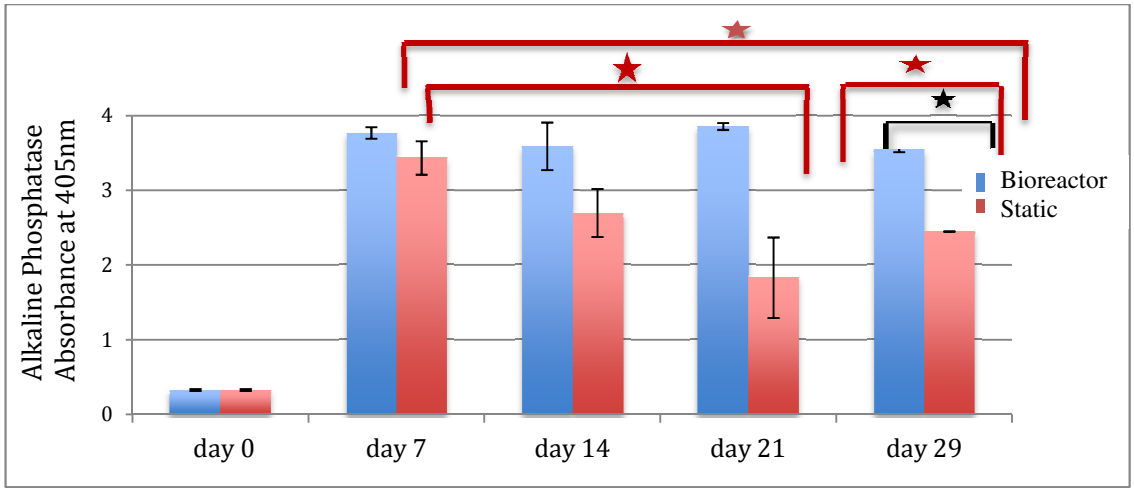


Figure 4.8: ALP activity throughout the 29 days of the experiment. Comparison was performed between static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested platforms within the same time point whereas asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested time points within static platform. Values are mean ± SD of N=3.

On the other hand, after normalisation with cell numbers, the trend was different (Figure 4.9). In perfusion culture, normalised ALP activity reached highest level by day 7 and maintained it until day 21, while on day 29, there was a statistically significant decrease. The reason for this outcome was the observed increase in cell numbers on day 29 in the bioreactor culture using the Dex protocol.

In static culture, ALP activity was maximised by day 7 and maintained the same level until day 14. There was a subsequent decrease by day 21 and then ALP activity was increased again by day 29 in the end of the experiment.

In most of the time points of the experiment, ALP activity, after normalised with cell numbers, was higher in static than perfusion.

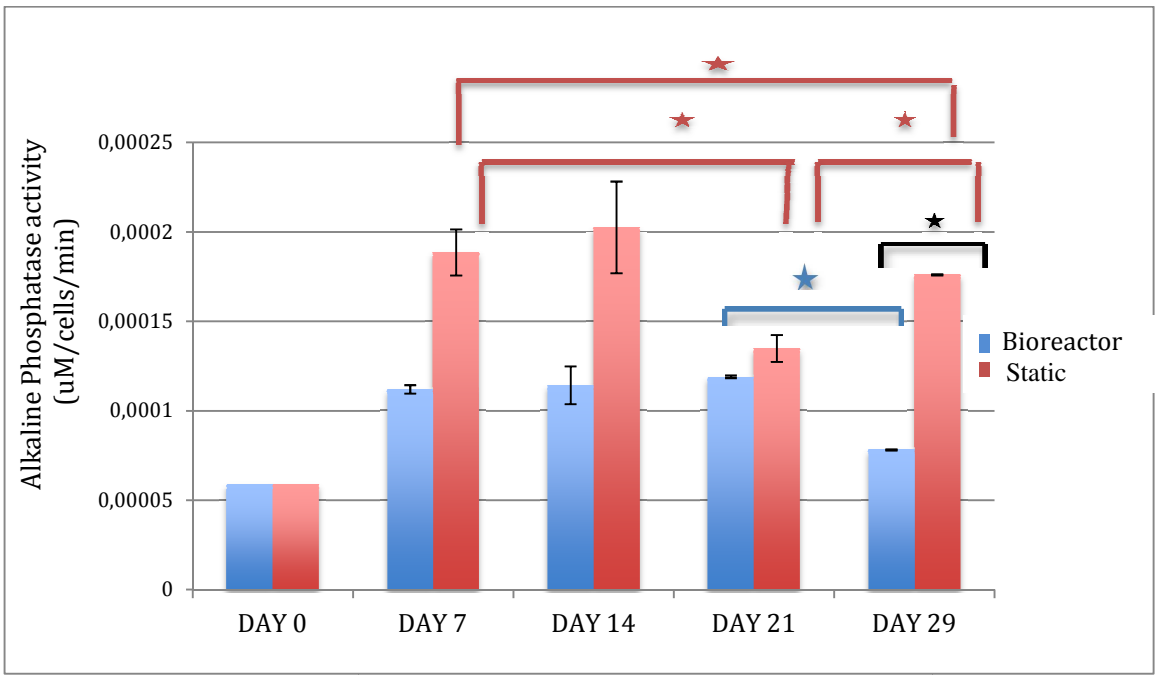


Figure 4.9: ALP activity normalized with cell numbers obtained from DNA quantification and expressed as μM/cells/min throughout the 29 days of the experiment. Comparison was performed between static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested platforms within the same time point. Asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between these time points within the same platform.

ALP was measured biochemically during the 29 days of the experiment and comparison was made between perfusion and static 3D culture configuration. By day 7, both platforms achieved similar and maximum amount of ALP activity. After that, in perfusion culture, ALP activity maintained the same high level until day 21 and marked a small decrease by day 29. In static culture, ALP activity gradually decreased until day 21 and marked a small increase by the end, on day 29.

DNA quantification was also used to estimate cell numbers throughout the 29 days of the experiment. In both platforms, experiments started with the same number of cells. By day 7, both cultures proliferated and reached maximum cell density with perfusion achieving double the amount of cells compared to static. In perfusion configuration, cell numbers remained the same as day 7 throughout the whole experiment. In static configuration, cell density maintained the same high amount until day 21 and marked a small increase by day 29.

ALP activity was subsequently converted to concentration units and expressed as μM and divided with the cell numbers at each time point. Based on the previous mentioned results, normalized ALP throughout the experiment indicated the expression profile presented in Figure 4.9 where in perfusion bioreactor, ALP was maximized by day 7 and then maintained the same amount until day 21 and marked a small drop in the end on day 29 whereas in static culture, ALP reached maximum activity by day 7 which maintained until day 14, followed by a drop on day 21 and marking again an increase in the end by day 29.

In order to make ALP results more clear, it is important to examine the tested cell population. To be more specific, initial cells were pluripotent mESCs characterized by high proliferative capacity and high ALP activity. These cells decrease ALP expression as they proceed towards differentiation. Moreover, the aim of this project was to differentiate mESCS into osteoblast cells, which are characterized by high ALP expression. Based on this information, different cell populations can be distinguished in Figure 4.9. To be more specific, in the bioreactor cells maintained their pluripotent stage for longer and by day 29 decreased ALP activity suggesting the development of a more progenitor phenotype. On the other hand, in static platform cells started decreasing ALP expression earlier, by day 21, indicating the transition to the progenitor stage and subsequently, by day 29 ALP was increased again suggesting the progression to the osteoblast lineage.

The above results showed the existence and maintenance of progenitor osteoblastic cells in perfusion culture while cells in static configuration were in a more advanced stage of differentiation towards maturation.

Mineralisation capacity was the next thing to be analysed. ARS was utilised to indicate calcium deposition (Figure 4.10). For both culture configurations, there was statistically significant increase from day 21 to day 29 as expected. Both tested platforms started with similar calcium deposition on day 21 but perfusion culture indicated significantly increased mineralisation by day 29 compared to static configuration.

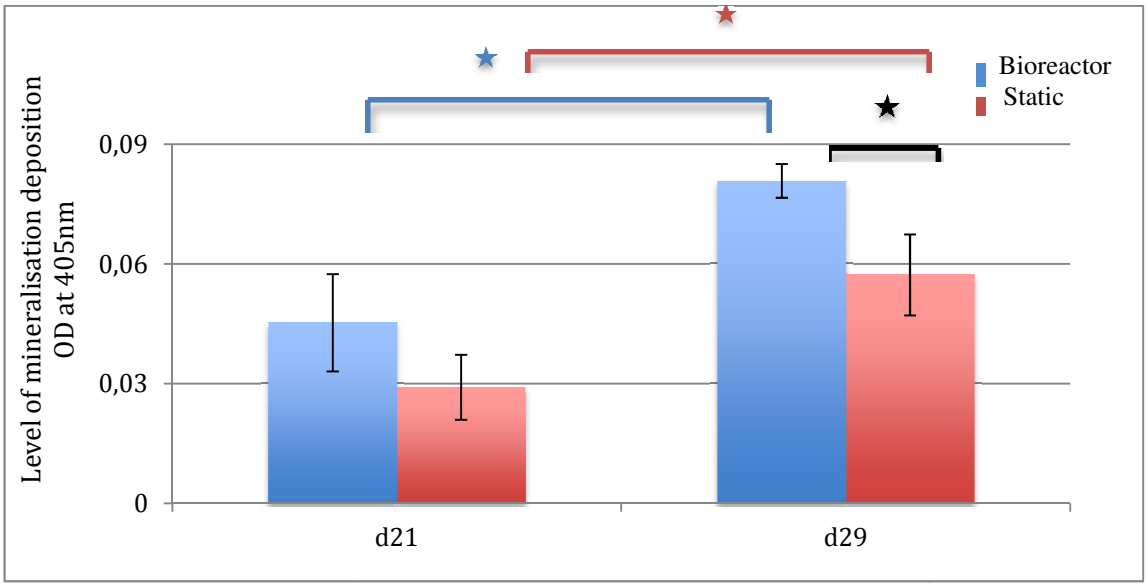
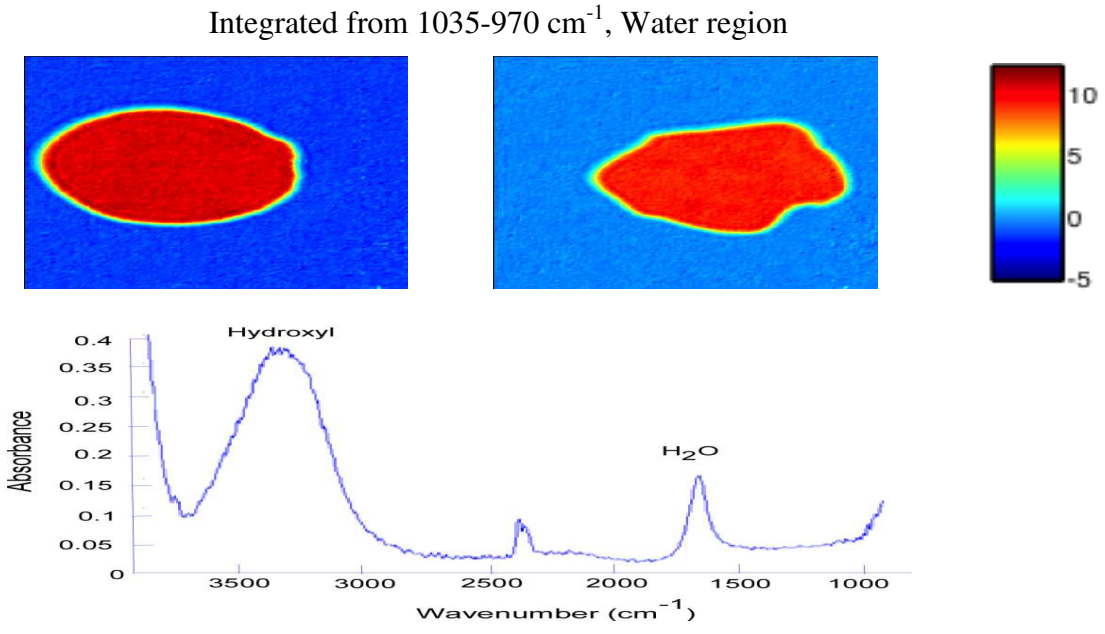


Figure 4.10: ARS was used to evaluate calcium deposition. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested platforms within the same time point. Asterisks ★ ★ indicate $p < 0.05$ and shows that there is statistically significant difference between these time points within the same platform. Values are mean ± SD of N=3.

Having the desire to get some extra information and to confirm mineralisation of the osteogenic constructs, the use of ATR-FTIR Spectroscopic imaging was an ideal solution to analyze spatial HA composition in the mineralised hydrogel beads (Chan and Kazarian, 2003).

With the help of Jennifer Dougan, a post-doctoral fellow in Professor's Sergei Kazarian Vibrational Spectroscopy and Chemical Imaging of Complex Materials and Processes laboratory within the Department of Chemical Engineering, analyses of several samples was conducted and results showed the presence of phosphate in the form of HA, a finding confirming bone mineralisation (Figure 4.11).

Samples from day 29 were examined and results are presented in the following representative ATR-FTIR images.



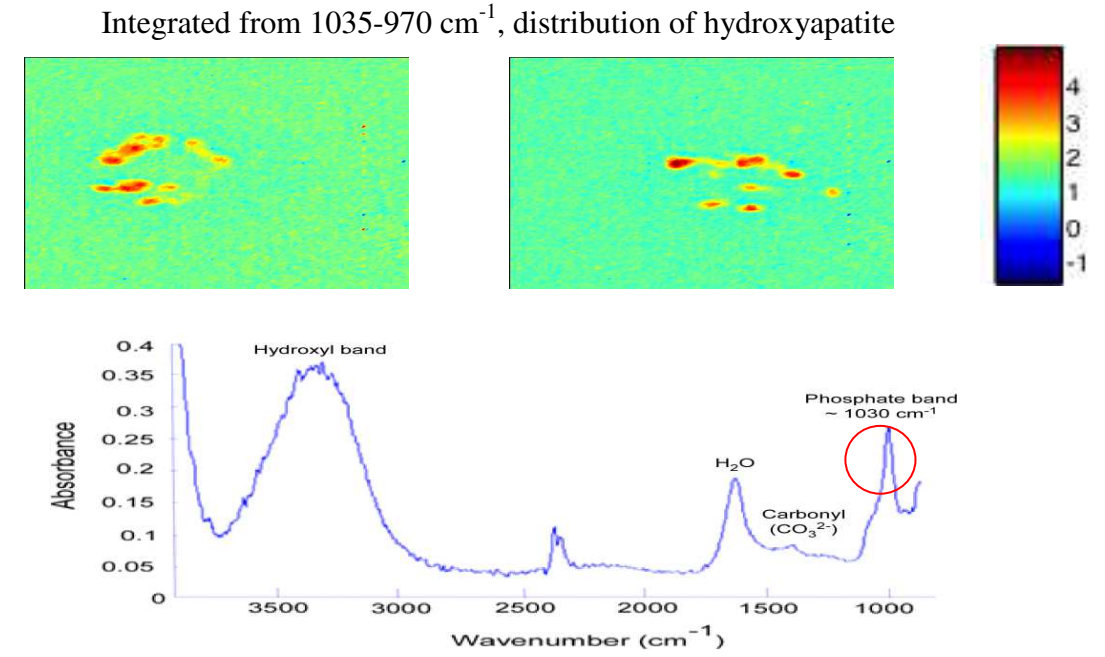


Figure 4.11: Analysis of the mineralisation of the 3D constructs. The FTIR images and ATR-FTIR spectra of mineralised constructs generated by plotting the integrated absorbance of PO4 (between 970 and 1035 cm^{-1}) and amide I (between 1565 and 1500 cm^{-1}) over the imaged area. The registered area within the spectral region was represented with the colour gradients from yellow to red approaching to the peak in the spectra. Images were obtained with the help of the post-doctoral fellow Jennifer Dougan.

HA deposition by day 29 was confirmed by FTIR imaging. Images present the distribution of the integrated absorbance of the HA band at ~ 1030 cm^{-1}, where **red indicates higher abundance**. Quantitative comparisons could not be made at this point. ATR-FTIR analysis showed distinct peaks of phosphate and carbonyl, indicating mineralised calcium/phosphate formation, which is major component of bone mineral (Hunter and Goldberg, 1993).

FTIR spectroscopic imaging has significant advantages compared to many other imaging methods for the characterisation of biomedical materials because it relies on the characteristic absorbance of corresponding molecular vibrations in the sample. Therefore, FTIR imaging does not require the use of added dyes or labelling methods for visualisation of different chemical components in the sample.

The three major IR-spectroscopic sampling modes are transmission, transflection and attenuated total reflection (ATR). Each mode offers convenience for some samples and challenges for others (Baker et al., 2014).

FTIR imaging using ATR mode represents a complementary approach to the use of FTIR imaging in transmission or reflection modes. One of the key advantages of ATR-FTIR imaging is that it requires minimal or no sample preparation prior to spectral measurements. This is due to the fact that the penetration depth of IR light in the sample for ATR measurements is independent of sample thickness. Consequently, this approach is particularly suitable to measure substances with strong infrared absorption such as water. Significantly enhanced spatial resolution was also achieved (Kazarian and Chan, 2006).

Furthermore, in order to observe cell distribution within the constructs, photos of two hydrogels, one from perfusion and one from static culture, both using Dex in their differentiation protocol, were acquired under the light microscope on day 21 (Figure 4.12) and are presented in the following section.

From the photos presented in Figure 4.12, bigger and more homogenously distributed colonies can be distinguished in the perfusion culture compared to static. Moreover, fewer cells were observed in static configuration, probably due to the existence of necrotic parts, which are not easy to see in the above photos.

However, results from live-dead analysis, obtained by Dr Wesley Liam Randle indicated that in 3D static culture configuration, there were large pieces of viable tissue that had numerous necrotic areas by day 29 (Randle, 2006) (Appendix 4).

Moreover, results from different research groups indicated that engineered constructs cultured under static conditions, where culture media is not properly mixed, are frequently

inhomogeneous in structure and composition, **containing a hypoxic necrotic central region** and dense layers of viable cells encapsulating the construct periphery (Wendt et al., 2005). When formed in static cultures, usually in tissue culture flasks, agglomerated large EBs revealed **extensive cell death and eventually large necrotic centers** due to mass transport limitations (Gerecht-Nir et al., 2004).

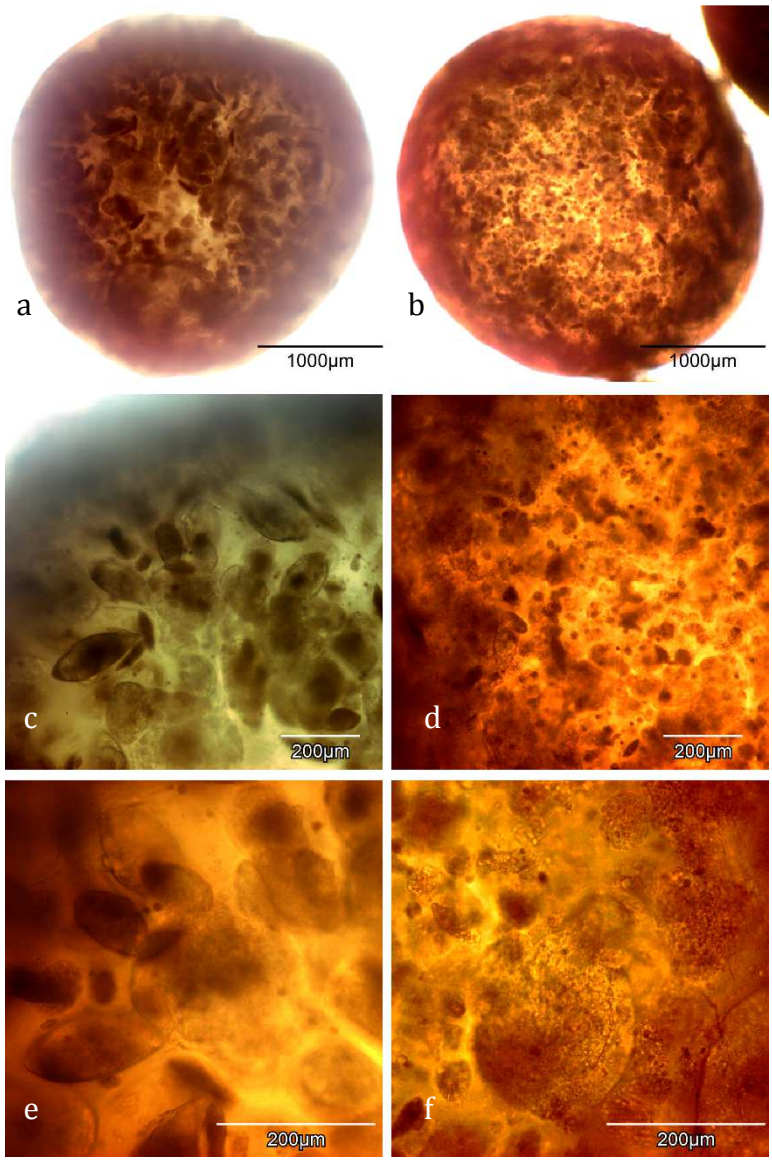


Figure 4.12: Light microscopy images of encapsulated mESCS within one alginate hydrogel from perfusion bioreactor (a, c, e) and one from static configuration (b, d, f) at different magnifications acquired on day 21. Scale bar is 1000µm for the images a and b and 200µm for the images c, d, e and f.

Finally, gene expression analysis was conducted using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The housekeeping gene RPL27 was used for normalization. The gene expression of several bone markers was tested in order to get some quantitative information and obtain a complete image of the osteogenic differentiation process followed by the mESCs when cultured under the influence of Dex.

ALP is one of the most important enzymes to participate in osteogenic differentiation and subsequent mineralisation. For this reason, apart from the biochemical analysis, it was also tested fot its gene expression profile (Figure 4.13). Following and confirming the biochemical results, ALP gene expression was significantly higher in the perfusion culture in comparison to static for both time points tested. In particular, ALP expression in the perfusion cultured increased significantly from day 21 to day 29 while in static culture, expression was very low on day 21 and remained in the same low level without any statistically significant difference until day 29.

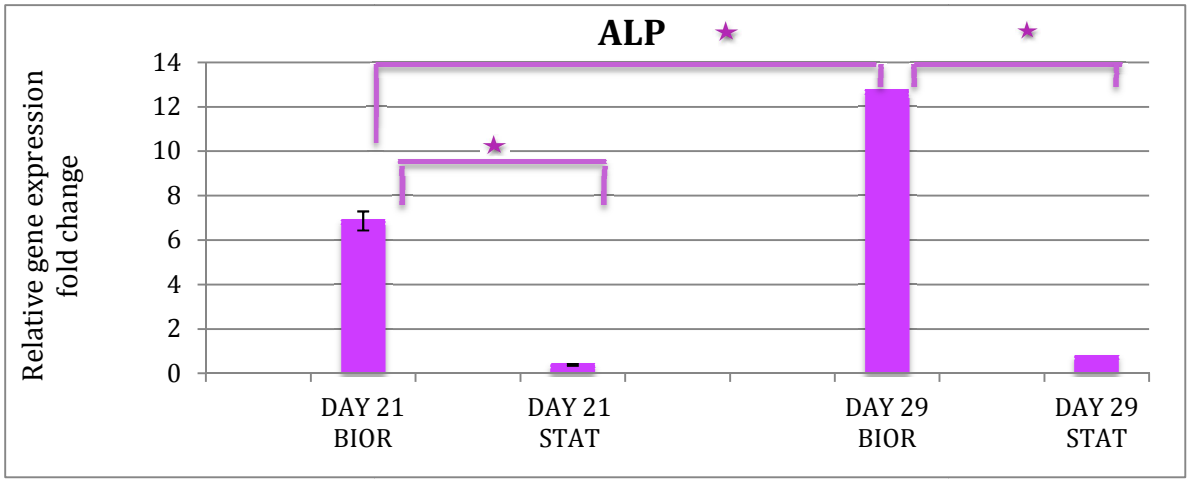


Figure 4.13: Gene expression of ALP. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

Similar findings, indicating that perfusion culture enhanced ALP activity and maintained it in a high level, have been previously shown by other groups as well (Bernhardt et al., 2011; Cartmell et al., 2003). This observation highlighted the important role that the favorable environment of perfusion culture plays in the gene expression.

Gene expression analysis continued with the examination of Runx2 and OSX, two significant transcription factors, which are considered master regulators of osteoblast differentiation and bone formation. Research has shown that OSX acts downstream of Runx2. Moreover, Runx2

augments OSX activity by activating a specific region on its promoter (Nishio et al., 2006). In the performed experiments (Figure 4.14), Runx2 expression was very low on day 21 and then slightly increased by day 29 for both culture configurations. OSX expression was also very low and to similar levels with Runx2 on day 21. On day 29, however, there was a statistically significant increase in OSX expression, 7-fold in the bioreactor and 10-fold higher in the static culture.

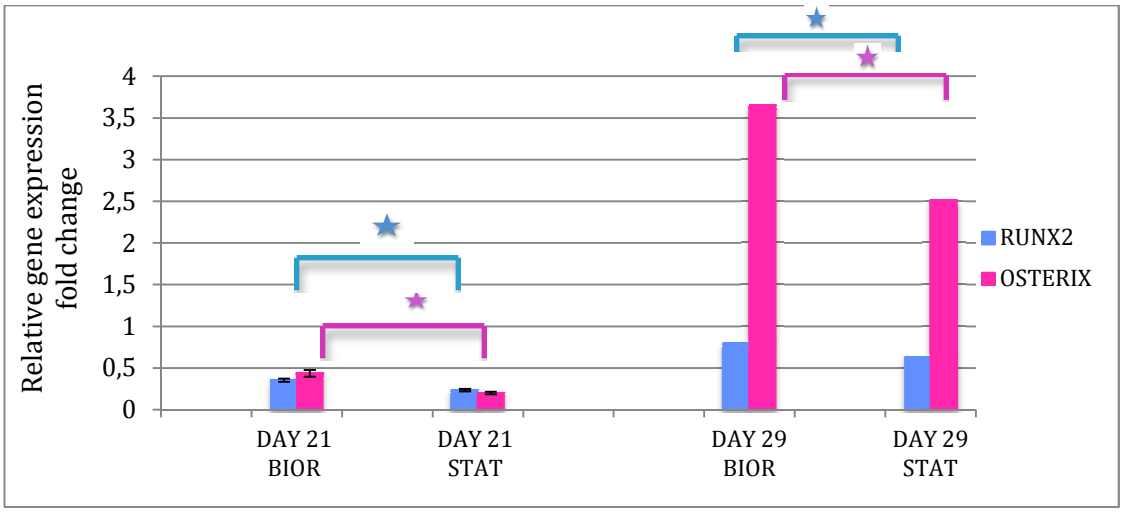


Figure 4.14: Gene expression analysis of the transcription factors Runx2 and OSX. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested samples in Runx2 expression while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested samples in OSX expression. Values are mean ± SD of N=3.

The above findings indicated that osteogenic differentiation was strongly enhanced by day 29 on both culture platforms and also the environment created by the perfusion bioreactor could support better quality osteogenic differentiation as proved by the enhanced gene expression of bone markers.

Another two important markers of osteogenic differentiation were tested, COL 1 and BMP2 (Figure 4.15). COL 1 is considered an early stage marker of bone formation. It is the most important and most abundant bone matrix protein (Wendel et al., 1998; Young, 2003a; Young, 2003b). From the obtained results, COL 1 was expressed in a higher level in static culture compared to perfusion for both time points tested. In particular, COL 1 expression was high on day 21 and increased significantly by day 29 in static configuration. In bioreactor culture, the

expression of collagen was lower than the static on day 21 and maintained the same gene expression level with no statistically significant difference until day 29 of the experiment.

BMP2 is the strongest osteoinductive factor and a characteristic marker of osteogenic differentiation. BMP2 is expressed relatively early during bone formation. Its expression was tested and revealed that on day 21, it was highly expressed in both configurations, with static demonstrating statistically higher fold increase compared to perfusion. On day 29, perfusion culture indicated a very small reduction in BMP2 expression and supported almost the same high level as day 21 while in static culture, BMP2 expression demonstrated a six-fold decrease. This finding supported the idea that the favorable culture conditions of the perfusion bioreactor maintained a high level of BMP2 expression for longer and confirmed results from other groups, which noticed that perfusion culture enhanced BMP2 expression and also prolonged the time of expression (Hosseinkhani et al., 2006a; Hosseinkhani et al., 2005a; Hosseinkhani et al., 2005b; Hosseinkhani et al., 2006b).

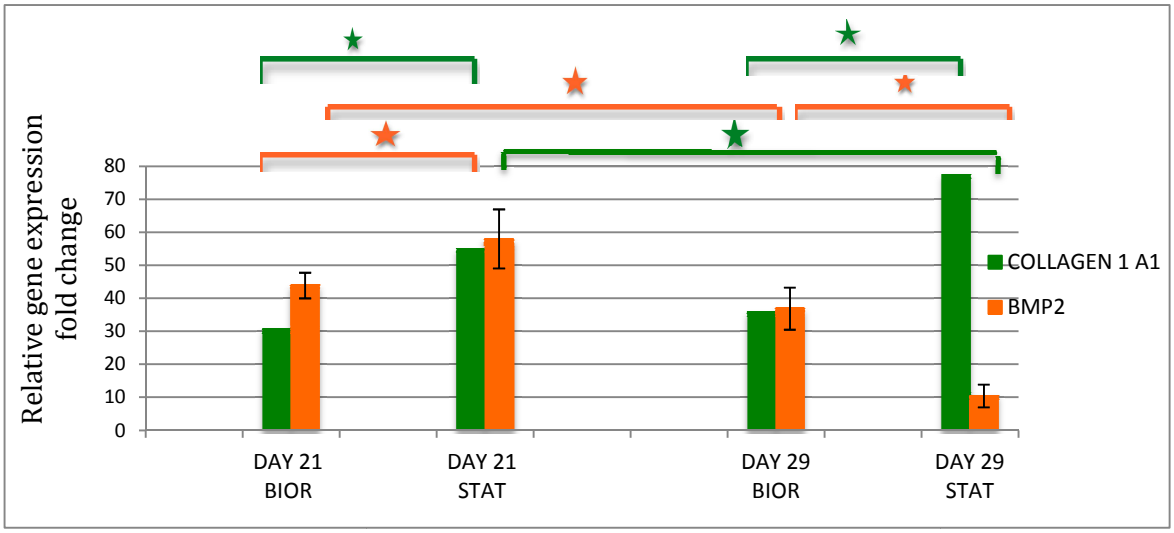


Figure 4.15: Gene expression of COL 1 and BMP2. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples in COL 1 expression while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in BMP2 expression between the tested samples. Values are mean ± SD of N=3.

Bglap is the gene encoding for the protein OCN, one of the few osteoblast specific proteins of bone matrix. OCN is expressed in mature osteoblasts and it is considered a late stage marker of osteogenic differentiation (Sila-Asna et al., 2007). BSP is another bone matrix protein characterised as late stage marker of bone formation (Gordon et al., 2007).

In the results acquired from the experiments (Figure 4.16), it was observed that Bglap was highly expressed on day 21 in the perfusion culture and then its expression slightly decreased by day 29. The same profile was followed by static culture with Bglap expression being high on day 21 and then subsequently decreasing by day 29. For both time points tested, the relative Bglap expression was significantly higher in perfusion culture in comparison to static. In particular, on day 21 in static configuration the expression was three times lower and on day 29, about ten times lower compared to the bioreactor. BSP expression, similar to the previous results, was significantly higher in perfusion configuration for both time points tested compared to static culture. To be more specific, there was a two-fold increase in BSP expression in the bioreactor culture from day 21 to day 29. In static culture, there was very low expression on day 21, which maintained at the same level until the end of the experiment, on day 29.

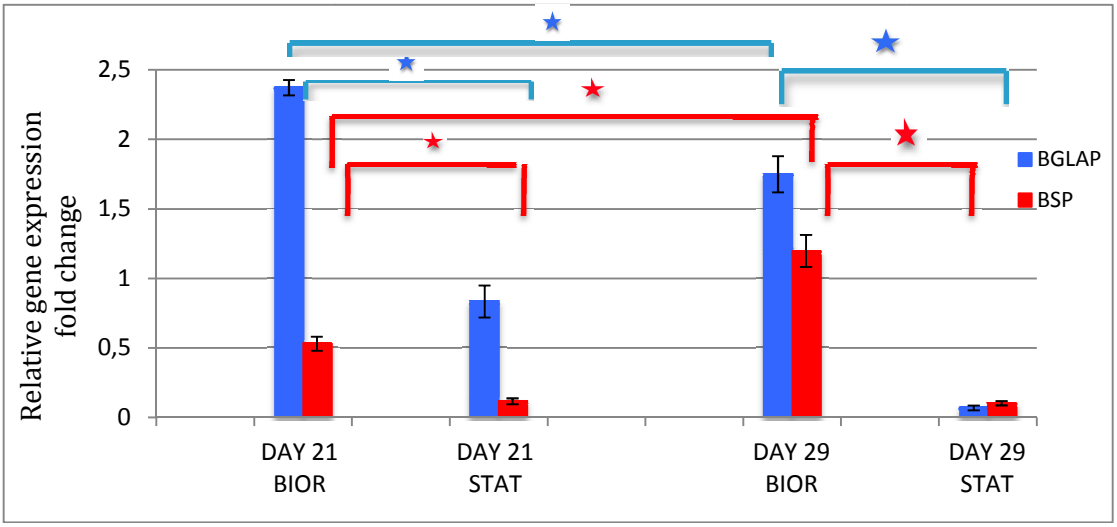


Figure 4.16: Gene expression of Bglap and Bsp. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in Bglap expression between all the tested samples. Values are mean ± SD of N=3.

These results indicated that perfusion culture better supported the expression of bone matrix proteins and progression to osteogenic phenotype in comparison to static configuration as indicated by the enhanced gene expression of these late stage markers.

Gene expression analysis continued with the examination of a couple of significant bone matrix proteins, in particular OPN and ON.

OPN is a major non-collagenous bone ECM protein. It has significant role in bone remodelling (Denhardt and Noda, 1998; Standal et al., 2004). OPN has been shown to exhibit two distinguish peaks during bone development, one early in the proliferation period and a second one late during mineralisation (Aubin, 2001).

Obtained results (Figure 4.17) showed the same low expression level of OPN on day 21 for both culture configurations. By the end of the experiment, on day 29, OPN expression increased significantly demonstrating a 4.5 fold increase in the bioreactor culture and an almost three-fold increase in static configuration in comparison to day 21.

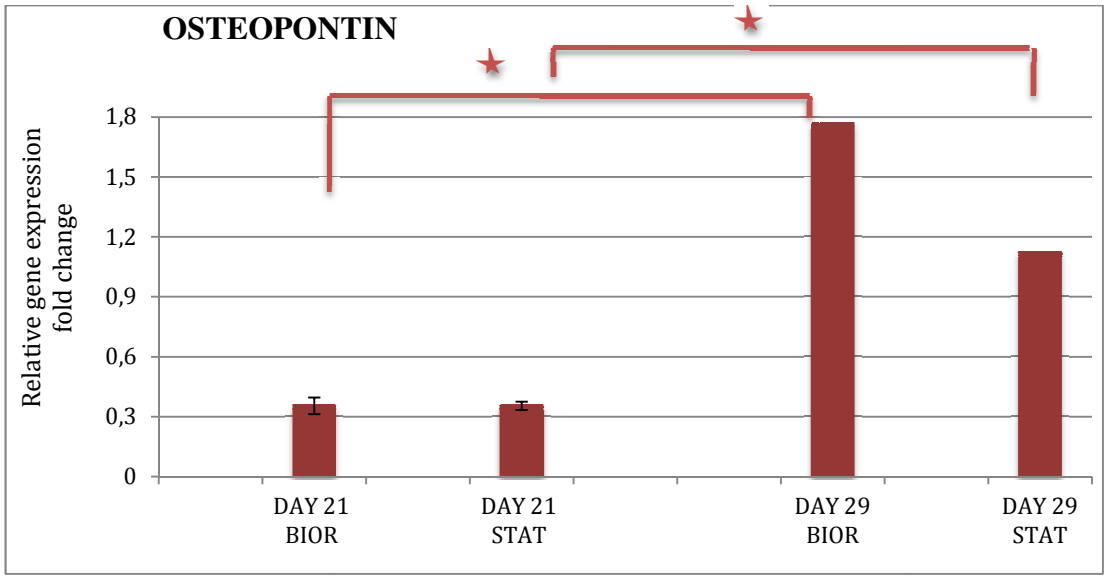


Figure 4.17: Gene expression of OPN. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

As mentioned previously, OPN is highly expressed during the late stage of osteogenic differentiation, the mineralisation period (Aubin, 2001). This observation can be further supported by the previous results, which indicated a high OPN expression on day 29, by the end of the culture, in both tested platforms and with higher expression marked in the bioreactor.

ON also known as SPARC (secreted protein acidic and rich in cysteine) is a major non-collagenous bone matrix protein (Termine et al., 1981). It has been shown to modulate cell-ECM interactions (Barker et al., 2005) and also to interact with COL 1 (Bornstein and Sage, 2002). It

is critical for normal bone remodeling. Sparc is highly expressed early during differentiation but decreases as cells acquire more osteoblastic characteristics (Delany and Hankenson, 2009).

Based on the calculated results (Figure 4.18), ON expression indicated a very high level of expression on day 21 in both platforms, with higher fold increase in the perfusion. By the end of the experiment, on day 29, SPARC expression decreased significantly in both culture configurations and acquired the same low levels for both.

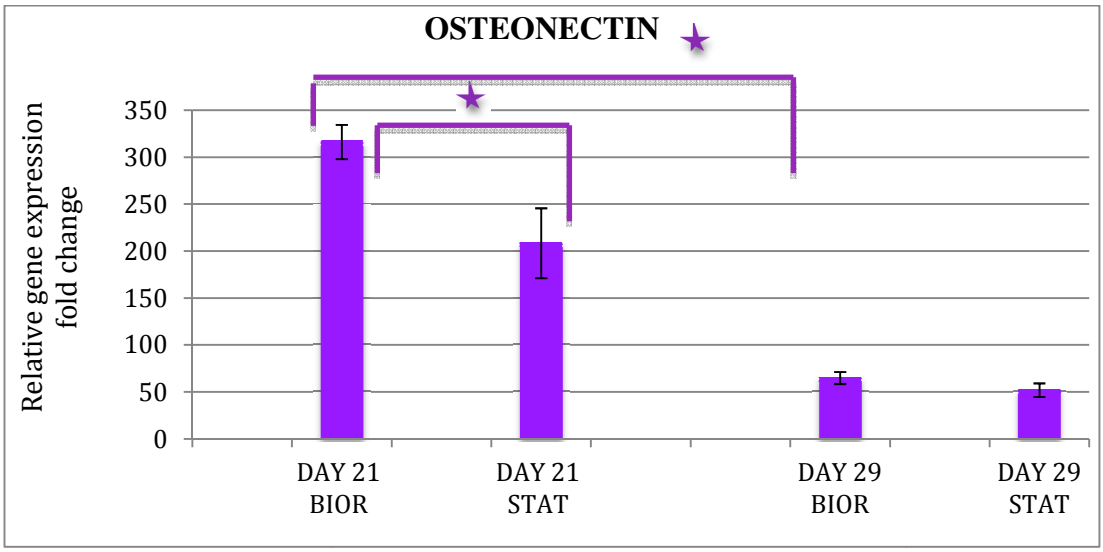


Figure 4.18: Gene expression of ON. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

The above findings confirmed observations from other groups which noticed that ON is highly expressed early in the differentiation process and is decreasing when cells acquire a more mature phenotype (Delany and Hankenson, 2009). Both platforms indicated high level of ON expression on day 21, with perfusion showing higher level compared to static and the expression reduced significantly for both by day 29. To summarize, perfusion bioreactor indicated higher level of expression compared to static for the tested matrix proteins, OPN and ON and at different time points as expected. This finding suggested the generation of better quality osteogenic cells from the perfusion culture.

4.5 Discussion and conclusions

The primary aim of this chapter was to test traditional and widely employed, by many research groups, protocols for osteogenic differentiation of mESCs (Buttery et al., 2001; Sottile et al., 2003; zur Nieden et al., 2003). The majority of these protocols, after initial expansion, goes through the step of EB formation and ends up with bone nodule formation and mineralization.

EBs are aggregates grown in suspension and contain cells from all the three germ layers (Figure 4.2). They recapitulate early aspects of embryonic development. EB formation is considered an essential part for the proper osteogenic differentiation and it constitutes one of the major steps in various protocols. However, it has been recognised that it poses a high risk of spontaneous differentiation and makes difficult to direct cell differentiation towards the desired cell type (Kurosawa, 2007).

For this reason, the recent years, many research groups looked for alternative ways to avoid the EB formation step in order to reduce the risk of spontaneous differentiation into cell types beside the desired one. In particular, it has been shown from previous people in the group that the use of HepG2-CM could bypass the EB formation and could enhance osteogenic differentiation (Hwang et al., 2006). HepG2-CM has been also indicated to enhance both cell proliferation and cell growth (Hwang et al., 2006; Kim et al., 2012).

A commonly used protocol for osteodifferentiation is the DAG, consisting of Dex, AA and b-GP. The steroid Dex is a widely used chemical to promote osteogenic differentiation and mineralisation. It has been shown to be more efficient when supplied at a late time during the differentiation (Buttery et al., 2001).

Based on the above information, a novel protocol was generated by previous colleagues in the laboratory and was employed for the osteogenic differentiation of mESCs during the performed experiments (Figure 4.5). 3D configuration was tested and comparison was performed between static and dynamic culture. In particular, cells were encapsulated in alginate hydrogels and cultured either in tissue culture flasks or in a custom made RWV perfusion bioreactor developed in the Biological Systems Engineering Laboratory (BSEL) at Imperial College London.

DNA quantification, confirming previous observations by other research groups (Akmal et al., 2006; Crabbe et al., 2015; Song et al., 2008), supported enhanced proliferation rate of the cells cultured within the RWV bioreactor compared to the ones in static, resulting in higher cell densities achieved by the end of the experiment (Figure 4.7). This is due to the environment provided by the perfusion bioreactor, by continuous supplying fresh media and removing metabolites. It is important to take into account the fact that the experimental procedure started

with a pluripotent population of stem cells characterized by high proliferation capacity while later during the culture, cells began acquiring the osteoblast phenotype which has limited proliferation ability if it includes progenitor cells or even no proliferation capacity referring to the mature phenotype (Bernhardt et al., 2011).

ALP activity was evaluated to estimate osteogenic differentiation and results indicated that continuous perfusion maintained high and similar ALP activity throughout the whole experimental procedure (Figure 4.8), thus confirming previous findings from other research groups which noticed that perfusion culture enhanced ALP activity in comparison to static control (Bernhardt et al., 2011; Wang et al., 2003), a fact that confirms the validity and accuracy of the obtained results.

Following that, ARS analysis indicated significantly higher calcium deposition in the bioreactor platform compared to static configuration, something that has been previously supported by other research groups as well (Yu et al., 2004; Zhang et al., 2009) (Figure 4.10). Further confirmation of the apatitic nature of the observed mineral was provided through ATR-FTIR analysis, which indicated the presence of HA in the hydrogels (Figure 4.11).

Finally, gene expression analysis during the early and late stage of mESCs osteogenic differentiation provided significant information regarding the gene profile during the followed procedure. It has been indicated that removal of LIF from the culture media allowed the generation of precursors representative of all three germ layers (Desbaillets et al., 2000; Mansergh et al., 2009). Cells gradually became less naïve and more primed by reducing the expression of pluripotent markers and at the same time increasing the expression of markers of the first germ layers that would be formed (Karwacki-Neisius et al., 2013).

Following the above information, similar findings were observed in the performed experiments (Figure 4.6).

Pluripotent markers Nanog and Rex1 were highly expressed during the first days of the culture and they gradually downregulated their expression. At the same time, epiblast marker Fgf5 and germ layer markers Gata4 and Brachyury T started to be expressed followed by markers of early bone formation, such as COL 1.

The expression of characteristic bone markers was also evaluated during the last week of culture and comparison was performed between 3D static and dynamic culture configuration.

Following biochemical analysis, the gene expression of ALP was initially tested (Figure 4.13) and results indicated significantly higher expression level in the bioreactor compared to the low level in static configuration at both tested time points. These findings followed the trend of the biochemical results, but it is important to consider that changes in gene expression would need

more time to occur (Chechik and Koller, 2009) and they are also influenced by the potential existence of metabolic stress (Yeo et al., 2013). Similar results supporting the idea that perfusion culture enhanced ALP activity in comparison to static were observed previously by other research groups (Bancroft et al., 2002; Gomes et al., 2003; Sikavitsas et al., 2003).

Moreover, it is important to consider that different cell types appear during the experiment. Pluripotent mESCs, which are characterized by high ALP, exist in the beginning of the culture. There is a gradual transition to the progenitor phenotype and subsequent differentiation towards osteoblastic cells, which are also characterized by high ALP. Discrepancies between biochemical and genetic results of ALP have been observed by other research groups as well. To be more specific, Liao and his colleagues tested ALP activity on day 10 and day 20 and while there was an upregulation in the gene expression, the biochemical results marked a decrease during the same time (Liao et al., 2013). Similar findings supported by Datta and his collegues who examined ALP on day 3 and day 7 between two different scaffolds and they observed an increase in the biochemical activity and a reduction in the gene expression during the same time points. They suggested that genetic expression occurred earlier in one of the scaffolds compared to the other (Datta et al., 2013). Finally, Hoemann and his colleagues noted that levels of ALP activity are not proportional to the mineralisation process (Hoemann et al., 2009).

Gene expression of the transcription factors Runx2 and OSX was then examined (Figure 4.14). They are considered the most important factors which drive osteogenic differentiation and research suggested that OSX acts downstream of Runx2. Results acquired from the experiments indicated expression of these genes on day 29 for both configurations, with higher level in the bioreactor in comparison to static culture. Similar observations, indicating increased expression of these early stage markers in dynamic culture have been noticed previously by other groups (Cartmell et al., 2003; Mygind et al., 2007).

The expression of COL 1 and BMP2 early stage bone markers was then tested (Figure 4.15). COL 1 is the most abundant bone matrix protein while BMP2 is considered the strongest osteoinductive factor. The expression of COL 1 was significantly higher in static culture compared to the bioreactor. Similar observation was made by another research group which noticed significant decrease of COL 1 expression in the groups cultured in the bioreactor in comparison to the static control group (Plunkett et al., 2010). BMP2 expression was upregulated in static culture at an earlier time point, something that has been observed previously by another research group (Bjerre et al., 2008; Stiehler et al., 2009).

Gene expression analysis continued with the late stage markers of mature osteoblasts, OCN and BSP (Figure 4.16) which both indicated significantly higher expression in the bioreactor

compared to static culture. These results suggested a better support of osteogenic differentiation and an enhanced expression of the late stage markers by the perfusion bioreactor culture in comparison to the static, due to the significantly lower expression levels achieved. Similar findings were noticed by other research groups, which supported the increased expression of OCN and BSP in dynamic culture configuration (Mygind et al., 2007; Rath et al., 2012).

Finally, the gene expression of two major non-collagenous proteins of bone matrix, OPN (Figure 4.17) and ON (Figure 4.18) was tested. OPN has been shown to be expressed during the stage of active proliferation, decreases immediately after the post-proliferative stage and increases again at the onset of mineralisation, achieving the greatest level of expression during mineralisation (Lian and Stein, 1995). ON indicated early expression (Stiehler et al., 2009) and decreased when cells acquired more mature phenotype. Acquired results supported the late expression profile of OPN and the early expression profile of ON.

To summarize, gene expression outcome indicated a higher expression level of bone proteins in perfusion configuration in comparison to static, suggesting the better support of osteogenic differentiation in the favorable and well mixing environment created by the RWV perfusion bioreactor, with continuous supply of fresh media and removal of metabolites, creating metabolically less stressful conditions for cell culture (Yeo et al., 2013). Moreover, dynamic culture not only increased the expression of osteogenic markers but also caused a shift and genes in the dynamic environment of the bioreactor were peaked earlier than in static cultivation (Mygind et al., 2007). The bioreactor also supported high cell numbers and enhanced bone formation and mineralisation.

To summarize, the important conclusions that can be drawn from this chapter are the following ones:

- 3D bioprocess of mESCs using Hepg2-CM allowed more uniform cell differentiation towards mesoderm and production of a more homogeneous cell population.
- Perfusion platform supported and allowed the generation of high cell numbers.
- Gene expression analysis suggested that Dex significantly enhanced the expression of important bone markers in 3D dynamic culture environment.
- The well mixing environment offered by the bioreactor enhanced bone formation and mineralisation.

5th CHAPTER

5. EVALUATION OF THE USE OF SIM AS AN OSTEOINDUCTIVE FACTOR: INITIAL PROBLEMS, OPTIMIZATION OF TIME OF SUPPLEMENTATION AND COMBINATION WITH ADHESION MOLECULE

5.1 Introduction

Considerable progress has been made in directing the osteogenic differentiation of ESCs. However, the exact mechanism of action is not fully elucidated and makes urgent the need to develop well-defined protocols that would improve the efficiency of osteogenic differentiation and reduce incidents of spontaneous differentiation. Moreover, gene and protein expression profiles differ between cell lines making it difficult to create a protocol that could be potentially applied to all different cell types (Hwang et al., 2007).

Widely used osteoinductive factors are BMPs, in particular BMP2 and BMP7. These two proteins are also FDA approved for clinical treatment of open tibial shaft fractures and long bone nonunions respectively. The problem with these proteins is that in order to be effective *in vitro*, they are supplied in very high super-physiological concentrations. They have also suspicion of carcinogenicity and they need appropriate carriers. Moreover, one significant issue with these proteins is their high cost due to the recombinant technology applied for their production. For these reasons, an alternative osteoinductive factor is needed that would replace the use of BMPs.

Dex is a steroid commonly used for osteogenic differentiation and it has been shown to enhance osteogenic differentiation and mineralisation (Mikami et al., 2007; Song et al., 2009). It is not as efficient as BMPs though and as a steroid, it can have some uncontrolled side effects.

Based on these observations, Mundy and his group in 1999 had the idea to try to find a small molecule that could activate the promoter of BMP2 gene and enhance osteogenic differentiation. They examined a collection of natural products and identified the statin lovastatin as the candidate to have this function. Statins are widely used drugs to lower cholesterol and prevent cardiovascular diseases. They inhibit the synthesis of the enzyme HMG Co-A reductase. There are lipophilic and hydrophilic statins. Lipophilic statin Sim was used in these experiments because it could cross the cell membrane with passive diffusion.

Statins have another interesting characteristic that should be taken into account. They have been shown to inhibit cell cycle progression by blocking events critical for G1/S transition (Sala et al., 2008). In the literature, there are examples of this mechanism on mouse fibroblasts (Vogt et al.,

1996) and on glioma cells (Crick et al., 1998). Another possible mechanism is also the induction of apoptosis, for example in vascular smooth muscle cells (Blanco-Colio et al., 2002; Buemi et al., 1999). In a more recent study, statins inhibited the proliferation and led to loss of self-renewal capacity of mESCs (Lee et al., 2007b). For this reason, statins need to be supplemented in the culture media at specific time point during cell culture, after cells have completed proliferation and reached the desired number.

Statins have been also used as anticancer agents based on preclinical evidence of their anti-proliferative, pro-apoptotic, anti-invasive and radio-sensitizing properties (Chan et al., 2003). They have been shown to induce anti-proliferative effects in various cancer cells (Ishikawa et al., 2014) or to inhibit tumor cell proliferation and to induce apoptosis (Graaf et al., 2004). These antitumor effects result in retardation of tumor growth and/or inhibition of the metastatic process (Jakobisiak and Golab, 2003). To summarize, antitumor effects of statins include growth inhibition caused by cell cycle arrest and the induction of apoptosis, the reduction of metastatic potential, the inhibition of angiogenesis and the differentiation of tumors (Osmak, 2012).

Finally, cell adhesion molecules are well known factors used for coating plates in order to improve cell adherence and help to study *in vitro* cell-ECM interactions (Kuschel et al., 2006). Controlled cell adhesion to the ECM is essential for coordinated morphogenesis and growth of functional tissue during embryonic development, tissue differentiation and regeneration (Albelda and Buck, 1990). ECM receptors of the integrin family have been implicated as important regulators of the proper development of various tissues. Integrins have as main function to properly regulate the balance between proliferation and differentiation (Watt, 2002). Integrins have been implicated in bone development, regulating bone remodeling and influencing the expression of differentiation-related genes (Danen and Sonnenberg, 2003).

One of these molecules belonging to the integrin family is fibronectin. It is widely used for coating surfaces for various adherent cells. Fibronectin contains the RGD peptide, which is the main area that cells bind to. Fibronectin has been shown recently from previous people in the group to also enhance osteogenic differentiation of mESCs (Kang et al., 2015). To take it one step further, fibronectin coating in 2D culture plates was tested together with Sim media to evaluate if this protocol could ameliorate osteogenic differentiation.

5.2 Aim and Objectives

The aim of this chapter was to try the efficacy of Sim as an osteoinductive factor in performing osteogenic differentiation of mESCs.

In particular:

1. To establish a new protocol for osteogenic differentiation using the small molecule Sim.
2. To combine the Sim protocol with fibronectin adhesion molecule and evaluate if there is improvement in osteogenic differentiation.

5.3 Methodology

Following previously published data from the group (Pagkalos et al., 2010), Sim was tested in 2D and 3D static culture in six well plates. Different analyses to evaluate osteogenesis were performed including ALP for osteodifferentiation and ARS for mineralisation. The next step was to find the optimal time to supplement Sim in the culture media in order to get higher osteogenic differentiation and mineralisation without significant reduction in cells numbers. Two different time points were tested and evaluation was performed based on ALP and ARS analysis both in 2D and 3D static configuration. The time points tested were day 21 and day 14. The choice of day 21 was based on the currently used traditional protocol where Dex was supplied in the media on day 21 because it has been shown to have better outcome when supplemented in the culture media later in the differentiation process. Day 14 was chosen based on the general osteogenic process followed by mESCs, the different stages and time needed for each of them.

The idea was based on the three stages followed by the cells in order to differentiate. Cell expansion was initially performed, followed by matrix formation to support subsequent osteogenic differentiation and mineralisation. Based on previous results from DNA quantification, mESCs proliferated and reached maximum numbers after one week of culture and then started synthesising bone matrix to support subsequent bone formation and mineralisation. Thinking that cells were quite stable after having started synthesising bone matrix, this was the reason for the choice of day 14.

Finally, following recently published data from the group (Kang et al., 2015) and with the intention to improve more this osteogenic differentiation protocol, coating with the adhesion molecule fibronectin was tested in 2D culture and compared with the currently used molecule for coating, gelatin. Media containing Sim was also employed in the process. The choice of fibronectin was based on the analysis of the conditioned media derived from HepG2 cells, which indicated the significant role this molecule played in mesoderm induction (Kang et al., 2009b).

5.4 Results

5.4.1 CHEMICAL OSTEOINDUCTION USING SIM IN 2D AND 3D STATIC CONFIGURATION: EMERGING PROBLEMS

In this chapter, the aim was to try a new promising osteoinductive factor, Sim and evaluate the possibility to replace Dex. Following published data (Pagkalos et al., 2010) as a first step, 2D culture of mESCs took place in six well plates where the 29 days osteodifferentiation protocol was followed. The first three days, cells incubated with HepG2-CM and then media was replaced with osteogenic media supplemented with b-GP and Sim. At the same time, standard differentiation protocol with Dex was used as a control (Figure 5.1).

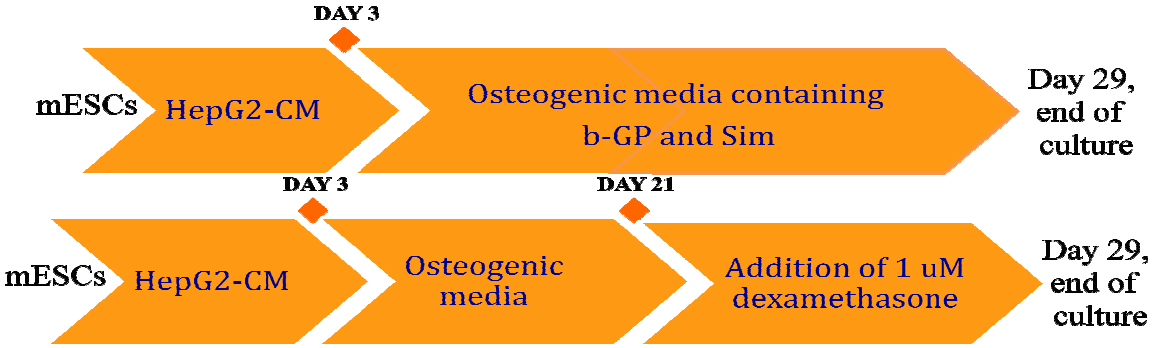


Figure 5.1: The two protocols followed in these experiments: the novel with Sim and the control with Dex.

The first analysis that was performed was ARS in order to evaluate mineralisation (Figure 5.2). It was easy to observe with naked eye but it was confirmed after quantification as well that calcium deposition was much lower in the protocol using Sim compared to the control. This interesting finding was noticed from a photo taken from the plate after ARS staining and before

quantification where it seemed there were bone nodules but cell numbers were very low and there was no matrix formation.

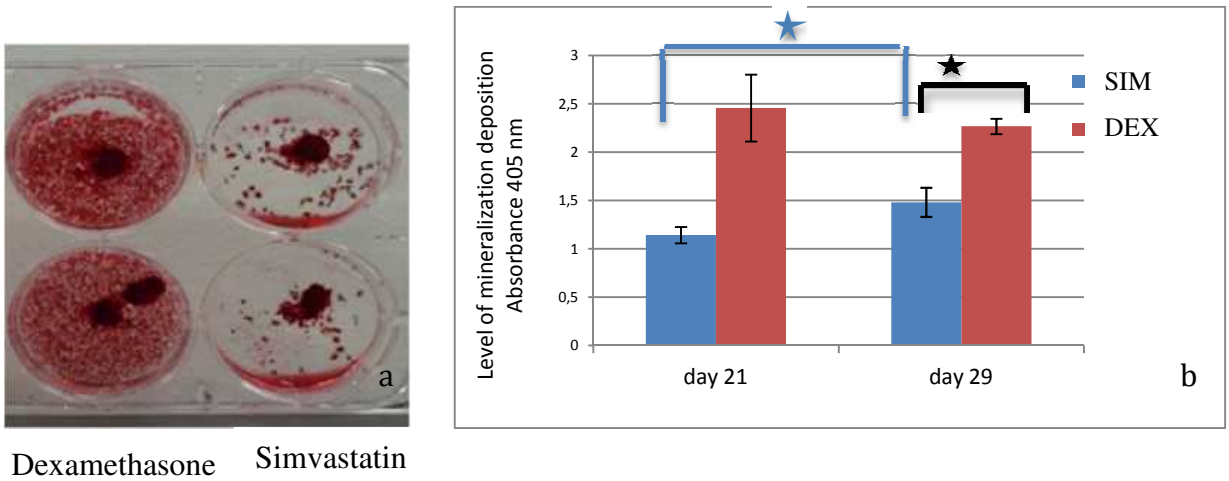


Figure 5.2: ARS in 2D culture configuration (a). Obtained results after quantification of ARS (b). Comparison was performed between Sim and Dex protocol, on day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two chemicals within the same time point while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested time points within Sim group.

Using the same protocol, 3D static culture was also performed. The results of ARS were similar to the 2D culture after quantification; calcium deposition in the Sim protocol was lower in comparison to the Dex protocol (Figure 5.3).

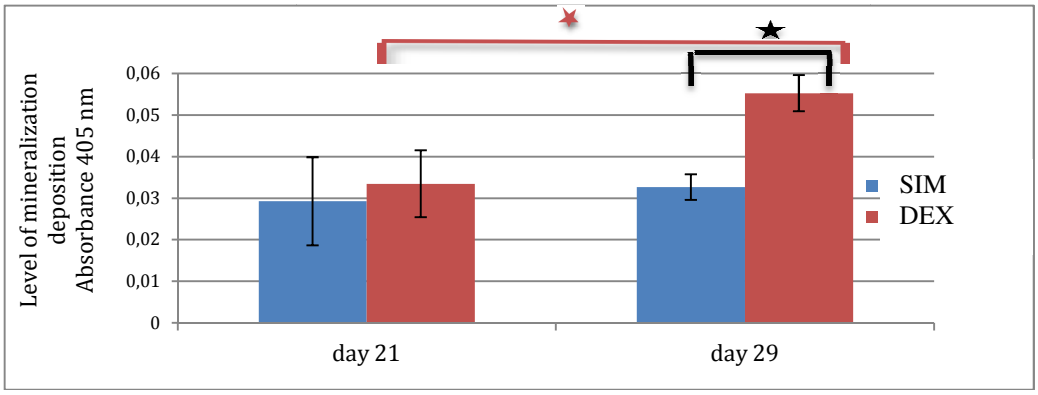


Figure 5.3: Quantification of ARS in 3D static configuration. Comparison was performed between Sim and Dex protocol, on day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two chemicals within the same time point while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested time points within Dex group.

Next analysis was to test for ALP activity, the most important marker of bone formation (Figure 5.4). Biochemical analysis revealed that ALP in the Sim-containing wells increased the first week of culture but after that exhibited a significant reduction until the end of the experiment on

day 29. On the other hand, in the Dex-containing wells, ALP activity increased after one week and maintained the same high level throughout the whole experimental period.

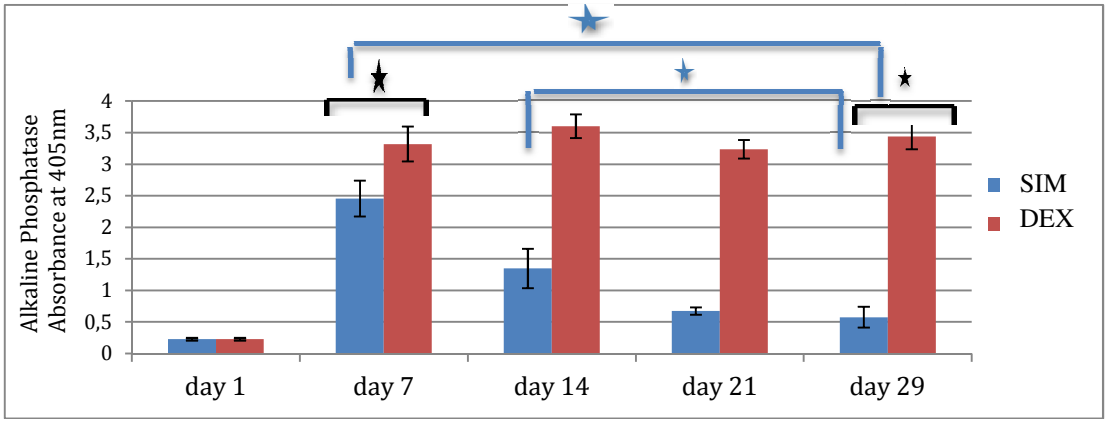


Figure 5.4: ALP activity in 2D culture configuration throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two chemicals within the same time point while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested time points within Sim group.

ALP activity was also tested in 3D static culture for both protocols (Figure 5.5). The results in 3D configuration were following in a better way the change of ALP profile during the different stages of differentiation. ALP increased the first week of culture and then decreased by the end towards mineralisation. On the beginning, on day 7, both protocols achieved similar high ALP levels and also in the end, by day 29, both protocols exhibited the same low ALP level. The main difference was observed during the culture, on day 14 and day 21 where beads cultured with Dex indicated significantly higher ALP levels in comparison to those cultured with Sim.

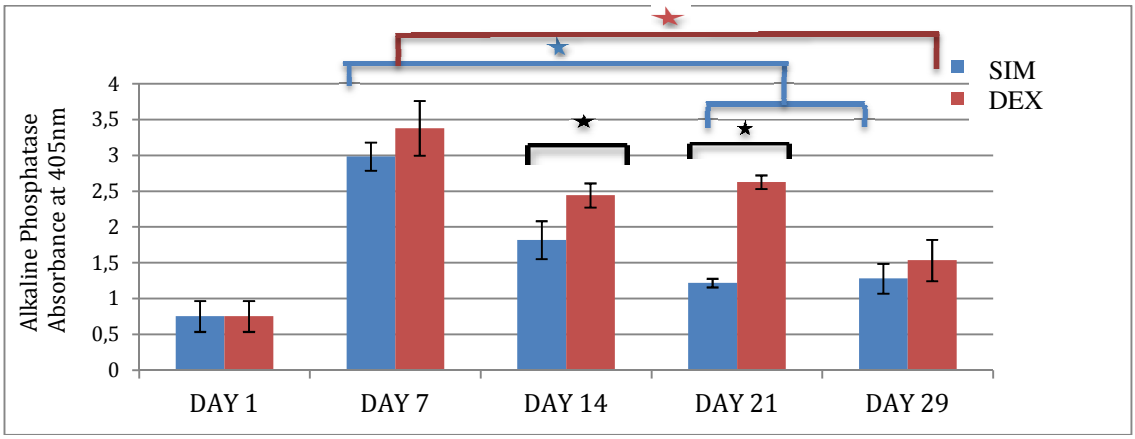


Figure 5.5: ALP activity in 3D static configuration throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol. Two-way ANOVA was used for statistical analysis. Asterisk indicates ★ $p<0.05$ and shows that there is statistically significant difference between the tested chemicals within the same time point and asterisks ★★ indicate $p<0.05$ and show that there is statistically significant difference between the tested time points within the same platform.

Dex was provided in the osteogenic media on day 21 and for the last week of culture. Such protocol allowed time for the cells to proliferate and gradually change their phenotype to the osteoblastic one, which was induced even more by day 21 when Dex was supplemented in the culture media. This means that on the obtained results, on day 14 and day 21 there was no Dex in the osteogenic media and as mentioned previously, ALP activity was due to the extracellular phosphate provided in the culture media through b-GP. On the other hand, Sim was provided in the culture media from day 4 of culture, following a previously published protocol (Pagkalos et al., 2010) and induced earlier osteogenic cell differentiation without allowing cells to reach high cell numbers and acquire a more stable phenotype after matrix synthesis. Sim has been also indicated to act as cell-cycle inhibitor and it is also employed for its anticancer properties (Ahmed et al., 2013; Crosbie et al., 2013; Goard et al., 2010; Pisanti et al., 2014; Relja et al., 2010). The obtained results indicated possible cell death in part of the cell population.

The previous finding suggested that 3D culture configuration better supported the osteogenic differentiation of the cells and helped them not to experience the negative effects of Sim in a severe and probably lethal way.

Finally, to evaluate the effects of Sim on cell proliferation, MTS was applied on 2D culture (Figure 5.6) and DNA quantification on 3D static (Figure 5.7).

Cells cultured with Sim exhibited lower proliferation rate compared to cells cultured with Dex throughout the whole duration of the experiment as shown from the lower values obtained from optical density measurements.

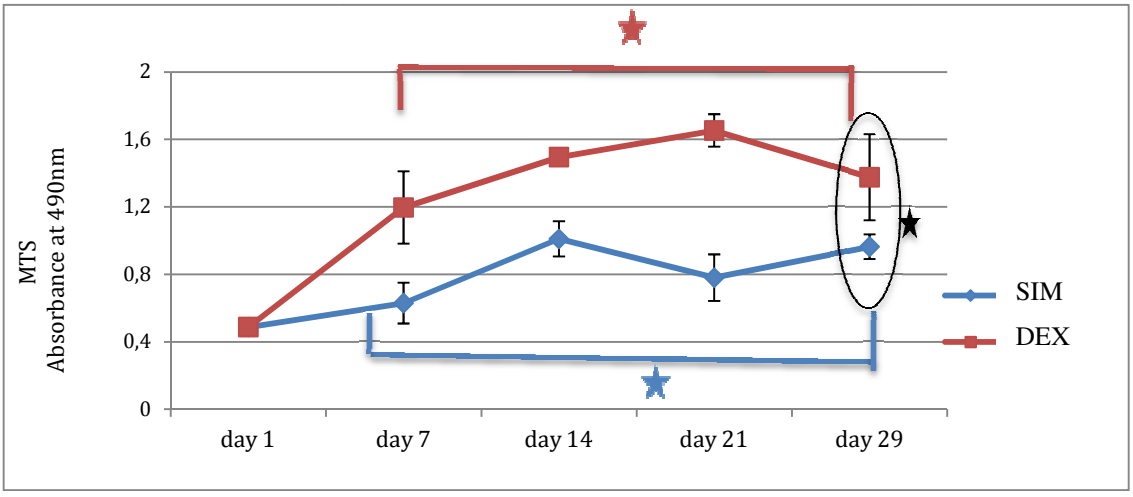


Figure 5.6: MTS in 2D culture configuration throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two chemicals within the same time point while asterisks ★★ indicate that there is statistically significant difference between the tested time points within the same chemical.

In the beads from 3D static culture DNA quantification was performed as a more accurate and reliable measurement of cell growth. DNA is more stable compared to the metabolic changes that used in MTS measurement. Similar to the proliferation results in 2D, cell numbers increased after one week in both protocols with Dex reaching 120.000 cells per bead while Sim 90.000 cells per bead. After that point, cell numbers started decreasing in the Dex protocol reaching around 100.000 cells per bead by the end of the culture. However, there was a significant decrease in the Sim protocol and cell numbers were around 40.000 cells per bead by the end of the experiment indicating extensive cell death.

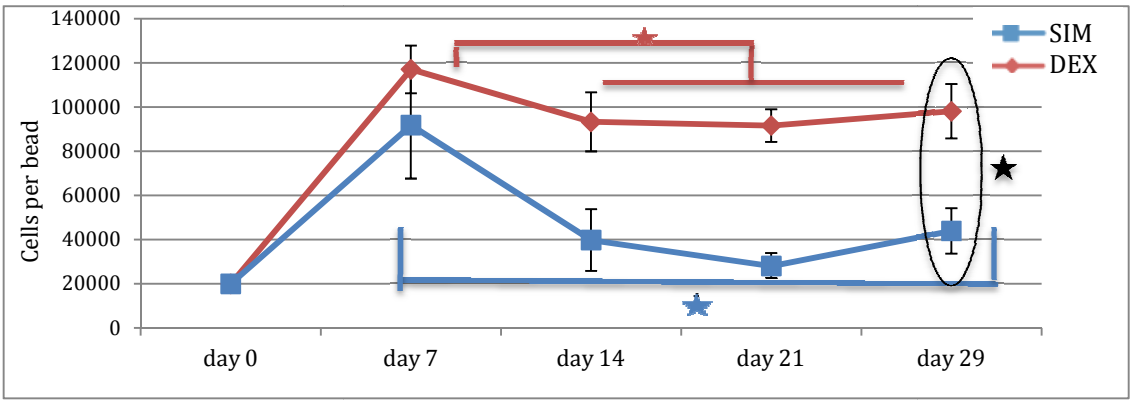


Figure 5.7: DNA quantification in 3D static configuration throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two chemicals within the same time point while asterisks ★★ indicate that there is statistically significant difference between the tested time points within the same group.

Apart from day 0, there is statistically significant difference between Dex and Sim protocol for all time points tested. Within Sim group, there was no statistically significant difference between day 0 and day 21 and also between day 14 and day 29. Within Dex group, there is no statistically significant difference between day 14, day 21 and day 29.

In the 5th chapter of the results, Sim was supplemented in the osteogenic media on day 4 until the end of the experiment on day 29, following a previously published protocol (Pagkalos et al., 2010). The reason for the extensive cell death is attributed to one important property of Sim which is the fact that it acts as a cell cycle inhibitor and for this feature is widely employed in cancer treatment. Early addition of Sim didn't allow the cells to proliferate and adapt a stable phenotype and they were forced to differentiate. During that process, a number of cells from the sensitive population of mESCs died. Following the previously mentioned protocol, concentration of 0.1 nM Sim was employed. To be more specific, Pagkalos results indicated that Sim was effective in a dose range of 0.1 to 100 nM, which corresponds to serum levels of Sim (Cmax1⁄410 to 34ng/mL) when administered by mouth to exert its antilipidemic effect in

humans (Corsini et al., 1999) and to the concentrations that have been used previously in *in vitro* studies. Specifically, Sim at 10^{-6} to 10^{-9} M has been tested on MC3T3-E1 cells (Hwang et al., 2004; Maeda et al., 2001), human osteoblast-like (hOB) and MG-63 cells (Ruiz-Gaspa et al., 2007) and bone marrow stromal cells (Baek et al., 2005).

The reason for this choice was also the fact that based on published data from other research groups (Kupcsik et al., 2009; Li et al., 2003; Viereck et al., 2005), the use of higher doses of statins, in the micromolar range, was found to be toxic for the cells and to have a negative effect on cell viability and proliferation, an effect also observed in Pagkalos experiments (Pagkalos et al., 2010). On the other hand, the employed concentration was effective for osteodifferentiation as indicated by ARS and ALP results. The tested concentration in the nanomolar scale was low enough to induce differentiation without eliciting cell death. Lower amount would probably be ineffective.

To summarize, literature data suggested that statin concentrations in the 10^{-9} M range should not inhibit cellular proliferation and were not toxic to the cells, which corresponded well with the optimal Sim concentration (0.1 nM) obtained in the study performed by Pagkalos and his collegues. The problem with the observed cell death was not the employed concentration of the small molecule but the early time supplemented in the media. For this reason, a short optimization on the time of supplementation was performed and the updated protocol included addition of Sim on day 14 until the end of the culture.

5.4.2 OPTIMISATION OF TIME OF SUPPLEMENTATION OF SIM IN THE MEDIA

Currently employed osteogenic differentiation protocol of mESCs using Dex lasts for 29 days. Culture begins with 3 days incubation in HepG2-CM, followed by osteogenic media supplemented with b-GP and ascorbate-2-phosphate until day 21, when Dex is added to the culture media for the last week of the experiment (Figure 4.5). Based on that protocol, in Figure 5.8, the timeline of the tested osteogenic differentiation protocol of mESCs with Sim is presented. The first 14 days of the protocol are the same. Optimization is related with the addition of Sim in the culture media either on day 14 or on day 21 (Table 5.1). An extended literature research was performed in order to try to explain the observed outcome. Significant information regarding the cell cycle effects that Sim has and its use for cancer cells because of this characteristic feature were obtained. The acquired information gave the idea that probably Sim should be supplemented in the culture media at a later time point, after cells had proliferated and reached high cell numbers, similar to Dex. In order to define the optimal time, a short

optimisation experiment with 2 time points was performed both in 2D and 3D static configuration. Similar to the Dex protocol, day 21 was chosen, where Sim was supplied in the media for the last week of culture. Day 14 was chosen as an earlier time point that left enough time for the cells to proliferate, reach high cell density and start synthesising bone matrix, indicating a more stable status. To evaluate the performance of the two protocols, ARS and ALP analysis were performed as a fast and easy way to get quick and reliable answers.

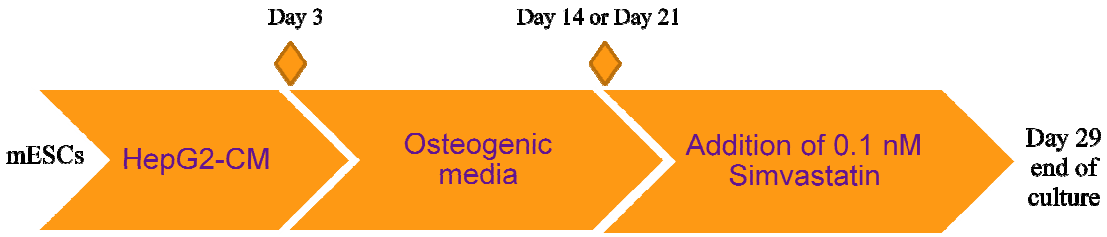


Figure 5.8: The 29 days osteogenic differentiation protocol of mESCs

Table 5.1: The two-tested time points to optimize the supplementation of Sim in the culture media

PROTOCOL	SIMVASTATIN
Protocol 2	Day 14
Protocol 3	Day 21

Analysis of 2D experiment was first performed. ARS was tested on day 21 and day 29 in 2D experiment and results revealed similar levels of calcium deposition and mineralisation for both time points and for both protocols tested (Figure 5.9).

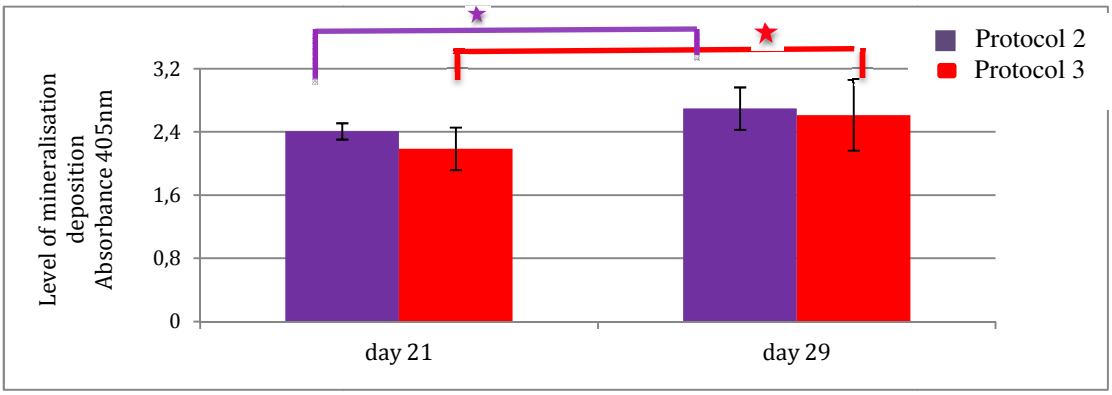


Figure 5.9: Quantification of ARS in 2D culture configuration. Comparison was performed between protocol 2 and protocol 3 of Sim optimization. One-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and shows that there is statistically significant difference between the samples of day 21 and day 29 within the same protocol.

ALP activity was also tested in 2D configuration (Figure 5.10). In general, the profile was the same for both protocols tested. There was only one difference on day 21 where ALP activity was

significantly higher in the protocol 2, where Sim was supplemented on day 14 in comparison to protocol 3, where Sim was supplemented on day 21.

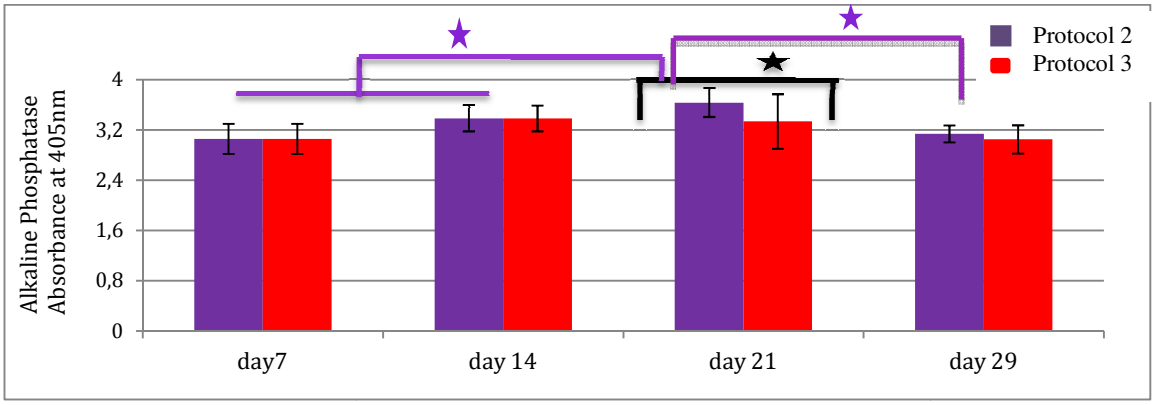


Figure 5.10: ALP activity in 2D culture configuration. Comparison was performed between protocol 2 and protocol 3 of Sim optimization. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested time points within the same group while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between day 21 and the other time points within protocol 2.

Based on these two analyses in 2D culture, both protocols indicated similar results without any major differences. This outcome suggested that earlier supplementation of the osteoinductive factor, Sim in that case, generated similar results as if it was provided at a later time point. This observation could potentially lead to the generation of a new, shortened protocol for osteogenic differentiation. For this reason, day 14 looked as a good time point to be tested for Sim supplementation in the culture media and an optimal protocol to be applied in the future. Analysis in 3D static samples was then performed. ARS showed similar pattern of mineralisation and calcium deposition between the two tested protocols (Figure 5.11) indicating that earlier supplementation of Sim can have the same outcome and lead to a potentially shortened protocol for osteogenic differentiation.

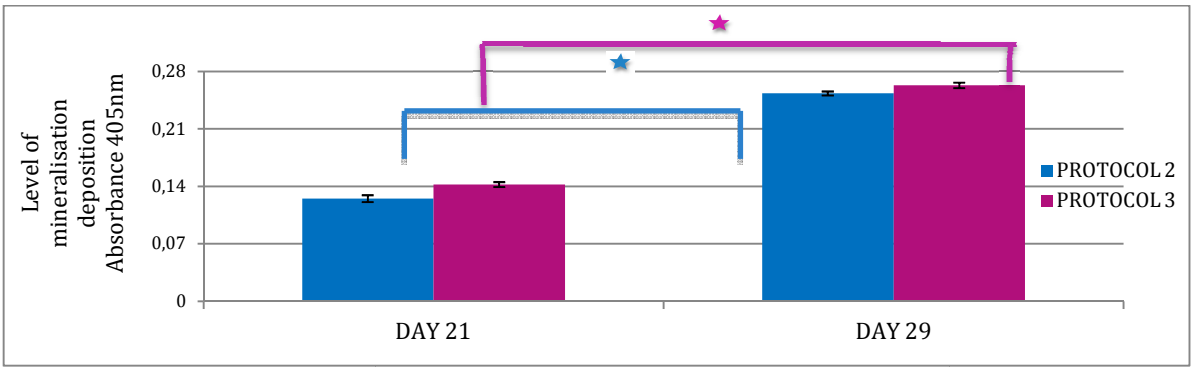


Figure 5.11: Quantification of ARS in 3D static configuration. Comparison was performed between protocol 2 and protocol 3 of Sim optimization. One-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between day 21 and day 29 within the same protocol.

In 3D static culture, ALP was also tested between the two protocols (Figure 5.12). In this configuration, ALP activity was the same between the two protocols until day 21.

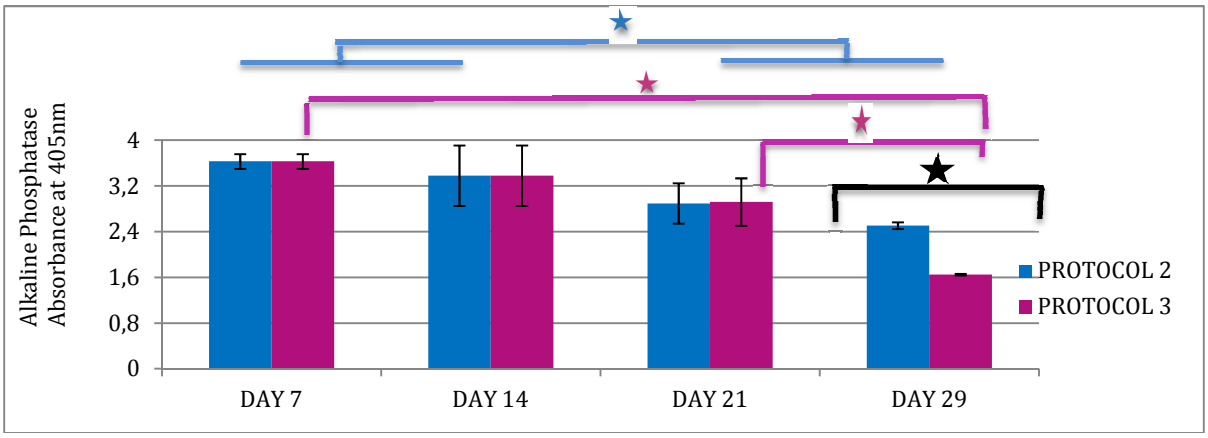


Figure 5.12: ALP activity in 3D static configuration throughout the 29 days of the experiment. Comparison was performed between protocol 2 and protocol 3 of Sim optimization. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two protocols within the same time point while asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between the tested time points within the same protocol.

On day 29, ALP expression was different between the two protocols with protocol 2 maintaining the same high expression as day 21 while ALP expression reduced significantly in protocol 3 where Sim was supplied on day 21. Higher ALP activity suggested better support of osteogenic differentiation.

Based on the above results in 3D culture, the protocol where Sim was supplied on day 14 of culture looked like the optimal to be used. ARS results didn't reveal any significant differences between the two protocols and ALP suggested a potentially better performance with Protocol 2.

To summarize, the short optimisation in 2D and 3D static configuration indicated that addition of Sim in the culture media on day 14 could lead to improved osteogenic differentiation and mineralisation of mESCs without affecting cell numbers and by avoiding negative effects on the cell cycle.

5.4.3 EVALUATION OF THE ABILITY OF FIBRONECTIN COATING TO ENHANCE OSTEOGENESIS OF MESCS INDUCED BY SIM IN 2D CULTURE CONFIGURATION AND COMPARISON WITH CURRENTLY USED GELATIN COATING

After defining the optimal time to supplement Sim in the culture media, next step was to take it one step further and try to improve even more the protocol and get higher osteogenic differentiation. Based on recently published results from previous people in the lab (Kang et al., 2015), the adhesion molecule fibronectin has been shown to enhance bone formation of mESCs. A small trial took place to test this finding, combine it with the new optimised Sim protocol and observe the possible outcome. To test this hypothesis, 2D culture in six well plates was performed. The plates were coated with **5 µg/cm^2** fibronectin and also with **0.1% (w/v)** gelatin as a control and were seeded with mESCs. The cells were incubated with HepG2-CM for the first three days. HepG2 was then changed to osteogenic media supplemented with b-GP and AA until day 14 when the osteogenic media was further supplemented with Sim until day 29. ARS and ALP analysis were used to evaluate osteogenesis and mineralisation.

ARS test indicated that calcium deposition was significantly higher in the fibronectin group compared to gelatin both on day 21 and day 29 (Figure 5.13).

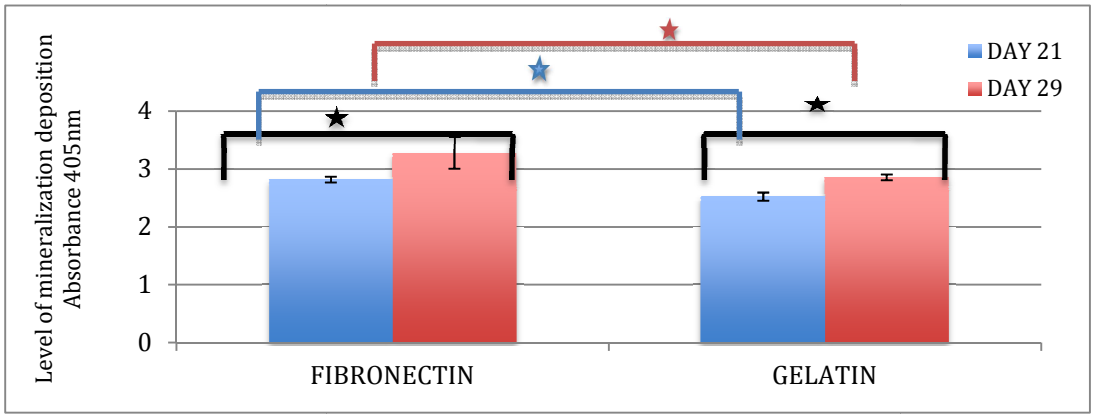


Figure 5.13: Quantification of ARS in 2D culture configuration. Comparison was performed between fibronectin and gelatin coating and for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested samples within the same coating group while asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between the tested samples within the same day.

ALP activity was also measured (Figure 5.14) but there was much heterogeneity between the wells, possibly because not the same cell numbers attached to all of the wells in the beginning of the experiment. Experiment would need to be repeated to reach final conclusions and have a more clear view of potential osteogenic ability of fibronectin. Optical densities presented in the following graph indicated that gelatin coating maintained a high level of ALP throughout the whole experiment. Fibronectin coating indicated similar findings apart from the last time point, on day 29 where there was reduction in ALP activity.

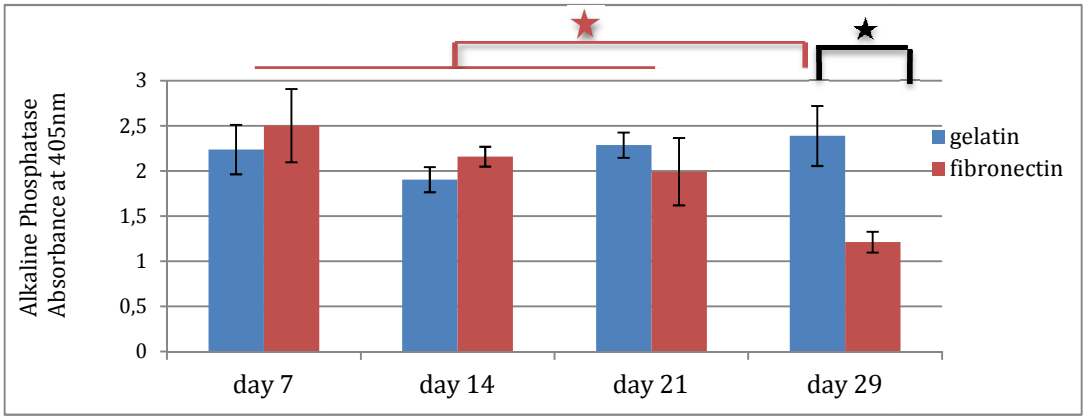


Figure 5.14: ALP activity in 2D culture configuration. Comparison was performed between fibronectin and gelatin coating and for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two adhesion molecules within the same time point while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the tested time points within the same group.

The obtained results suggested that there was statistically significant difference among Sim and Dex protocol only within day 29 while the level was similar for the rest time points. Within gelatin group, the level was constant during the experiment and there was no statistically significant difference between the tested time points. Within fibronectin group, there was no statistically significant difference between day 7, day 14 and day 21. There was statistically significant difference between the previously mentioned time points and day 29.

The above preliminary results indicated that fibronectin coating followed the expected ALP profile where decreased by day 29 during mineralisation while gelatin coating maintained the same high level throughout the whole duration of the experiment.

ALP is a characteristic early marker of osteogenic differentiation. It has been indicated the existence of a temporal expression of ALP which is upregulated at the onset of differentiation and gradually decreasing as differentiation progresses (Rawadi et al., 2003). To be more specific,

it has been supported by several groups previously that ALP expression augments during osteoblast maturation but subsequently declines during the osteoid mineralisation phase (Kulterer et al., 2007; Liu et al., 2003; Malaval et al., 1999). ALP is often used as an indication that cells are undergoing osteogenic lineage specification. The ALP enzyme is an important component of osteogenesis, which typically peaks in expression prior to mineralisation (Arpornmaeklong et al., 2009; Lian et al., 2006).

Based on the above information, it can be jutified why fibronectin indicated reduced ALP activity and thus, supported better osteogenic differentiation. Moreover, gelatin coating used as a control because this is the standard way mESCs were cultured in the lab so there wouldn't be a proper comparison without a coating material. Finally, it was mentioned that there was much heterogeneity between wells based on personal observation and in order to justify the need for repeating the analysis. However, results were acquired and analysed only from the wells with similar cell attachment.

5.5 Discussion and conclusions

In this chapter, the novel osteoinductive factor Sim was evaluated for its ability to enhance osteogenesis and potentially replace the use of the steroid Dex. Sim has been shown to increase BMP2 expression. It is also a relatively safe drug that is widely used to reduce cholesterol. It has a number of pleiotropic effects not related to its anti-cholesterol action. Sim has been used in clinical trials for reducing cholesterol levels and it has been shown to decrease the risk of bone fracture and to enhance bone formation. Following initially a published protocol (Pagkalos et al., 2010), Sim was supplemented in the culture media on day 4 of culture until the end, on day 29. The protocol was tested in 2D and 3D static configuration and comparison was performed with the previously described Dex protocol (Figure 5.1).

Several problems appeared after performing a number of analyses to evaluate the final product. ARS indicated the existence of stained bone nodules in the Sim-containing wells but there were very few cells in comparison to the Dex-containing wells and also there was no matrix formation (Figure 5.2). To further support this outcome, ARS quantification in static culture indicated lower calcium deposition in the beads cultured with the Sim protocol compared to those cultured with the Dex protocol (Figure 5.3). ALP activity was then evaluated and obtained results indicated a significant low level of ALP after Sim treatment compared Dex treatment suggesting a non-favorable environment created by Sim (Figures 5.4 – 5.5).

MTS in 2D (Figure 5.6) and DNA quantification in 3D (Figure 5.7) were employed to evaluate cell proliferation. In both culture configurations, there was significantly lower proliferation rate in the cells cultured with Sim compared to the cells cultured with Dex throughout the whole experiment. It is important to take into account two facts. In 2D culture, there was not much space for the cells to grow and they reached confluence much faster compared to 3D culture. 2D configuration could not support high cell numbers. Moreover, MTS is a test based on metabolic activity and the initial cell population was comprised of mESCs with high proliferation potential while in the end, there should be osteoblast-like cells, which had very low or no proliferation capacity at all. Metabolic activity would probably be different between these two cell types. Another important thing to notice was the fact that static culture was not promoting high cell numbers because of the limitations related to fresh media supply and removal of metabolites.

Extended literature research was performed to identify the source of the problem. It has been indicated that statins inhibit cell cycle (Forero-Pena and Gutierrez, 2013; Liao, 2002; Sala et al., 2008). They are also employed with cancer cell lines. However, statins have been also shown to enhance bone formation both *in vivo* and *in vitro* (Edwards and Spector, 2002; Garrett and Mundy, 2002). Based on the above information, it seemed a good idea to first allow cells to grow and proliferate reaching a high cell density and start synthesizing bone matrix before providing the necessary signals to guide them towards osteogenic differentiation.

As it was described in the Materials and Methods section, a short optimization was performed to define the optimal time of supplementation of Sim in the culture media, with the use of two time points, day 14 and day 21; their choice was explained previously. Evaluation of the outcome was based on ARS and ALP analysis both in 2D and 3D static configuration (Figures 5.9, 5.10, 5.11, 5.12). The obtained results suggested that there was not a big difference between the two time points and the supplementation of Sim on day 14 of culture seemed a good time point that allowed enough time for the cells to grow, proliferate and start matrix synthesis before force them to go through differentiation and mineralisation. In this way, the time of osteodifferentiation could be reduced to less than 29 days, but this is something to be tested in the future if there is adequate time.

Finally, to take the protocol one step further and try to improve more osteogenic differentiation, recently published data from previous people in the lab were evaluated (Kang et al., 2015). These data suggested that the adhesion molecule fibronectin enhanced osteogenic differentiation of mESCs. Trying to confirm those results and improve them even more, 2D culture was performed using the new Sim protocol and coating the six-well plates with fibronectin. There were also wells coated with gelatin as a control. ARS indicated higher calcium deposition

(Figure 5.13) and ALP showed the expected time-dependent expression profile (Figure 5.14) on the fibronectin-coated wells suggesting potential improvement in the osteogenic differentiation.
The composition of the ECM substrate and the existing integrins *in vitro* can determine cell fate and in particular, the important role of ECM cell adhesion molecules in osteogenic differentiation has been previously indicated (Gattazzo et al., 2014; Lavenus et al., 2011).
It has been indicated that plating ES cells on laminin or fibronectin induces differentiation, whereas self-renewal is maintained when the cells are plated on type I or type IV collagen substrates (Hayashi et al., 2007). Laminin-322 stimulates osteogenic differentiation of MSCs, whereas laminin-111 promotes neural differentiation (Klees et al., 2005; Klees et al., 2007; Mruthyunjaya et al., 2010). In adult stem cells, fibronectin can promote differentiation along skeletal lineages while suppressing adipogenic differentiation (Martino et al., 2009; Ogura et al., 2004; Wang et al., 2010). Moursi and his colleagues indicated that the a5b1 integrin mediates the binding of osteoblasts to fibronectin and is required for osteogenic differentiation, suggesting a pivotal role of fibronectin in osteoblast differentiation (Moursi et al., 1997).
Salasznyk and his colleagues demonstrated that even though hMSCs can adhere to various ECM-coated substrates (COL 1, fibronectin, vitronectin and COL IV), the greatest osteogenic differentiation occurs among cells plated on vitronectin and COL 1 (Salasznyk et al., 2004). Rowlands and his colleagues (Rowlands et al., 2008) used MSCs and investigated the osteogenic potential of various substrates coated with covalently bound tissue-specific ECM proteins (COL 1, COL IV, laminin or fibronectin). Higher osteogenic differentiation was supported by COL 1 coating probably due to the fact that it better mimics the *in vivo* environment of bone formation.
To summarize, a new protocol for osteogenic differentiation of mESCs was developed in this chapter. The protocol comprised 29 days in total, starting with 3 days incubation in HepG2-CM followed by osteogenic media supplemented with b-GP and ascorbate-2-phosphate and further supplementation with Sim on day 14 until the end of the culture. Moreover, there were indications that potential combination of Sim in the culture media with the adhesion molecule fibronectin could enhance osteogenic differentiation.

To summarize, the major conclusions that could be drawn from this chapter are the following ones:

- Sim can be an effective osteoinductive factor when applied at specific time points during cell growth and differentiation.
- The employment of fibronectin as an adhesion molecule shows indication of enhanced osteogenic differentiation.

6th CHAPTER

6. 3D BIOPROCESS OF MESCS TOWARDS OSTEOGENESIS USING SIM: COMPARISON OF FED-BATCH VS PERFUSION CULTURE

6.1 Introduction

Sim is a commonly prescribed drug to lower serum cholesterol. It acts by inhibiting HMG-CoA reductase, which is the main enzyme involved in the isoprenoid biosynthetic pathway and leads in the production of cholesterol. Sim prevents cholesterol synthesis in the liver by increasing the number of LDL receptors, which in their turn remove higher amount of plasma LDL cholesterol from the circulation (Stancu and Sima, 2001). In addition to the depletion of cholesterol, the inhibition of HMG-CoA reductase by statins leads to decreased production of the isoprenoids farnesylpyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). Depletion of FPP and GGPP, leads to several other pleiotropic effects, which are not related with the anti-cholesterol action (Liao and Laufs, 2005; Wang et al., 2008). It interferes with cell cycle and inhibits tumour growth and metastasis and for this reason, it has been used for cancer studies. It has been also reported to promote angiogenesis. Mundy et al. demonstrated that statins stimulate osteoblast differentiation and bone formation through the induction of BMP-2 expression. These results have been subsequently supported by several *in vitro* and *in vivo* studies, which showed that administration of lipophilic statins has been positively correlated with higher BMD in post-menopausal women (Safaei et al., 2007; Uzzan et al., 2007). Sim has been shown to promote fracture healing by enhancing osteoblast formation (Maeda et al., 2001; Mundy et al., 1999; Ruiz-Gaspa et al., 2007; Tikiz et al., 2004) and by inhibiting osteoclast activity (McFarlane et al., 2002; Staal et al., 2003).

To our knowledge, this is where the novelty of this project lies. It is the first time where Sim is used as an osteoinductive factor supplemented in the osteogenic media in very low concentration of the nanomolar scale in a 3D culture for the osteogenic differentiation of mESCs encapsulated in alginate hydrogels and cultured in a custom made RWV perfusion bioreactor.

It is also the first time where extended gene expression analysis, including a number of recently discovered transcription factors, is performed in order to evaluate not only the osteogenic differentiation of the cells but also to get more insight information regarding the followed ossification pathway. The expression of genes from all the three germ layers was also tested for the first time to assess the potency of the embryonic cells and if they still possess the capacity as progenitors with the appropriate incentive to be differentiated to other cell types.

6.2 Aim and Objectives

The aim of this chapter was to test the osteoinductive capacity of Sim in 3D static and perfusion culture configuration using the new established protocol after optimisation of the time of supplementation of Sim in the culture media.

In particular:

1. To evaluate the efficiency of the newly established protocol with Sim in 3D configuration
2. To compare 3D bioprocess between static and dynamic culture configuration
3. To obtain information regarding the whole differentiation process followed, from the initial undifferentiated status, through germ layer formation, up to the final osteogenic phenotype.

6.3 Methodology

Evaluation of the new optimized protocol for osteodifferentiation using Sim was performed in 3D static and perfusion culture configuration. A number of analyses were employed to evaluate the outcome. DNA quantification helped to calculate the total number of cells at each time point. Calcium deposition and mineralisation were tested with ARS and ATR-FTIR. ALP activity was measured in order to confirm osteogenesis. Photos of the hydrogels under the microscope were

obtained to observe cell morphology. Finally, extended gene expression analysis was performed in order to evaluate osteogenic differentiation, to identify the pathway of ossification that was followed and also to get some more insight information regarding the expression of genes from the three germ layers.

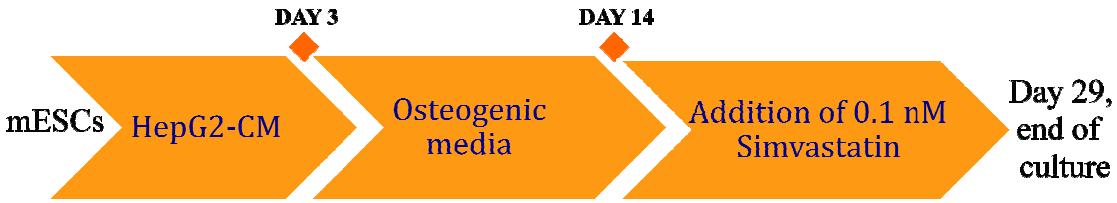


Figure 6.1: The optimized protocol for osteogenic differentiation using Sim

6.4 Results

After the short optimization performed in Chapter 5 in order to identify the best time to supplement Sim in the osteogenic media, experiments to test the efficiency of the new protocol were performed in 3D static and perfusion culture. The protocol that was followed consisted of 29 days, starting with incubation for 3 days in HepG2-CM, followed by osteogenic media containing b-GP and ascorbate-2-phosphate and finally, on day 14 further supplemented with Sim until the end of the experiment (Figure 6.1). A number of analyses were performed to evaluate the progress of osteogenesis, mineralisation and also possible effects on cell growth.

DNA quantification was used as a reliable method to indirectly calculate cell numbers at each time point (Figure 6.2). The experiment started with 20.000 cells per bead. In the perfusion culture, cells proliferated and reached maximum numbers by day 14, around 400.000 cells per bead and they maintained this number until the end of the experiment on day 29. There was a 20-fold increase in cell numbers compared to day 0. In static culture, cells reached maximum numbers by day 7, around 200.000 cells per bead, then there was a significant reduction on day 14 to less than 100.000 cells per bead but then cell numbers recovered and on day 29, there were 150.000 cells per bead. This indicated a 7.5 times fold increase compared to day 0.

These results followed similar observations by other groups, which noticed that the favorable environment of the perfusion bioreactor, with continuous supplementation of fresh media and removal of metabolites, increased viability and proliferation capacity of the cells (Cartmell et al., 2003; Liu et al., 2012; Wang et al., 2013; Xie et al., 2006).

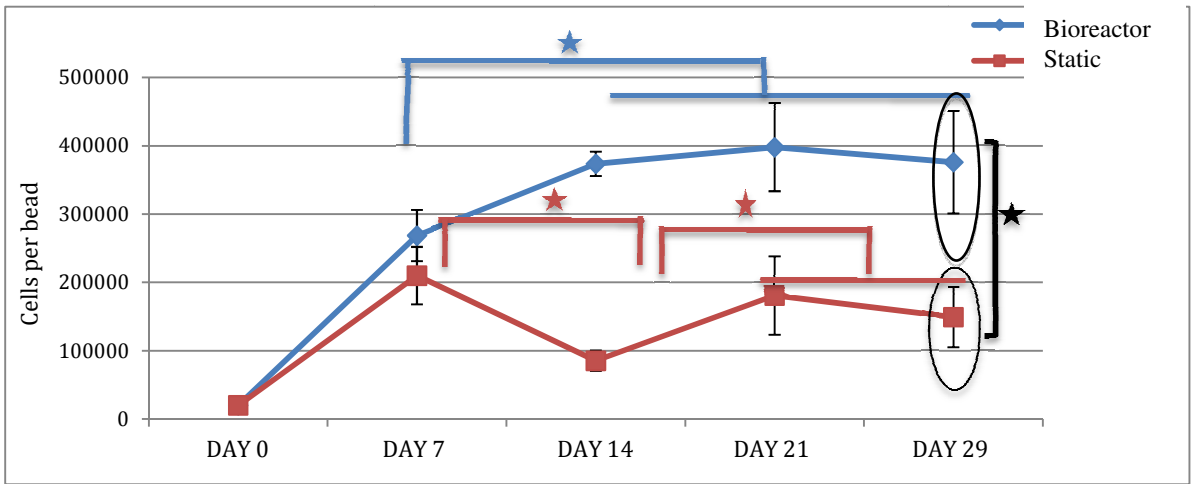


Figure 6.2: DNA quantification throughout the 29 days of the experiment. Comparison was performed among dynamic and static culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the platforms on the same time point while asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between different time points within the same platform.

One thing that could be noticed from the graph in Figure 6.2 and disrupted the growing profile of the cells was the significant reduction in cell numbers observed by day 14 in static culture. The assumption for this event was that by that day, cells in the hydrogels had already reached a confluent state. Due to the fact that media and oxygen transport only took place with simple diffusion, it has been proved that static configuration cannot support high cell numbers. Increased cell death combined with the existence of numerous necrotic areas in the interior of the hydrogels (Appendix 4) could explain the observed decrease in cell numbers by day 14. On that day, however, Sim was supplemented in the culture media and this event probably activated again the proliferation capacity of the cells which were still in a progenitor stage and did not fully mature, thus they reacted to Sim stimuli by starting growing again and subsequently differentiating.

ALP activity was determined biochemically throughout the experiment in order to evaluate the progress of osteogenesis (Figure 6.3). The obtained results showed that in the perfusion culture with Sim treatment, ALP was maintained in high and similar level throughout the whole experiment, from day 7, where it reached maximum activity, until the end, on day 29. On the contrary, in static culture, ALP activity was maximized by day 7 as well, but after that point, it dropped gradually until day 21 and slightly increased again by day 29.

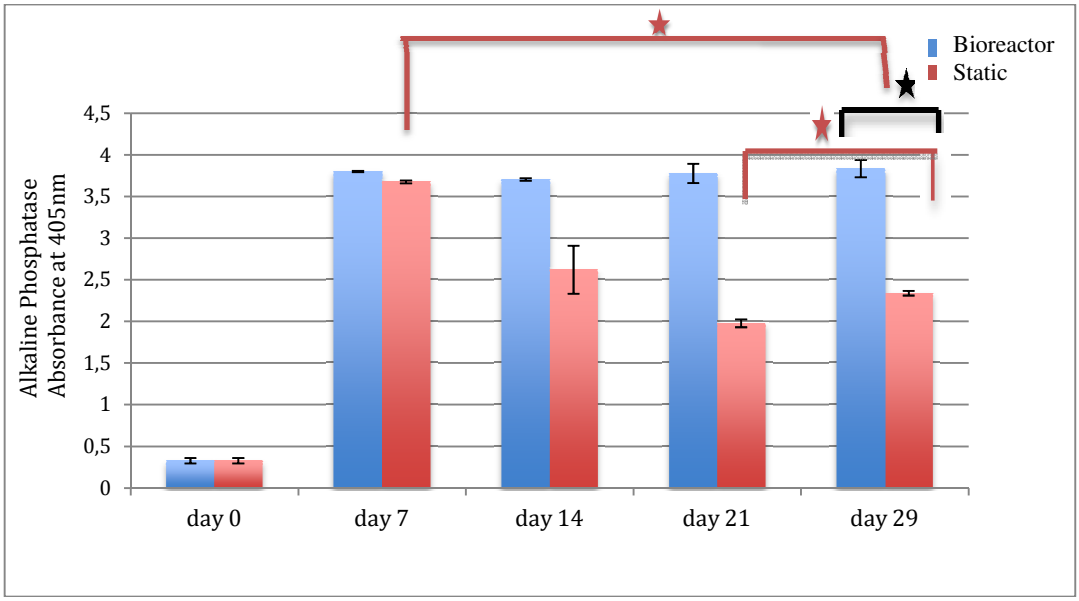


Figure 6.3: ALP activity throughout the 29 days of the experiment. Comparison was performed between static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the platforms on the same time point. Asterisk ★ indicates $p < 0.05$ and show that there is statistically significant difference between different time points within the same platform.

The obtained results indicated that continuous media perfusion maintained high ALP activity throughout the whole experimental procedure suggesting that cells in the bioreactor had a more progenitor like phenotype. In static culture, there was lower ALP activity but the profile followed during time was closer to what was expected to appear with ALP increasing during osteoblast differentiation and subsequently decreasing during mineralisation.

The acquired results were then normalized with cells numbers and expressed as μM/cells/min (Figure 6.4). The image of the ALP profile was different now. In many time points tested, static culture indicated higher activity because of the lower number of cells in comparison to the perfusion culture where increased proliferation resulted in higher cell numbers. On day 14, ALP exhibited significantly higher level in static culture in comparison to the bioreactor. This finding was expected if the DNA quantification results would be considered, where on day 14, there was a significant reduction in cell population in static culture. On day 0 and on day 29, ALP activity after normalization with DNA was in similar level for both tested platforms.

In perfusion culture, normalized ALP exhibited the highest activity on day 7 and then it was significantly reduced on the following time points. In static culture, normalized ALP reached the highest level, later, on day 14 and then significantly decreased. This finding suggested earlier progression to osteogenic differentiation in perfusion culture.

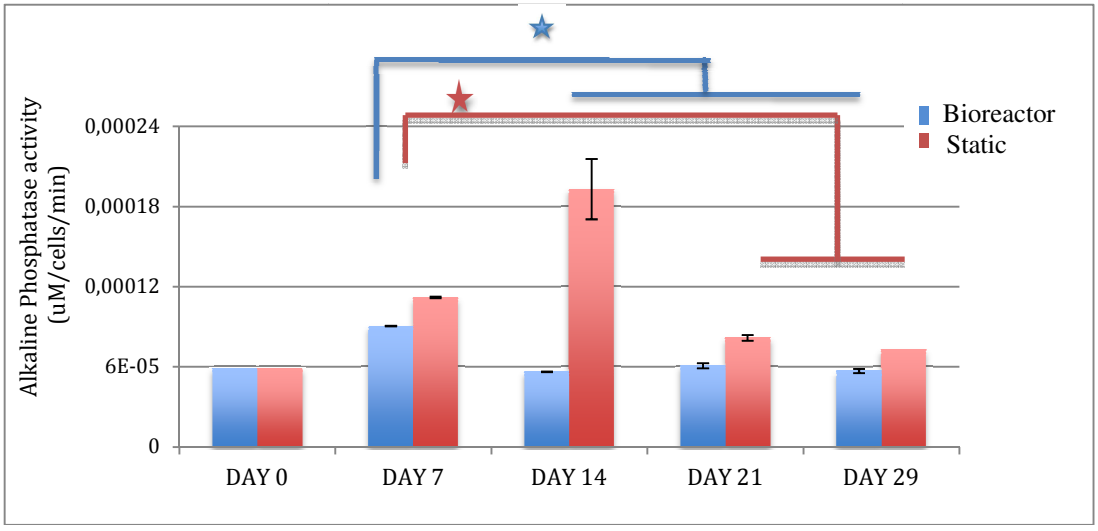


Figure 6.4: ALP activity normalized with cell numbers obtained from DNA quantification and expressed as μM/cells/min throughout the 29 days of the experiment. Comparison was performed between static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between different time points within the same platform.

Calcium deposition and mineralisation was quantified with ARS (Figure 6.5) and was also evaluated with ATR-FTIR (Figure 6.6).

ARS indicated that on day 21, both platforms deposited similar calcium levels. On day 29, both culture configurations exhibited increased staining; with perfusion bioreactor marking a significantly higher level of mineralisation in comparison to static culture.

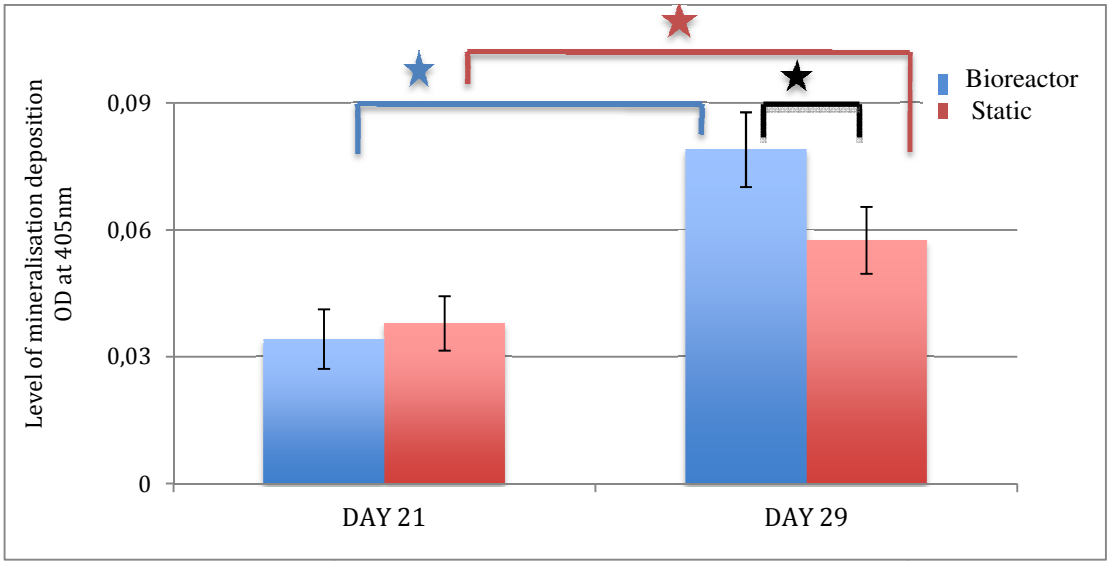


Figure 6.5: ARS was used to evaluate calcium deposition. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the platforms within the same time point, asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between day 21 and day 29 within the same platform.

In order to confirm the ‘‘functionality’’ of the mineralised constructs formed within the alginate hydrogels, HA deposition on day 29 of osteogenic culture was verified using the ATR-FTIR spectroscopic imaging method in combination with a focal plane array (FPA) detector (Chan and Kazarian, 2003).

With the help of Jennifer Dougan, a post-doctoral fellow in Professor’s Sergei Kazarian Vibrational Spectroscopy and Chemical Imaging of Complex Materials and Processes laboratory within the Department of Chemical Engineering, analysis of several samples was conducted and the FTIR imaging results verified the distinct presence of phosphate in the mineralised constructs as illustrated in the plots showing the integrated absorbance of PO_4 (between 970 and 1035 cm^{-1}) and amide I (between 1565 and 1500 cm^{-1}).

Samples from day 29 of the experiments were examined and are presented in the images on Figure 6.6. The important outcome was the presence of phosphate confirming mineralisation but quantitative comparisons could not be made at this point.

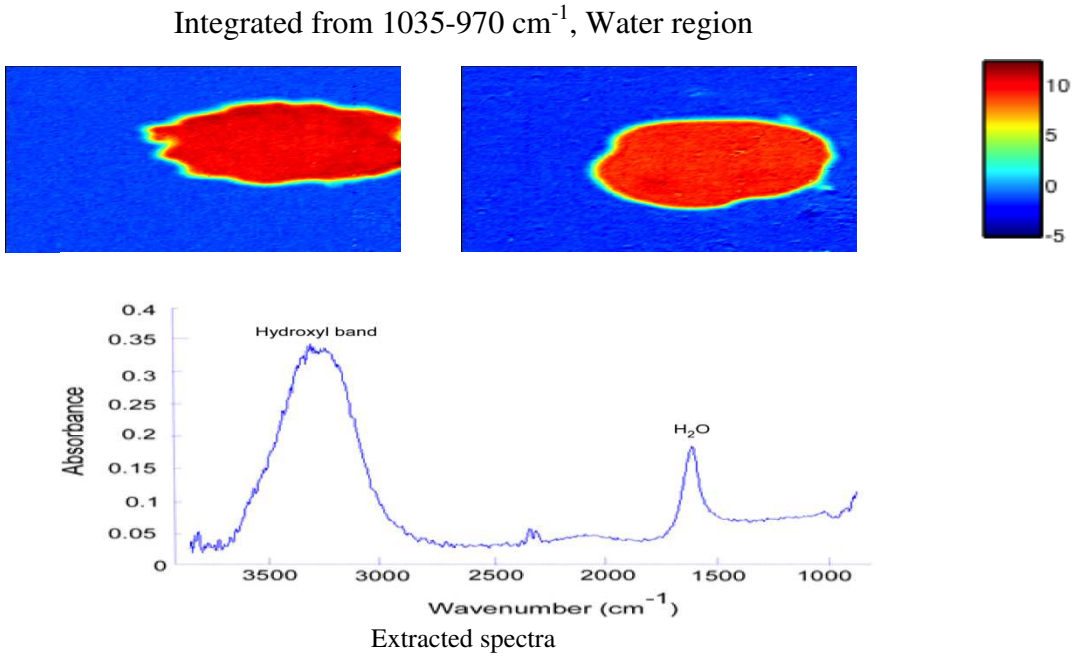


Figure 6.6: Analysis of the mineralisation of the 3D constructs. The FTIR images and ATR-FTIR spectra of mineralised constructs generated by plotting the integrated absorbance of PO_4 (between 970 and 1035 cm^{-1}) and amide I (between 1565 and 1500 cm^{-1}) over the imaged area. The registered area within the spectral region was represented with the colour gradients from yellow to red approaching to the peak in the spectra. Images were obtained with the help of the post-doctoral fellow Jennifer Dougan.

HA deposition was confirmed by FTIR imaging. Images present the distribution of the integrated absorbance of the HA band at ~ 1030 cm^{-1}, where **the red areas indicate field with higher concentration of phosphate**. Quantitative comparisons could not be made at this point. ATR-FTIR analysis showed distinct peaks of phosphate and carbonyl, indicating mineralised calcium/phosphate formation, which is major component of bone mineral (Hunter and Goldberg, 1993).

Photos of two hydrogels, one from perfusion and one from static culture, both using Sim in their differentiation protocol, were acquired under the light microscope on day 21 in order to observe cell morphology (Figure 6.7).

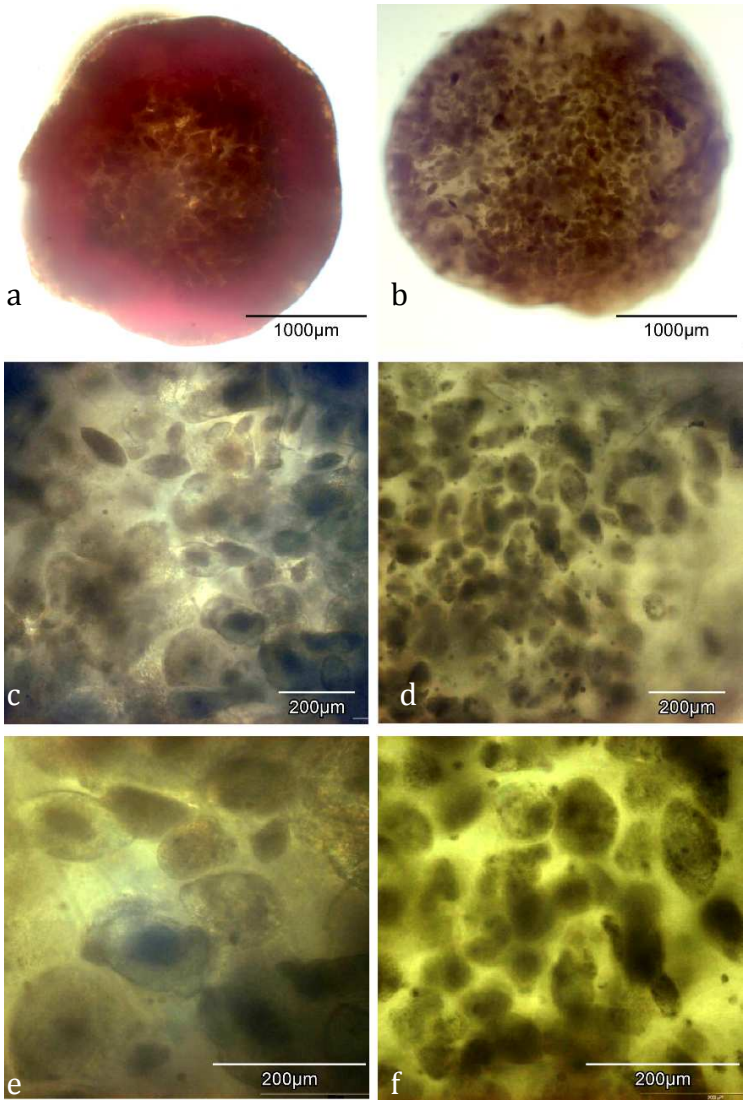


Figure 6.7: Light microscopy images of encapsulated mESCS within one alginate hydrogel from perfusion bioreactor (a, c, e) and one from static configuration (b, d, f) at different magnifications acquired on day 21. Scale bar is 1000µm for the images a and b and 200µm for the images c, d, e and f.

From the photos in Figure 6.7, it can be observed, similar to the previous findings, the better colony formation and cell distribution in the perfusion culture compared to static platform. By day 21, hydrogels cultured under Sim appeared to have already reached a confluent state, as observed under the microscope. To further support that observation, DNA quantification results (Figure 6.2) indicated that maximum cell density was achieved by day 14 and maintained until the end of the experiment in the bioreactor platform. Moreover, fewer cells were calculated in static configuration, probably due to the existence of necrotic parts, which are not easy to see in the above photos. However, as mentioned previously, it has been indicated through live-dead analysis by Dr Wesley Liam Randle the existence of large pieces of viable tissue that had numerous necrotic areas in 3D static culture configuration (Randle, 2006) (Appendix 4).

Moreover, results from different research groups indicated that engineered constructs cultured under static conditions, where culture media is not properly mixed, are frequently inhomogeneous in structure and composition, **containing a hypoxic necrotic central region** and dense layers of viable cells encapsulating the construct periphery (Wendt et al., 2005). When formed in static cultures, usually in tissue culture flasks, agglomerated large EBs revealed **extensive cell death and eventually large necrotic centers** due to mass transport limitations (Gerecht-Nir et al., 2004).

6.4.1 GENE EXPRESSION

Extended gene expression analysis was conducted using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) in order to get information regarding several different things and various events that took place throughout the 29 days of the experiment. The expression of bone markers was initially tested in order to evaluate the progress of osteogenesis using the Sim protocol, how efficient it was and how it could have affected gene expression in a positive or negative manner. The housekeeping gene RPL27 was used for normalization (de Jonge et al., 2007).

Moreover, it is well known that *in vivo* bone formation takes place via two possible pathways: either through intramembranous ossification where mesenchymal cells directly differentiate to osteoblasts or through endochondral ossification where mesenchymal cells first condensate and create a cartilage template. The template gets invasion from blood vessels, chondrocytes become hypertrophic and finally, they differentiate to osteoblasts and start synthesizing bone matrix. In order to get information on the pathway followed through 3D culture and with the use of Sim, the expression of a number of cartilage and hypertrophic chondrocyte markers was also tested.

Finally, to estimate the differentiation status and the potency of the cells on the last week of the experiment, on day 21 and day 29, various markers from the three germ layers, mesoderm, endoderm and ectoderm, were also tested. In this way, it was intended to elucidate if our cells were fully and homogenously differentiated towards a mature stage or they had acquired a more progenitor phenotype where they could still be tuned to differentiate to any cell type of the three germ layers.

The expression of several bone markers was first tested and the results are presented in the following graphs. Comparison was performed between day 21 and day 29 and also between the two culture platforms, static versus perfusion.

The expression of ALP gene was evaluated (Figure 6.8). ALP is considered one of the most significant factors affecting osteogenic differentiation. Experimental results in the bioreactor configuration showed that ALP increased significantly from day 21 to day 29. In static culture, ALP expression on day 21 was similar to the perfusion but decreased significantly by day 29.

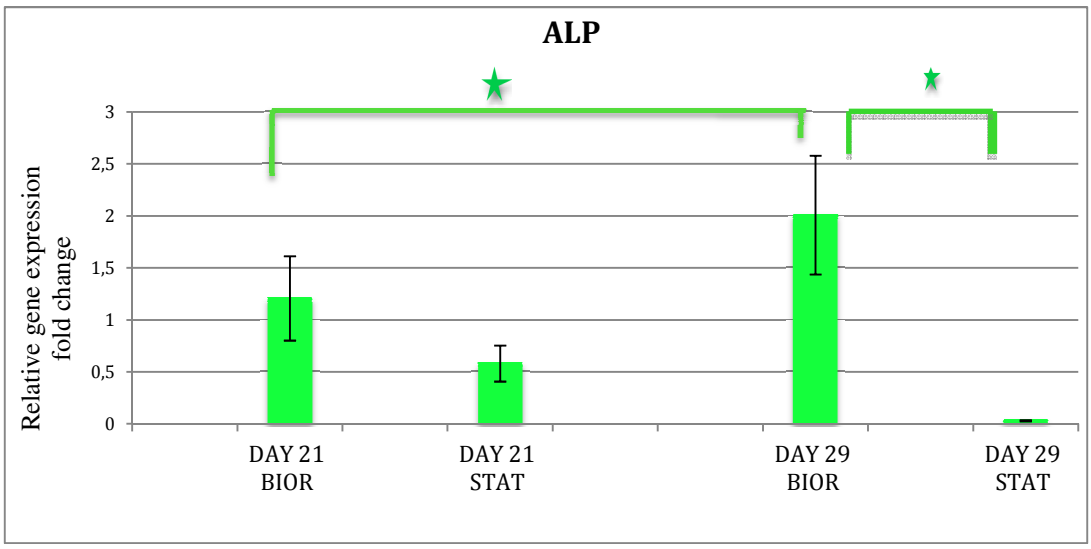


Figure 6.8: Gene expression of ALP. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± S) of N=3.

Following previous observations, the favorable environment of perfusion bioreactor induced a high level of ALP activity throughout the whole experiment (Barron et al., 2012). In particular, another research group realized that cells in the reactor consistently maintained higher ALP activity and calcium deposition than those in static cultures, suggesting that perfusion culture favours the subsequent osteogenic differentiation upon induction (Zhao et al., 2009).

It has been suggested that Runx2 regulates early differentiation of mesenchymal cells into preosteoblastic cells, whereas OSX controls the differentiation of preosteoblastic cells into immature osteoblasts (Komori, 2006; Nakashima et al., 2002). The expression of Runx2, the most important factor that guides bone formation and activates other bone markers as well, was first calculated (Figure 6.9). Runx2 expression exhibited low expression at both time points and both platforms tested. To be more specific, expression was the same between day 21 and day 29 for each platform and perfusion bioreactor had slightly higher expression level compared to static.

OSX is an important transcription factor essential for osteoblast differentiation that has been shown to act downstream of Runx2 (Nakashima et al., 2002). Runx2 regulates OSX expression (Karsenty, 2008). OSX expression indicated a different profile (Figure 6.9). First of all, OSX exhibited relatively high expression levels compared to the ones observed in Runx2. To be more specific, in the bioreactor, OSX showed a 1.8 fold increase from day 21 to day 29. In static culture, OSX expression was high on day 21 and then decreased significantly to very low level by day 29.

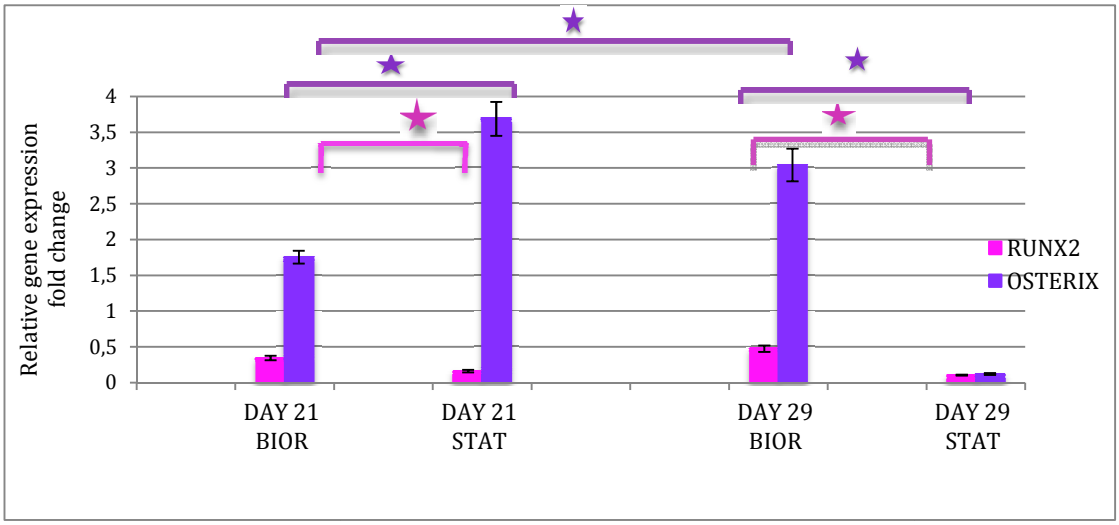


Figure 6.9: Gene expression of the transcription factors Runx2 and OSX. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between the tested samples. Values are mean ± SD of N=3.

Runx2 expression showed very low levels, with higher values observed in the bioreactor in comparison to static configuration. This could indicate that treatment with Sim induced cells towards differentiation indicated by the low expression level of the early stage marker.

OSX expression reached maximum level in static culture by day 21 and then decreased while in bioreactor platform, the expression increased from day 21 to day 29. This finding indicated an earlier expression of this marker in static culture, showing probably an earlier progression to the mature phenotype compared to the bioreactor, which probably maintained cells in a more progenitor state for longer time.

Gene expression analysis continued with the examination of several bone markers.

COL 1 is the most abundant protein of bone matrix. It is expressed early during osteogenic differentiation (Figure 6.10). In the perfusion culture, COL 1 was expressed in a low level on day 21 and then marked a 5-fold increase by day 29. In static culture, COL 1 was highly expressed on day 21 and then was significantly reduced by day 29.

The expression of BMP2 was also tested (Figure 6.10) and the profile was similar to the one of COL 1 but with significantly lower levels achieved. In particular, BMP2 expression in the bioreactor was low on day 21 but showed a 2.5 fold increase by day 29. In static configuration, BMP2 expression was relatively high on day 21 and then decreased significantly to a very low level by day 29.

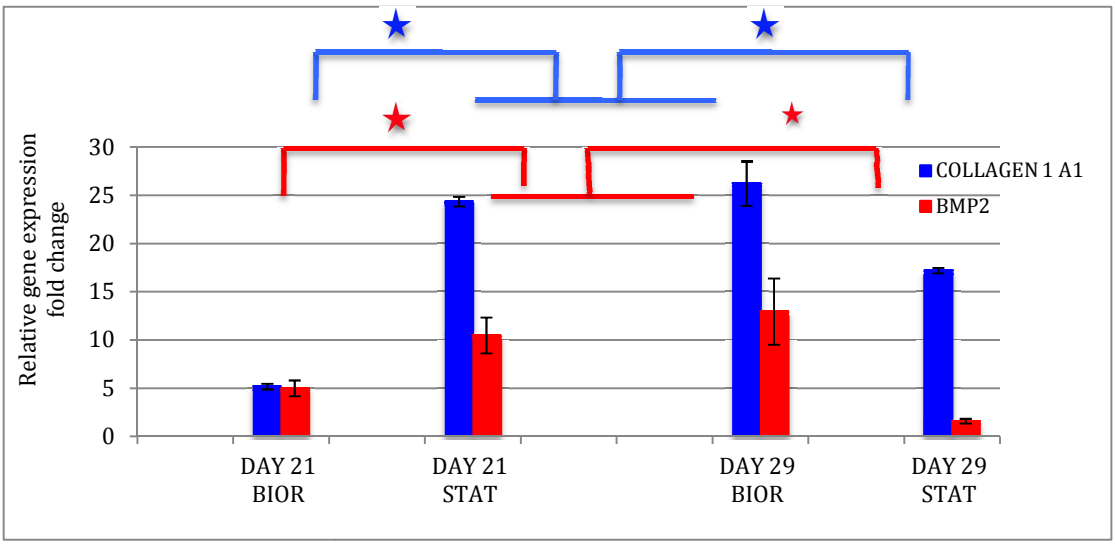


Figure 6.10: Gene expression of COL 1 and BMP2. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

COL 1 and BMP2 are both early stage markers of osteogenic differentiation. Their expression profile was similar in the above experiments, with the only difference being the lower expression level of BMP2 compared to COL 1. To be more specific, the expression levels for both tested genes were similar between day 21 in static culture and day 29 in perfusion culture.

This finding suggested an earlier maturation stage in static culture, which achieved higher gene expression level at an earlier time point in comparison to perfusion. Based on those findings, it could be assumed that the environment created by the perfusion bioreactor maintained the cells in a more progenitor-like stage, maybe pre-osteoblast cells, which are not fully differentiated, for longer time and more time was needed to achieve a fully mature phenotype. On the contrary, cells in static culture were in a more mature stage.

OCN and BSP are two late state markers of bone formation and mineralisation (Figure 6.11). In the obtained results, OCN was highly expressed on day 21 in the perfusion culture and increased even more by day 29. In static configuration, OCN expression was much lower on day 21 and slightly increased by day 29, remaining however to significantly lower level compared to perfusion.

BSP expression presented a similar profile with OCN but the level of expression was many times lower at both time points tested. In particular, on day 21 both platforms had very low and similar level of expression. Then, by day 29, perfusion culture indicated a 4-fold increase while static culture maintained the same low level of expression.

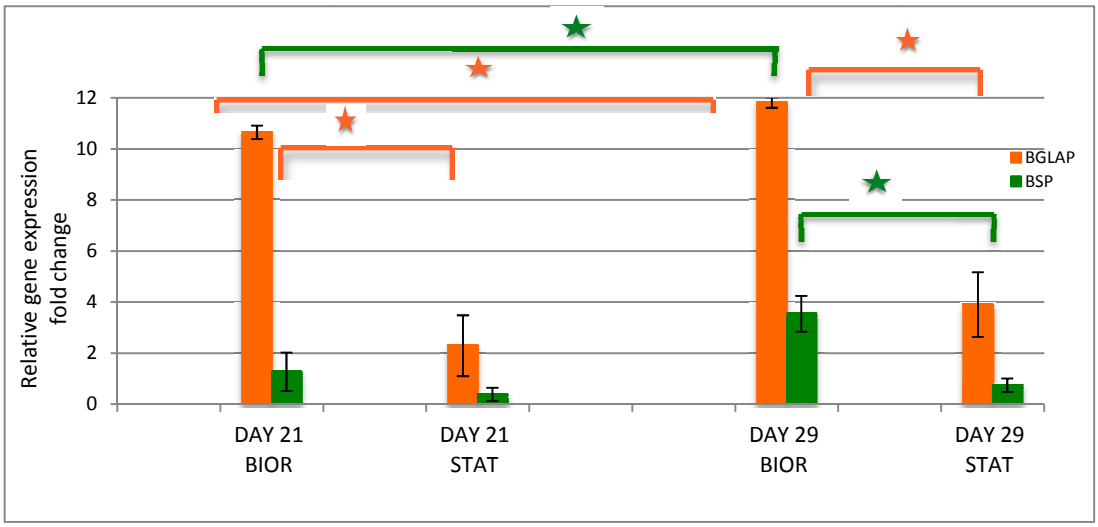


Figure 6.11: Gene expression of Bglap and BSP. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples. Values are mean ± (SD) of N=3.

The above findings indicated a preference for the expression of late stage markers of bone formation in the perfusion culture whereas a very low level presented in static culture. It is likely that the favorable environment created by the bioreactor could better support osteogenic phenotype with the generation of appropriate bone matrix to further support bone formation and mineralisation.

OPN is a non-collagenous protein of bone matrix. It is usually expressed during the proliferative phase, and then is downregulated and it is again upregulated during mineralisation (Lian and Stein, 1995; Neve et al., 2011). In the results obtained from the experiment (Figure 6.12), OPN expression was low on day 21 in the perfusion platform and then showed a significant 5.5 fold increase by day 29. In static culture, OPN expression was relatively high on day 21 and then marked a 6-fold decrease by day 29. In general, low expression of OPN was exhibited at both time points and platforms tested.

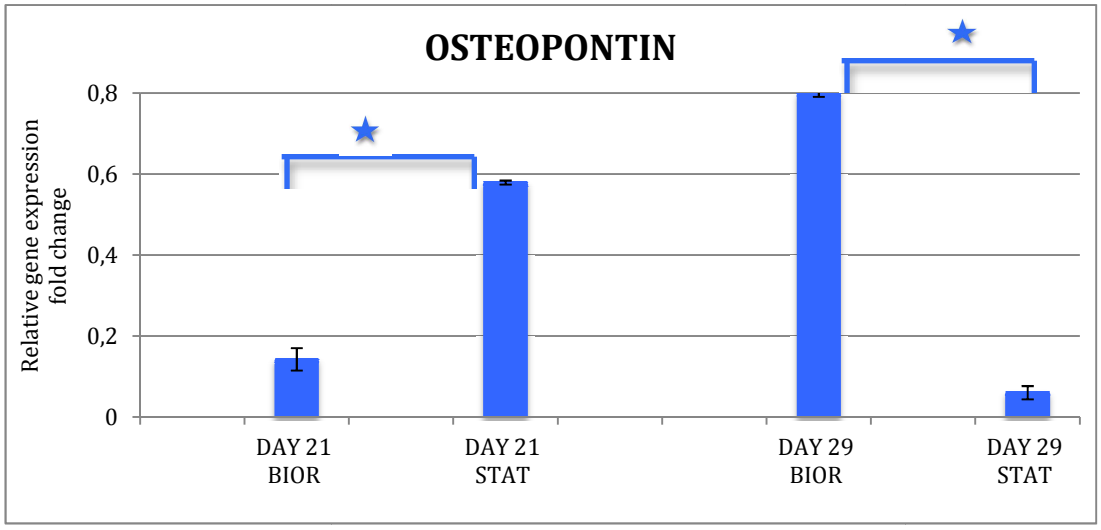


Figure 6.12: Gene expression of OPN. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

The above finding indicated once more the earlier expression of a bone marker in static configuration in comparison to the bioreactor reinforcing the idea that the use of Sim in the perfusion culture may maintained cells in a more progenitor state and they needed more time in order to reach maturation.

ON is the major non-collagenous bone matrix protein. It is highly expressed early during osteogenic differentiation and decreases when cells acquire more mature phenotype (Delany and Hankenson, 2009; Kusafuka et al., 1999). In the obtained results (Figure 6.13), ON was highly expressed on day 21, showing about three times higher fold increase in static configuration in comparison to the bioreactor. On day 29, there was a statistically significant downregulation for both platforms reaching the same very low level of expression.

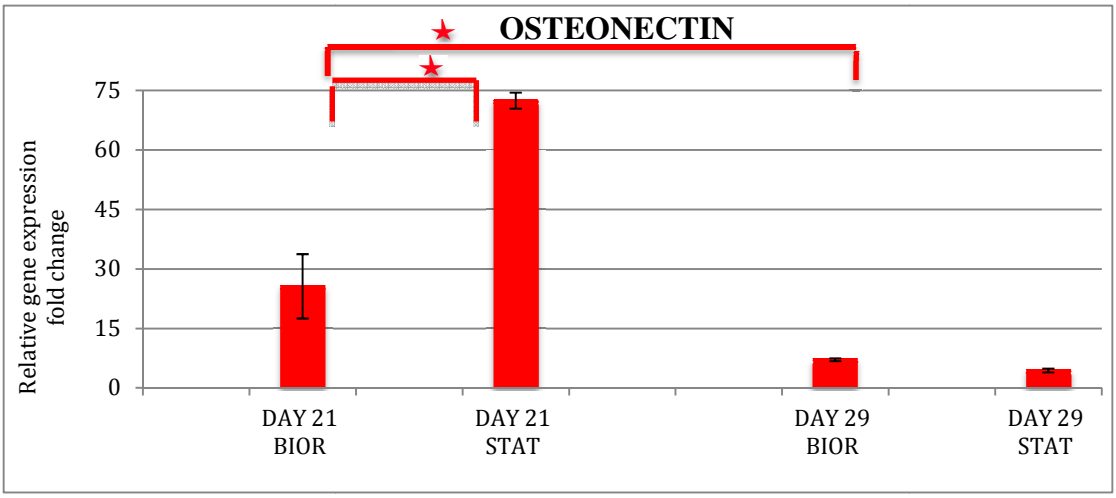


Figure 6.13: Gene expression of ON. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=3.

ON was highly expressed early on day 21 and subsequently decreased by the end of the experiment. Downregulation of ON expression on day 29 suggested that cells were progressing towards a maturation stage at that time point. It was also interesting the fact that the level of ON expression was much lower in perfusion culture compared to static. It was possible that perfusion culture indicated high ON expression at an earlier time point.

To summarize, results from the expression of bone markers indicated the more progenitor stage maintained in the cells cultured in the bioreactor after Sim treatment while static conformation was in a more mature stage but the low gene expression of bone markers questioned the proper osteogenic differentiation.

Apart from the tested osteogenic markers to evaluate osteogenic differentiation, a couple of cartilage markers and markers of hyperthrophic chondrocytes were tested in order to get information regarding the followed ossification process and if it was endochondral or intramembranous.

SOX9 is well known to drive cartilage formation and is the first marker to be expressed (Bi et al., 1999). SOX9 is expressed in all chondroprogenitors and all differentiated chondrocytes, but not in hypertrophic chondrocytes during chondrogenesis (Ng et al., 1997; Zhao et al., 1997). It particularly inhibits the transition of chondrocyte into hypertrophic chondrocytes and in this way, it controls subsequent endochondral ossification. SOX9 is needed for the commitment of undifferentiated mesenchymal cells to a cell type that is both a chondroprogenitor and an osteoprogenitor (Akiyama et al., 2002). Results obtained from the experiment (Figure 6.14) indicated high SOX9 expression in perfusion culture, which maintained on the same high level until the end of the process, on day 29. In static culture, there was significantly much lower level of SOX9 expression compared to the bioreactor, which was similar for both time points tested.

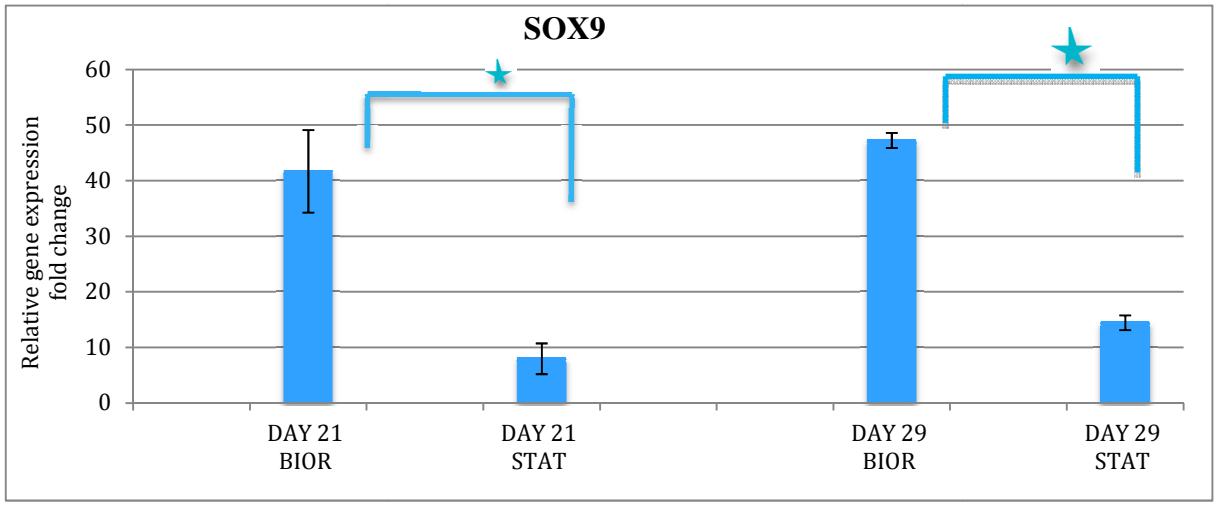


Figure 6.14: Gene expression of the transcription factor SOX9. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. One-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between these samples. Values are mean ± SD of N=2.

This finding provided another proof of evidence that cells on day 29 in the bioreactor configuration were progenitor cells, probably pre-osteoblasts, while in the static culture they were in a more advance stage towards osteoblast cells and they had low level of SOX9 expression. SOX9 marks the mesenchymal progenitors that give rise to all osteoblasts. It is not expressed by mature osteoblasts (Long, 2012). This observation suggested that they were not mature osteoblasts in the bioreactor.

Aggrecan is one of the major components of cartilage ECM. The ability of articular cartilage to resist compression is primarily due to the presence of aggrecan aggregates (Chen et al., 2007). Experimental analysis (Figure 6.15) indicated that aggrecan expression was relatively high on day 21 and then increased more by day 29 in the perfusion configuration. In static culture, there was very low to insignificant level of aggrecan expression on day 21 which noted a 4-fold increase by day 29.

COL10a1, a marker of hypertrophic chondrocytes was also evaluated (Figure 6.15). Its expression was high on day 21 in the perfusion culture and decreased by day 29. In static culture, on day 21 there was very low to insignificant COL 10 expression, which increased significantly by day 29.

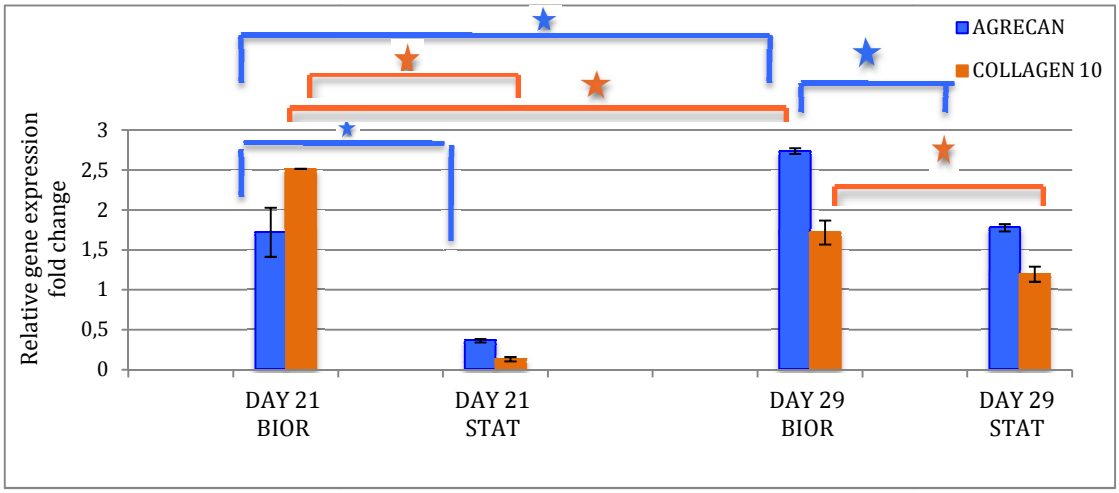


Figure 6.15: Gene expression of Agrecan and COL 10. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference on Aggrecan gene expression between these samples while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference on COL 10 gene expression between all the tested samples. Values are mean ± SD of N=2.

The expression of hypertrophic chondrocyte marker and cartilage matrix protein suggested that differentiation was taking place through endochondral ossification and the high expression level in the perfusion culture indicated that cells were in the common progenitor stage expressing markers from both bone and cartilage while cells in static culture indicated lower level of expression of these markers suggesting they had proceed towards a more mature stage of bone differentiation.

Two transcription factors, which are not osteoblast specific and may act through OSX, are Dlx5 and Msx2. These proteins affect the differentiation and regulation of the osteoblast phenotype. Low levels of expression are observed early in the differentiation and their expression seems to increase in a later stage. DLX5 has been shown to possess an important role in up-regulating the expression of the master gene Runx2 while MSX2 has the opposite function and inhibits the expression of Runx2 (Holleville et al., 2007; Ryoo et al., 2006; Shirakabe et al., 2001).

In the results obtained from the experiments (Figure 6.16), MSX2 expression exhibited the same relative mediumlevel of expression at both time points measured in the bioreactor culture. In static culture, the level was again the same at both time points, but it was about 7 times lower compared to the bioreactor. DLX5 expression in the perfusion culture was low on day 21 but increased significantly, exhibiting a 2.5 fold increase by day 29. In static configuration, DLX5 expression was very low on day 21 and indicated a 6-fold increase by day 29, where the level of expression was again many times lower in comparison to the perfusion.

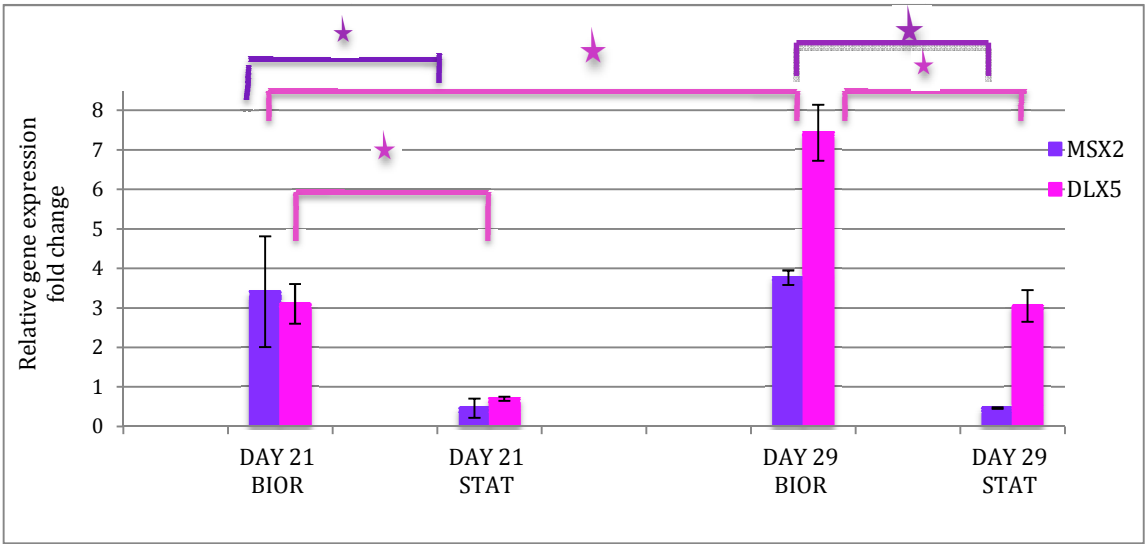


Figure 6.16: Gene expression of transcription factors Msx2 and Dlx5. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples. Values are mean ± SD of N=2.

The above results indicated a better support of osteogenic differentiation in the bioreactor platform in comparison to static as proved by the higher expression levels of the DLX5 transcription factor, which has been shown to enhance osteoblast differentiation. However, it could be observed that MSX2 expression, which acts as an inhibitor, was also higher in perfusion culture but in a significantly lower level compared to DLX5. This could suggest that in

the competition between the two transcription factors, DLX5 expression would probably dominate if the previous results on enhanced OSX expression were taken into consideration.

Parathyroid hormone 1 receptor (PTH1R) is expressed on the surface of osteoblasts. It has been revealed to have important physiological role in bone development and bone cell differentiation (Datta and Abou-Samra, 2009). Specifically, it has been suggested that PTH1R is expressed at low levels relatively early in the differentiation cascade, with increasing expression as osteoblasts mature (Liu et al., 2003; Rodan and Noda, 1991). PTHR1 has been described as a **"**globally**"** expressed marker for osteoblastic cells (Candeliere et al., 2001).

In the results obtained from the use of Sim (Figure 6.17), the expression of the PTH1R indicated a mediumlevel of expression on day 21 with similar level of expression at both platforms. The significant difference appeared on day 29, where the expression of the receptor increased significantly in the perfusion culture while it decreased significantly in static culture.

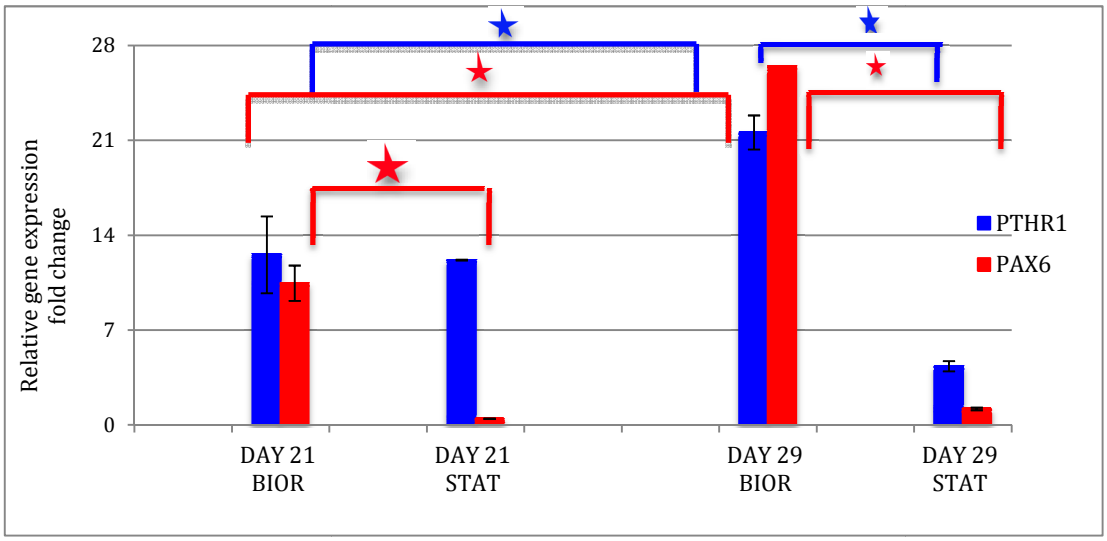


Figure 6.17: Gene expression of PTH1R and the transcription factor Pax6. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples. Values are mean ± SD of N=2.

PTH1R expression is very important for normal osteoblast differentiation. The acquired results indicated a better and more proper differentiation profile in the perfusion culture proved by the high gene expression in comparison to static configuration.

As it was mentioned previously in this chapter regarding gene expression, markers of the three germ layers were also tested in order to primarily evaluate the differentiation status and secondary, understand more about the progenitor stage of the cells.

PAX6 is a transcription factor considered as a marker of neuroectoderm and has a vital role in early regulation of neurogenesis (Zhang et al., 2010a). Results from the experiments (Figure 6.17) indicated high level of PAX6 expression on day 21 in the perfusion culture, which significantly increased by day 29. On the other hand, in static culture, there was very low to insignificant level of PAX6 expression at both time points tested.

The above results suggested that cells in the perfusion culture were not yet fully committed to the osteoblastic lineage and they were in a progenitor status where they were still expressing markers of the three germ layers.

Nestin is another marker of ectoderm (Figure 6.18). Following the results of the transcription factor Pax6, Nestin expression was higher in perfusion culture at both time points tested compared to static. In particular, Nestin expression was high on day 21 and then marked a small decrease by day 29 in the bioreactor. On the other hand, in static culture, Nestin expression was low and maintained the same level until the end of the experiment.

In general, between the two tested ectoderm markers, it could be noticed that Pax6 expression was much higher than Nestin expression and this difference was easier to observe in the perfusion bioreactor gene expression results.

Next step was the evaluation of the expression of two mesoderm markers, the BMP4 transcription factor and the Desmin protein (Figure 6.18).

The expression of the gene encoding for Desmin protein was relatively high on day 21 in the perfusion culture and then decreased by day 29. In static culture, its expression profile followed the one of perfusion, with Desmin expression getting significantly downregulated from day 21 to day 29. The expression level in static culture was always lower in comparison to the bioreactor.

BMP4 has been indicated to be significant for mesoderm formation (Winnier et al., 1995). BMP4 expression was relatively high on day 21 in the perfusion culture, following the expression level of desmin and decreased significantly by day 29. In static culture, there was low expression on day 21 which marked a 2.5 fold increase by day 29.

The expression of the two mesoderm markers was relatively high on day 21 in the perfusion culture indicating an earlier induction towards mesoderm in that configuration compared to static, where in general the expression level of the two tested mesoderm markers was low for both time points tested.

In Figure 6.18, the expression of the ATF4 was also evaluated. ATF4 has been shown to be crucial and essential for bone formation and to be required specifically for the late-stage osteoblast differentiation (Yang et al., 2004). It has been shown to be a critical regulator of osteoblast proliferation and differentiation. In particular, it has been proved that ATF4 favors bone formation by promoting osteoblast (OBL)-specific gene expression, amino acid import and the synthesis of Col I and proliferation and survival of OBLs (Yang and Karsenty, 2004; Zhang et al., 2008). Recent findings also suggested that ATF4 is important for osteoblastic responses to PTH to increase bone formation (Yu et al., 2009).

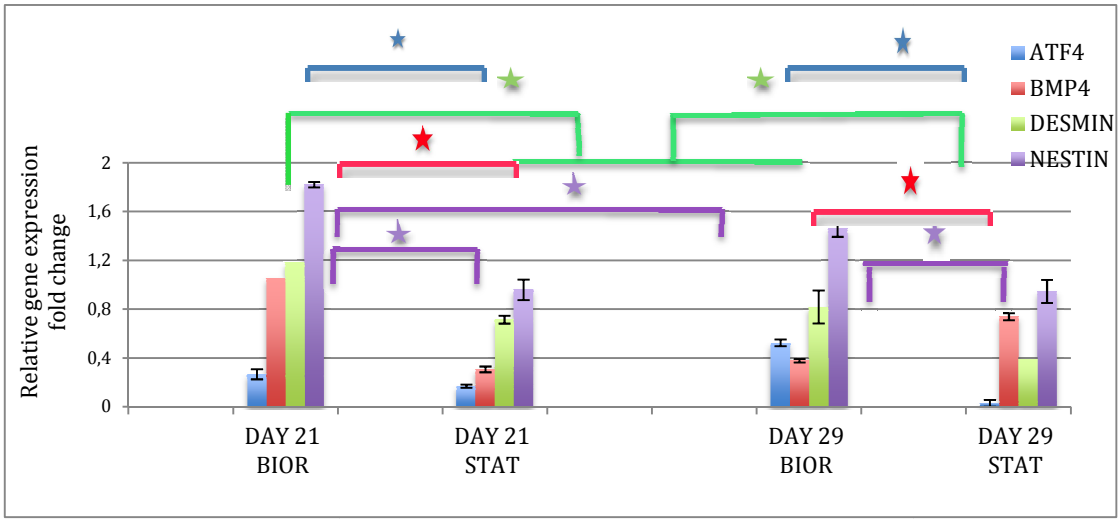


Figure 6.18: Gene expression of ATF4, BMP4, Desmin and Nestin. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★ ★ ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples in the expression of each of the four tested genes. Values are mean ± SD of N=2.

The results collected from the experiments indicated generally a very low level of ATF4 expression. Specifically, in the bioreactor, ATF4 expressed in a low level on day 21 and then marked a small increase by day 29. In static culture, there was very low expression on day 21, which almost disappeared by day 29. This outcome, indicating very low expression level of ATF4, reinforced the idea that there were not mature cells by the end of the experiment at both culture platforms.

Continuing the analysis of germ layer markers, the transcription factors Gata4 and Sox17, markers of endoderm and the transcription factor Hand1, marker of mesoderm, were tested (Figure 6.19).

Transcription factor Gata4 belongs to a family of zinc finger proteins involved in lineage determination. It is first expressed in yolk sac endoderm of the developing mouse and later in

cardiac tissue, gut epithelium and gonads (Soudais et al., 1995). Gata4 expression increases when ESCs differentiate into endoderm (Fujikura et al., 2002; Schuldiner et al., 2000). Gata4 expression was low day 21 and slightly increased by day 29 in the perfusion culture. In static culture, on day 21 there was relatively low level of expression, which was, however, 2 times higher compared to perfusion and decreased significantly by day 29.

Transcription factor Sox17 has been shown to facilitate and induce the differentiation program of mESCs towards primitive and definitive endoderm *in vitro* (Qu et al., 2008). It functions as an endoderm determinant factor (Niakan et al., 2010; Wang et al., 2011). Sox17 expression exhibited a mediumlevel on day 21 for both tested platforms. Its expression maintained on the same level in the bioreactor until day 29 while increased significantly in static culture.

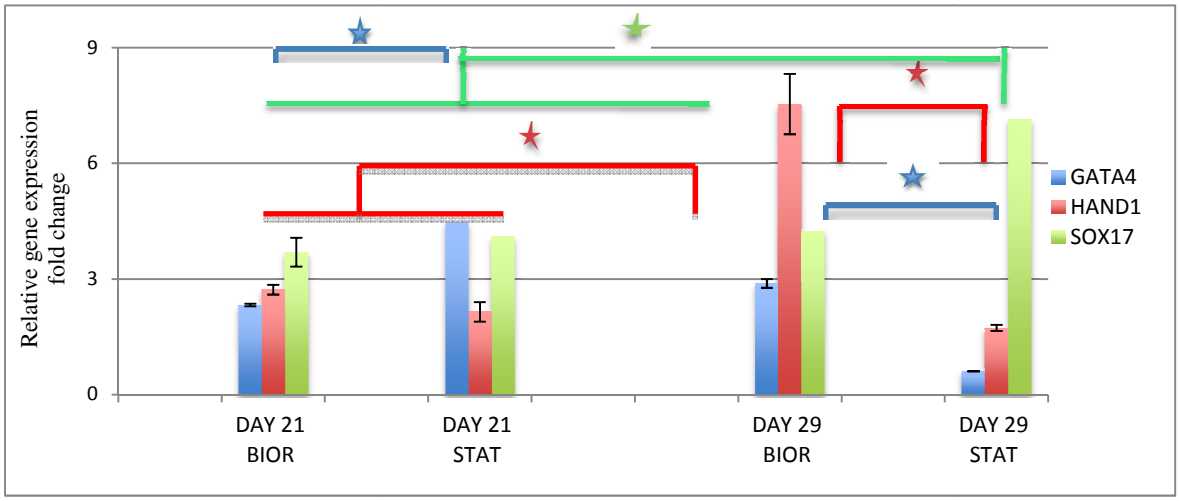


Figure 6.19: Gene expression of Gata4, Hand1 and Sox17. Comparison was performed between static and dynamic culture configuration and among day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★★★ indicate $p < 0.05$ and show that there is statistically significant difference between these samples in the expression of each of the three tested genes. Values are mean ± SD of N=2.

Finally, the transcription factor Hand 1 has been shown to have important role in the development of extraembryonic mesoderm and heart (Firulli et al., 1998). In the case of heart development, it has been shown to act as a regulator and maintain the correct balance between proliferation and differentiation (Risebro et al., 2006). It is used as a marker of mesoderm formation. Acquired results (Figure 6.19) indicated a low and similar expression level on day 21, at both platforms tested. However, by day 29, Hand1 expression exhibited a significant increase in perfusion culture while it remained on the same low level in static culture.

One important thing that could be observed from the graph in Figure 6.19 was that on day 29 in the bioreactor, there was statistically significant upregulation and high expression of the

mesoderm marker Hand1 while on day 29 in static configuration, there was significant expression of the endoderm marker Sox17. This finding indicated a better control of the environment in the bioreactor towards mesoderm formation and subsequent osteogenic differentiation whereas in static configuration, high expression of markers from other germ layers suggested uncontrolled and heterogeneous differentiation, which would probably result in low osteogenic differentiation efficiency.

Gene expression results for markers of the three germ layers suggested that the protocol used for osteogenic differentiation of mESCs employing b-GP, AA and Sim generated a cell population that retains in a very low level the expression of markers from all the three germ layers suggesting the more progenitor status of the cells and the possibility of having also other cell types before committing to fully mature osteogenic phenotype.

6.5 Discussion and conclusions

In this chapter, the new updated protocol with the optimised time for the supplementation of Sim in the culture media was tested and comparison was performed between 3D static and dynamic culture. Several analyses were performed to evaluate the efficiency of the protocol.

DNA quantification was used as an indirect method to calculate cell numbers (Figure 6.2). Perfusion bioreactor culture supported significantly higher cell numbers and achieved higher cell densities compared to static throughout the whole duration of the experiment due to the fact that the created conditions allowed the cells in that platform to proliferate for longer time. This outcome indicated that the favorable environment of perfusion culture with continuous supplementation of fresh media and removal of metabolites provided the cells with a better culture milieu to thrive. The perfusion bioreactor system enabled the support and culture of clinically relevant cell numbers needed for potential application in cell therapies. Static culture conditions possess the diffusion limitation of nutrients and oxygen transport (Buchwald, 2009; Lovett et al., 2009) leading to restricted proliferation of the cells and increased cell death observed usually with the creation of a necrotic core (Kasinskas et al., 2014; Mehta et al., 2012) in the middle of the hydrogel. Similar findings have been noticed by other groups, which supported that perfusion culture increased proliferation rate of the cells (Farack et al., 2011; Gomes et al., 2003; Sonnaert et al., 2014; Xie et al., 2006) and contributed to the achievement of higher cell numbers.

ALP activity was also tested to evaluate osteogenic differentiation (Figure 6.3). Following the previous findings of DNA quantification and as it was expected, the environment created by the perfusion bioreactor improved significantly the cell viability and at the same time increased ALP activity. In particular, literature suggests that Sim stimulates ALP activity (Liu et al., 2009; Park et al., 2012b).

Mineralisation was then evaluated using ARS and the obtained results indicated significantly higher calcium deposition in the bioreactor culture compared to static (Figure 6.5). Similar observations were made by other research groups, which noticed increased mineralisation after Sim application (Maeda et al., 2001; Pagkalos et al., 2010; Park et al., 2012b) and also with the use of perfusion bioreactor culture (Bancroft et al., 2002; Sikavitsas et al., 2003).

Further confirmation of the apatitic nature of the mineralized deposition was provided by ATR-FTIR analysis (Figure 6.6), which indicated the presence of HA, the main component of bone. The ATR-FTIR analysis suggested that the mineralised nodules were apatitic due to the characteristic PO_4 (at 1016 cm^{-1}) vibrations, which were typical of apatitic minerals. Two major additional peaks, hydroxyl and H_2O, centered at around 1650 and 3250 cm^{-1}, were observed in the FTIR spectra of the mineralised constructs. Moreover, the existence of the amide I band (between 1565 and 1500 cm^{-1}), which is related to the cellular origin of mineralised nodules, was observed. Finally, the carbonyl (CO_3^{-2}) minor peak provided an indication of the biological mineralisation of bone tissue (Aparicio et al., 2002).

Finally, extended gene expression analysis was performed in order to get more insight information regarding different processes involved in the osteogenic differentiation of mESCs. Several bone markers were first examined to confirm osteogenic differentiation and to observe the different time pattern followed in the perfusion culture compared to static. The different environment created by the two tested culture configurations resulted in different expression times of the various bone markers. One important thing to underline before the beginning of the gene expression analysis is the fact that in 3D static culture, diffusion was the only way for nutrient transport to the cells while in the perfusion bioreactor, convection supported the efficient transport of nutrients and oxygen inside the constructs. It is essential to refer to this difference between the two platforms, which suggests different metabolic stress that can potentially have an impact on the gene expression profile of the tested bone markers.

The expression of ALP was the first to be evaluated (Figure 6.8). The obtained results confirmed previous findings by other groups suggesting increased ALP expression during perfusion bioreactor culture (Barron et al., 2012; Campos et al., 2013; Gaspar et al., 2012; Gomes et al., 2003; Papantoniou et al., 2014).

It is well known and it has been previously shown that osteoblast differentiation is controlled by the sequential expression of several transcription factors (Franceschi et al., 2007).

Runx2 is considered one of the most important of these factors. It is a transcriptional activator essential for initial osteoblast differentiation from mesenchymal precursors and subsequent bone formation, development and matrix mineralisation (Ducy et al., 1997; Franceschi et al., 2003; Komori et al., 1997; Otto et al., 1997). Runx2 has been shown specifically to be necessary for the production and maintenance of progenitors (Long, 2012).

OSX is another transcription factor important for bone regulation and it has been shown to be located downstream of Runx2 (Nakashima et al., 2002). Runx2 is essential for the differentiation of mesenchymal cells into preosteoblasts. As a downstream gene of Runx2, Osx is required for the differentiation of preosteoblasts into mature osteoblasts (Zhang, 2010). Committed osteoprogenitors have been shown to express both Runx2 and OSX (Karner et al., 2009).

Both factors indicated a higher expression level in the bioreactor compared to static culture, with Runx2 indicating very low levels at both tested time points compared to OSX (Figure 6.9). The relatively low expression level of Runx2 has been noticed previously by other group, which observed that during culture of either primary osteoblasts or the MC3T3-E1 osteoblast cell line, there were no major changes observed in the level of Runx2 expression during *in vitro* differentiation (Franceschi et al., 2003).

There is one more possible reason for the significantly low Runx2 expression in comparison to OSX expression. It has been observed that PTH regulation of Runx2 transcription is predominantly mediated via protein kinase A (PKA) signaling, whereas the regulation of OSX might involve protein kinase C (PKC) activation as well, although it seems a pathway of minor importance compared with PKA signaling (Wang et al., 2006a). On the other side, there are several publications referring to the ability of statin to activate PKC (Lee et al., 2010; Sassano et al., 2012; Yang et al., 2010). This could indicate a possible reason for the significantly higher OSX expression level in comparison to Runx2.

Continuing with gene expression analysis, two important early stage markers of osteogenic differentiation were tested, COL 1 and BMP2 (Figure 6.10). COL 1 represents the predominant collagen in mineralised bone (Sodek and McKee, 2000) and together with ALP is considered an early marker of bone differentiation (Altmann et al., 2014; Orimo, 2010). BMP2 is known as the strongest osteoinductive factor. The expression of both genes was upregulated in the perfusion culture while it was downregulated in static culture during the tested time points suggesting an earlier transition of static culture to the more mature phenotype and a longer maintenance of a more progenitor pre-osteoblastic status of the cells in the perfusion culture.

Next step was the evaluation of the expression of two significant late stage markers of osteogenic differentiation, OCN and BSP (Figure 6.11). OCN is one of the few bone-specific proteins whereas BSP is the first late stage marker to be expressed indicating mature stage. In the performed experiments, both genes indicated a significantly higher expression level in the bioreactor culture compared to static at both tested time points. This finding suggested that the dynamic environment created in the bioreactor stimulated and supported better osteogenic differentiation.

Similar to that, another group also noticed that OCN was significantly increased in rotating bioreactors as compared to static controls, indicating that the phenotypic expression of osteoblasts under rotating conditions was enhanced. The higher expression level at an earlier time point could also support the idea that dynamic flow conditions stimulate osteoblastic cell function in rotating bioreactors (Yu et al., 2004).

The expression of two major non-collagenous proteins of bone matrix, OPN (Figure 6.12) and ON (Figure 6.13) was then examined. OPN expression profile suggested an earlier expression and maybe earlier maturation in static configuration. ON has a significant role in regulating bone-matrix mineralisation. Higher expression was indicated in static configuration, something that was observed previously by another group, which noticed high expression levels of ON in static microchips, while significant lower mRNA expression levels were noticed in bioreactors (Altmann et al., 2014).

To summarize, the majority of the important bone marker genes indicated higher expression level in the perfusion bioreactor culture compared to static configuration suggesting better support of osteogenic differentiation in the bioreactor platform.

Continuing with gene expression analysis and in order to elucidate what type of ossification took place, a couple of cartilage genes, genes of hypertrophic chondrocytes and matrix protein genes were evaluated. The expression of the transcription factor SOX9 (Figure 6.14), the cartilage matrix protein Aggrecan, which is expressed in mesenchymal condensations (de Crombrugghe et al., 2001) and the hyperthrophic chondrocyte marker COL 10 (Figure 6.15) was tested.

SOX9 is considered an essential transcriptional regulator of chondrocyte cell fate (Bi et al., 1999) and it is needed for formation of mesenchymal condensations (de Crombrugghe et al., 2001). SOX9 marks the mesenchymal progenitors that give rise to all osteoblasts. It is not expressed by mature osteoblasts (Long, 2012). SOX9 is expressed in all chondroprogenitor cells and all chondrocytes but its expression is abolished in hypertrophic chondrocytes and there is no expression of SOX9 in osteoblasts (de Crombrugghe et al., 2001). Moreover, it has been shown that SOX9 enhanced aggrecan promoter activity (Sekiya et al., 2000) and activated its gene

expression (Leung et al., 2011). Based on the above information, the two genes indicated similar profile of expression and they were highly expressed in the perfusion bioreactor compared to static configuration at both tested time points. The acquired results suggested that cells in the bioreactor were in a progenitor stage and had the possibility of becoming mature osteoblasts or chondrocytes. At the tested time points, there were probably no mature osteoblasts in the system. On the other side, in static configuration, SOX9 expression was very low suggesting the possible existence of a more mature phenotype.

The COL 10 gene is specifically expressed in hypertrophic chondrocytes and marks their transition stage to osteoblasts, favouring endochondral ossification (Linsenmayer et al., 1991). Previous studies suggested that the mechanism to facilitate endochondral ossification is performed through regulation of matrix mineralisation and compartmentalization of matrix components (Shen, 2005). Similar to the previous tested genes, its expression was significantly higher in the perfusion bioreactor at both time points compared to static.

The above results suggested the existence of hypertrophic chondrocytes and the proper progression of osteogenic differentiation through endochondral ossification in the perfusion bioreactor, as indicated by the gene expression levels of the tested markers. The low expression level in static configuration suggested that this platform didn't follow a proper osteogenic differentiation pathway.

Another two transcription factors, which affect osteoblast differentiation, are Dlx5 and Msx2 (Figure 6.16). Dlx5 has been proposed to up regulate Runx2 expression (Holleville et al., 2007) while Msx2 has the opposite effect (Kirkham et al., 2012; Lee et al., 2005). Following previous observations, the expression of both factors was higher in the bioreactor.

The expression of PTHR1 (Figure 6.17), another early marker of osteoblast differentiation (Kondo et al., 1997), was also evaluated. It is considered a "global" marker expressed by all osteoblasts *in vivo* (Aubin, 1998). It was highly expressed in the bioreactor, similar to the previously tested bone markers suggesting once more the more appropriate osteogenic differentiation performed within perfusion platform compared to static, as indicated by the gene expression level of important markers.

Finally, wishing to get more insight information regarding the differentiation process, markers from the three germ layers were tested to evaluate the efficiency of the differentiation. Starting with ectoderm, the expression of the transcription factor Pax6 (Figure 6.17), which has been shown to be a cell fate determinant of neuroectoderm (Zhang et al., 2010a) and the Nestin protein (Figure 6.18) was evaluated. Both markers were highly expressed in the bioreactor compared to static configuration.

Next step involved the examination of markers from the endoderm and in particular, the transcription factors Gata4 and Sox17 (Figure 6.19). It has been reported that Gata4 RNA is one of the transcripts induced during differentiation of embryoid bodies (Arceci et al., 1993). Both markers indicated a preference and were expressed in static culture but high levels appeared at different time points for each gene.

Continuing with gene analysis, the expression of three mesoderm markers, BMP4 and Desmin protein (Figure 6.18) and the transcription factor Hand 1 (Figure 6.19), was evaluated. All the three markers were highly expressed in perfusion culture suggesting an induction of mesoderm formation in perfusion bioreactor culture.

To summarize, results suggested there was a preference for mesoderm expression in perfusion culture and for endoderm in static culture.

This interesting observation suggested the more progenitor status of the cells in the bioreactor proved by the expression of markers of other germ layers in comparison to static which has been in a more advance stage expressing only osteoblastic markers.

The low expression level in static culture reinforced the previous results indicating probably a more mature phenotype on static configuration.

The obtained results suggested the better support of osteogenic differentiation in the perfusion bioreactor culture as proved by the enhanced expression of mesoderm markers and increased expression level of various important markers of osteoblastic differentiation. Results need to be repeated for further validation, as similar analysis hasn't been performed previously from other groups to make comparisons.

Several conclusions could be drawn from the gene expression analysis. Based on the expression of the various bone markers tested, it seemed that cells in static culture started to differentiate and to express the respective markers earlier compared to cells cultured within the RWV perfusion bioreactor.

To go one-step further, evaluation of a number of cartilage markers was performed and confirmed the fact that endochondral ossification pathway was followed through the Sim differentiation protocol. Together with the expression of bone markers, these results reinforced the idea that cells in static culture were in a more mature status compared to cells cultured within the bioreactor.

The obtained results suggested the existence of progenitor cells, pre-osteobleasts, which were not fully mature and could still proliferate and differentiate into more differentiated phenotype. These gene expression results indicated that Sim in combination with the perfusion culture could support osteogenic differentiation of the mESCs and there were strong indications that probably

this happened through endochondral ossification pathway. Moreover, obtained cells were not fully mature and had a progenitor phenotype; in this way, they could be used to activate endogenous mechanism of the body to regenerate the defect where they will be implanted.
To our knowledge, the important contribution of this chapter lies on the fact that this is the first time that an extended gene expression profile following osteogenic differentiation, ossification pathway and germ layer formation, was followed and major insight information can be obtained regarding the followed process. The majority of studies focus on the gene expression of the final desired product, ignoring the in between status of the cells.

To summarize, the conclusions that could be drawn from this chapter are the following ones:

- Sim enhanced cell proliferation and supported better and increased osteogenic differentiation and subsequent mineralisation in the 3D dynamic culture environment generated by the perfusion bioreactor.
- Gene expression indicated high level of bone markers in the dynamic environment of the bioreactor, thus confirming improved bone formation.
- Gene expression analysis suggested that endrochondral ossification was followed through the existence of an intermediate cartilage template.
- Germ layer analysis indicated high expression of mesoderm markers. There was, however, low expression of markers from the other two germ layers

7th CHAPTER

7. 3D BIOPROCESS OF MESCS: COMPARISON OF OSTEOINDUCTIVE MOLECULES SIM VS DEX AND CULTURE MODES FED-BATCH VS PERFUSION

7.1 Introduction

Chemical osteoinduction of mESCs is widely performed with the use of b-GP, AA and the steroid Dex. Dex has been shown to promote osteogenic differentiation of both embryonic (Buttery et al., 2001; Sottile et al., 2003; zur Nieden et al., 2003) and adult stem cells (Rogers et al., 1995).

In this PhD project, a new small molecule named Sim, that is a well-known and widely used drug to lower cholesterol, was tested as an osteoinductive factor. Sim has been shown to have anabolic effects on bone formation (Phillips et al., 2001) and also to upregulate BMP2 expression (Ohnaka et al., 2001; Sugiyama et al., 2000).

In the previous chapter, 3D osteogenic differentiation was tested in static and perfusion culture using Sim supplemented in the media at specific time point defined after a short optimisation. Results were encouraging showing upregulation of mineralisation, ALP activity and expression of several bone markers. In this chapter, the intention was to compare this newly developed protocol containing Sim with the standard protocol currently used with the employment of Dex. The aim was to evaluate the differences of the two protocols and which one supported higher cell numbers of better osteogenic quality. It was also important to identify the similarities and the differences between the two molecules in their mode of action and the different profile exhibited through gene expression analysis.

7.2 Aim and Objectives

The aim of this chapter was to compare the two molecules used for osteogenic differentiation of mESCs, Dex versus Sim.

In particular:

1. To evaluate similarities and differences between the two tested chemicals for osteogenic differentiation
2. To assess their efficiency and analyse the quality and the number of the obtained constructs
3. To compare 3D bioprocess between static and dynamic culture configurations among the two molecules

7.3 Methodology

Comparison of the two protocols employed for osteogenic differentiation of mESCs in 3D static and perfusion culture was performed using the previously mentioned 29 days differentiation protocols starting with HepG2-CM for the first three days, followed by osteogenic media supplemented with b-GP and ascorbate-2-phosphate and finally on day 14, supplementation with 0.1nM Sim for the one protocol and on day 21, supplementation with 1μM Dex for the other protocol (Figure 7.1).

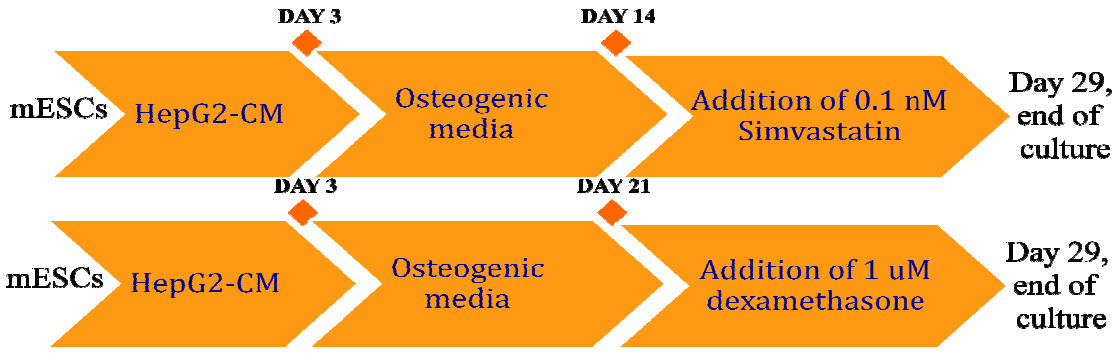


Figure 7.1: The two tested protocols employed and compared in this chapter

A number of different analyses were performed in order to evaluate the final product. DNA quantification was used to calculate cell numbers and measurement of ALP activity to evaluate osteogenic differentiation. ARS was utilized to indicate calcium deposition and mineralisation. Finally, gene expression analysis of important bone markers was tested to compare the two protocols, observe the possible differences in their profiles and identify the pros and cons of each of them.

Osteogenic differentiation, in general terms, is following three characteristic steps, starting with cell expansion, followed by matrix synthesis to support osteogenic diferentiation and subsequent mineralisation in the end. Based on this principle, both tested protocols for osteogenic differentiation in these experiments lasted for 29 days starting with 3 days incubation in HepG2-CM to expand cell numbers and enhance mesoderm formation. In the next step, cells were incubated in osteogenic media containing b-GP and ascorbate-2 phosphate to initiate matrix formation and osteogenic differentiation. For the last step, the osteoinductive agents Dex in one protocol and Sim in the other were provided to support and increase calcium deposition, at different time point in each case.

The above information indicated that both protocols followed the same principle idea for osteogenic differentiation and the only difference was the osteoinductive agent provided and the chosen time point. This suggests that results obtained after the application of the two tested protocols are comparable. Similar results comparing osteogenic differentiation protocols with the employment of different osteoinductive factors at distinct time points for each protocol, were performed previously (Pagkalos et al., 2010) and proper comparison was also carried out.

7.4 Results

DNA quantification was used as an indirect method to calculate cell numbers at each time point throughout the 29 days differentiation protocol (Figure 7.2). In the graph in Figure 7.2, comparison of two protocols for osteogenic differentiation, one that used Sim versus one that employed Dex was presented. Comparison between two culture platform configurations, static and perfusion, was also performed. It was easy to notice that perfusion culture, with both protocols, supported higher cell numbers throughout the whole experiment compared to static culture. To be more specific, all four different groups started with 20.000 cells per bead. In perfusion culture with Sim protocol, there was increased proliferation and cells achieved about 400.000 cells per bead by day 14 of culture and maintained the same number until the end of the experiment, on day 29. Perfusion culture with Dex supported about 200.000 cells per bead by day 7 of culture and maintained this density until day 21. There was an observed increase in cell numbers reaching about 250.000 cells per bead by day 29. In static culture with the Sim protocol, cells achieved about 200.000 cells per bead by day 7, then there was a decrease by day 14 and on day 21, they recovered back to the density of day 7, which they maintained until the

end of the culture. In static culture with the Dex protocol, cells achieved 100.000 cells per bead by day 7 and maintained this density until the end of the experiment.

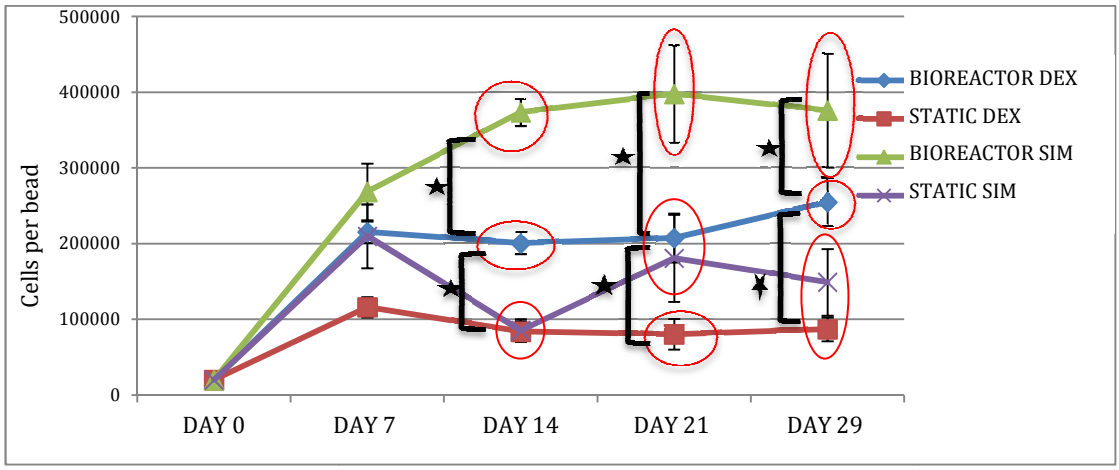


Figure 7.2: DNA quantification throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol and also among dynamic and static culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the groups with the red circle.

One significant thing to notice from the above results was the fact that Sim protocol after time optimisation supported higher cell numbers in comparison to Dex for both culture configurations. The reason for the above outcome was also the fact that experiments with Dex were performed during the early stages of the PhD project when I was still learning the proper operation of the perfusion bioreactor while Sim experiments were performed at a later time when perfusion operation was relatively optimised. This event could explain the different cell densities observed the first 14 days of culture where conditions were similar for both tested protocols. The important outcome from this analysis was the fact that perfusion bioreactor could support the generation of clinically relevant high cell numbers needed for potential application in cell therapies.

ALP activity is an important marker of osteogenic differentiation. From the obtained raw data of absorbance values (Figure 7.3), it could be observed that perfusion culture maintained high ALP activity throughout the whole 29 days duration of the experiment in comparison to static configuration and this finding was valid for both tested protocols. In static culture configuration, both protocols followed the same profile during time. In particular, ALP activity reached maximum level by day 7 and then gradually decreased until day 21. By the end of the

experiment, on day 29, ALP activity marked a small increase and achieved again the higher level of day 14.

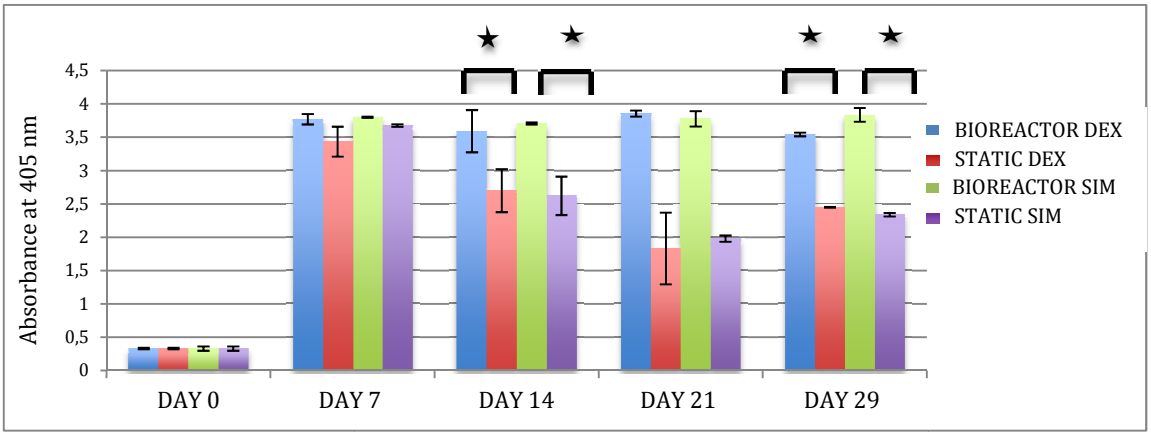


Figure 7.3: ALP activity throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested platforms within the same chemical group.

On the graph in Figure 7.4, ALP was normalised with cell numbers obtained from DNA quantification and results expressed as μM/cells/min (Figure 7.4).

In the presented graph, the influence of cell numbers on the ALP profile after the normalization was obvious. Static configuration exhibited higher values compared to perfusion in most of the time points during the experimental procedure due to the higher cell numbers supported by perfusion culture compared to static.

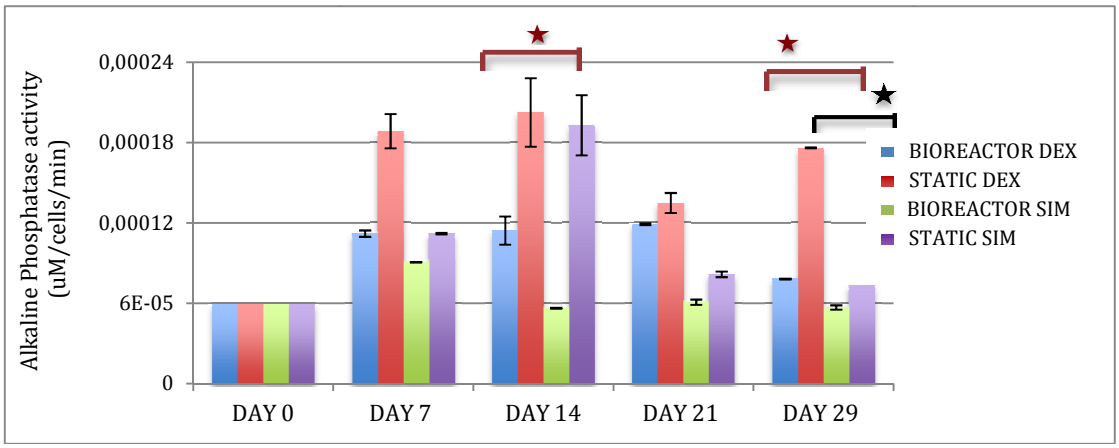


Figure 7.4: ALP activity normalized with cell numbers obtained from DNA quantification and expressed as μM/cells/min throughout the 29 days of the experiment. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between chemical within static group, asterisk★ indicates $p < 0.05$ and shows that there is statistically significant difference between chemical within bioreactor group

Both tested protocols, Dex and Sim, indicated similar ALP expression profile during time and according to the platform used. The ALP activity was accordingly expressed at similar levels. There was no pronounced difference between the mechanism of action of each compound that would influence differently ALP activity. Results supported the idea that perfusion platform can support the generation of precursor cells as indicated by the maintenance of high ALP activity throughout the 29 days of the experiment while in static configuration, the ALP profile indicated a progression towards more mature phenotype.

ARS was used to evaluate calcium deposition and mineralisation (Figure 7.5). From the acquired data, it can be observed that perfusion culture, especially by day 29, supported higher calcium deposition and mineralisation in comparison to static culture for both tested chemicals.

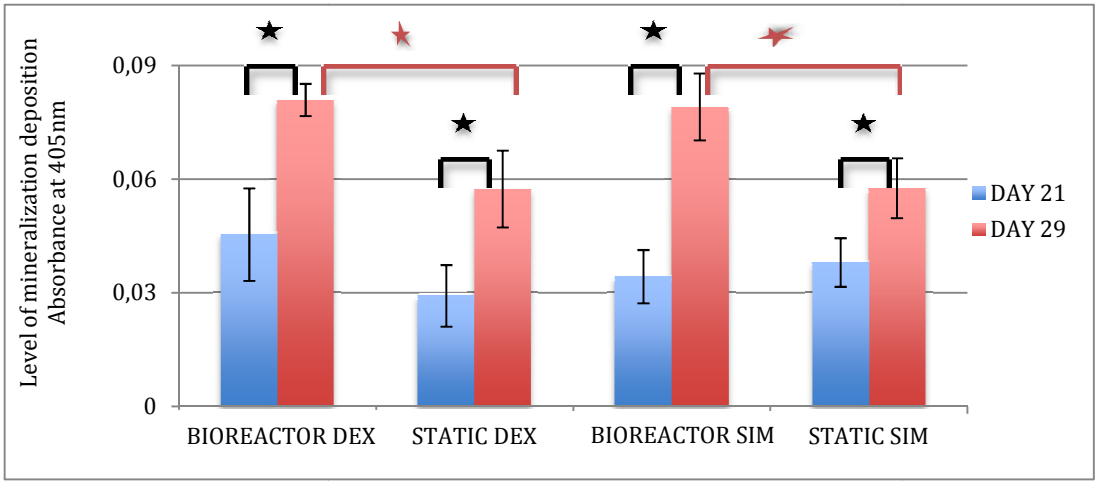


Figure 7.5: ARS was used to evaluate calcium deposition. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration and for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between day 21 and day 29 within the same platform. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between the two tested platforms on day 29 and within the same chemical group.

The above results suggested that calcium deposition was better supported in the perfusion bioreactor indicated by the higher staining quantified compared to static configuration. Between the two tested compounds, there were similar levels of calcium deposition at each of the platforms suggesting that mineralisation could be induced from either Sim or Dex without any pronounced differences.

7.4.1 GENE EXPRESSION

A number of important bone markers was tested and compared between the two protocols in order to evaluate and confirm osteogenic differentiation.

The earliest and most important osteogenic marker to be expressed and evaluated was ALP. From the following graph (Figure 7.6), it could be observed that ALP expression was significantly higher in the perfusion bioreactor culture for both tested protocols in comparison to static culture, where there was very low expression level. To be more specific, there is high ALP expression by day 21 in the Dex protocol, which increased more by day 29. Static culture using the same chemical indicated very low and similar expression level at both time points. On the other hand, in the bioreactor configuration using Sim, ALP exhibited very low expression level by day 21, which slightly increased by day 29. It was however significantly lower compared to the use of Dex in the same culture configuration. In static culture with the use of Sim, there was very low ALP expression by day 21, which significantly decreased to very low level by day 29. Following similar findings from other groups, ALP expression in the performed experiments was higher in the perfusion bioreactor culture for both protocols and at both time points measured compared to static culture (Bernhardt et al., 2011; Gomes et al., 2006). One interesting finding that could be noticed from the following graph was the fact that treatment with Dex enhanced significantly ALP expression while use of Sim didn't have such a strong influence on its expression.

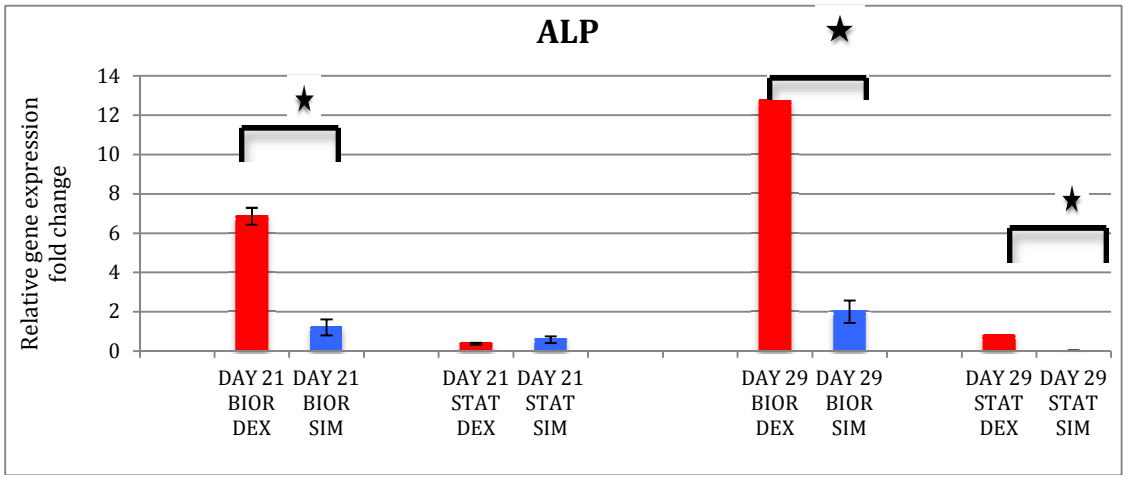


Figure 7.6: Gene expression of ALP. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference within the same sample at the same time point cultured with different chemical. Values are mean ± SD of N=3.

Gene expression analysis continued with the examination of two early bone markers. Runx2 and OSX are considered the master regulators of osteoblast differentiation. In the acquired results (Figure 7.7), it could be noticed that in general Runx2 expression indicated low expression in

both platforms and at both time points tested compared to OSX, which exhibited a relatively high level expression profile. To be more specific, with the employment of Dex in both culture configurations, Runx2 activity was very low on day 21 and marked a small increase by day 29. With the use of Sim, Runx2 indicated low expression by day 21 which slightly increased by day 29 in the bioreactor while in static culture, there was very low expression, which remained the same until the end of the experiment.

The situation with OSX expression was different with expression levels being much higher compared to the levels of Runx2. In the bioreactor, both protocols exhibited a significant increase from day 21 to day 29, with Dex being lower on day 21 and then getting higher that Sim on day 29. In static culture, samples treated with Dex increased their expression from day 21 to day 29 whereas samples treated with Sim had really high expression on day 21 which decreased significantly by day 29.

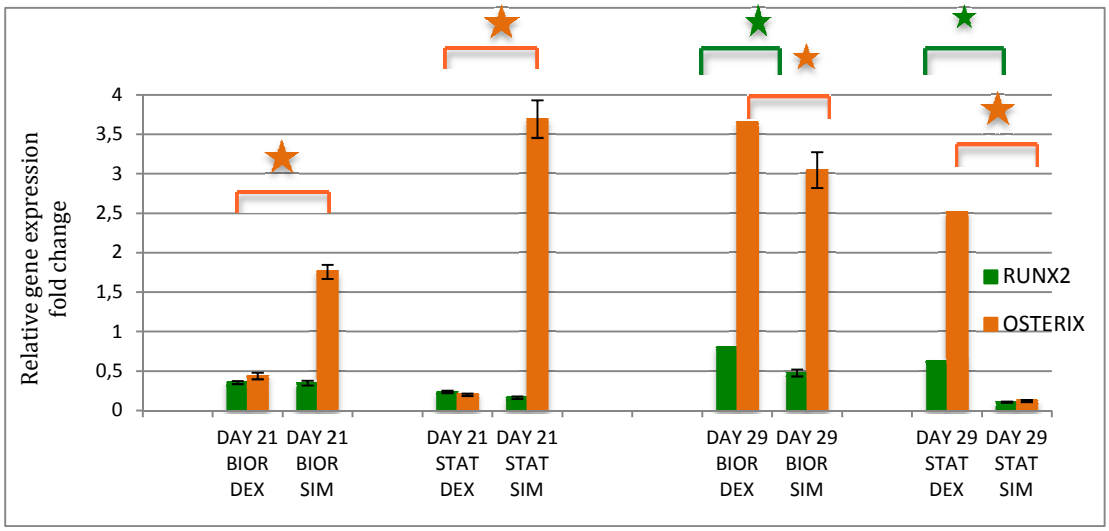


Figure 7.7: Gene expression of the transcription factors Runx2 and OSX. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in Runx2 expression between the tested chemical at the same time point and within the same platform whereas asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in OSX expression between all the tested samples. Values are mean ± SD of N=3.

COL 1 is the most abundant protein of bone matrix. It is expressed early and constitutes the majority of its organic component (Figure 7.8). In the protocol where Dex was employed, COL 1 expression was relatively high and maintained on the same level until day 29 in the bioreactor culture. In the protocol where Sim was employed in the same platform configuration, COL 1

expression was low on day 21 and marked a 5-fold increase by day 29. On the other hand, in static culture, in the Dex protocol, COL 1 had very high expression level on day 21 which increased significantly by day 29 while in the same culture configuration with Sim treatment, COL 1 indicated low expression level, which decreased by day 29. In general terms, COL 1 exhibited a higher expression level in static culture with the use of Dex protocol.

BMP2 is a characteristic marker indicating osteogenic differentiation and bone formation. In the obtained results (Figure 7.8), BMP2 expression was in general lower in Sim protocol in comparison to Dex. In particular, BMP2 was highly expressed on day 21 in perfusion culture using the Dex protocol and marked a small decrease by day 29. In static configuration using the same chemical, BMP2 expression was higher than perfusion on day 21 and then, by day 29, decreased significantly reaching a very low level of expression. In the protocol with Sim, BMP2 expression was very low on day 21 and slightly increased by day 29 in the perfusion configuration while in static culture, the expression level was low on day 21 but higher compared to the bioreactor and then significantly decreased to very low expression level by day 29.

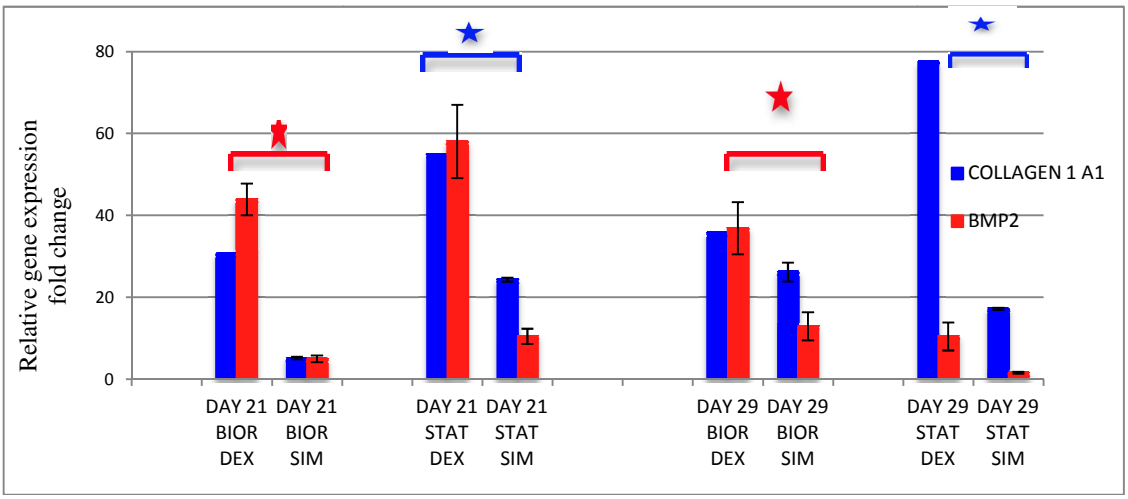


Figure 7.8: Gene expression of COL 1 and BMP2. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisks ★ ★ indicate $p < 0.05$ and show that there is statistically significant difference in COL 1 and BMP2 expression between all the tested samples. Values are mean ± SD of N=3.

COL 1 is the most abundant protein of bone matrix and a characteristic marker indicating bone formation. In the obtained results, COL 1 expression was significantly reduced in the RWV perfusion culture platform in comparison to the static configuration. Similar to the above

findings, Plunkett and his collegues noticed that all groups cultured in the bioreactor showed significant decreases in COL 1 expression compared to the static group (Plunkett et al., 2010). Bjerre and his collegues tested different sizes of a particular scaffold and observed that collagen expression in both cases showed a statistically significant preference for static culture at all time points (Bjerre et al., 2011).

Several groups attributed the collagen reduction to the microgravity condition simulated in a RWV bioreactor after performing several experiments. In particular, rotating wall bioreactor has been shown to decrease COL 1 expression (Wang et al., 2009). To be more specific, microgravity has been indicated to reduce COL 1 expression by disrupting integrin signaling (Meyers et al., 2004). Analysis during spaceflight or experiments in simulated microgravity indicated changes in bone metabolism and reduction in the gene expression of COL 1 (Carmeliet and Bouillon, 1999; Carmeliet et al., 1997; Nichols et al., 2006; Uddin and Qin, 2013).

As far as BMP2 results were concerned, the expression of BMP2 was significantly induced at early time points and after Dex treatment. In particular, it could be noticed that the expression levels were high on day 21 in the Dex group and reduced significantly by day 29. In the Sim group, there was low expression on day 21 which marked a small increase by day 29. Sim has been previously shown to induce BMP2 expression by other research groups (Baek et al., 2005; Chen et al., 2010).

The interesting finding was the high BMP2 activity on day 21 in the Dex group where actually Dex was provided in the culture media on day 21, which means there was another factor inducing BMP2 expression at that point. Looking in the literature, it has been observed that extracellular phosphate (Pi), provided in the osteogenic media through b-GP, increases BMP2 expression (Langenbach and Handschel, 2013; Tada et al., 2011). By day 29, when Dex has been supplied in the culture media, BMP2 expression reduced, following other people's observations (Chang et al., 2006). BMP2 expression was also maintained in relatively high level for longer in the perfusion culture while decreased by the end of the experiment in static, suggesting better support of osteogenic differentiation in the perfusion configuration.

Next markers to be tested were two bone matrix proteins, OCN and BSP considered as late stage markers of osteoblast differentiation (Figure 7.9).

Bglap is the gene encoding for the protein OCN, one of the few bone specific proteins characteristic of mature bone. In the performed experiments, Bglap was highly expressed by day 21 in the perfusion bioreactor using the Sim protocol and its expression was induced more by day 29. In static culture with the same chemical, Bglap indicated a similar expression pattern with increased activity from day 21 to day 29. The expression level, though, was on average 4

times lower in comparison to the bioreactor. On the contrary, in the Dex protocol for both culture configurations, Bglap expression was significantly low on day 21 and decreased more by day 29, with perfusion exhibiting slightly higher levels compared to static culture.

BSP is another late stage marker of bone formation and an important matrix protein. Results obtained from the experiments indicated generally a low expression level of BSP for both platforms and both chemicals tested. In particular, in the bioreactor configuration using the Sim protocol, BSP expression increased from day 21 to day 29 while in static culture using the same protocol, there was very low expression on day 21, which remained the same until day 29. In the Dex protocol, BSP expression was significantly low in the bioreactor until day 29 whereas in static culture, there was very low expression level.

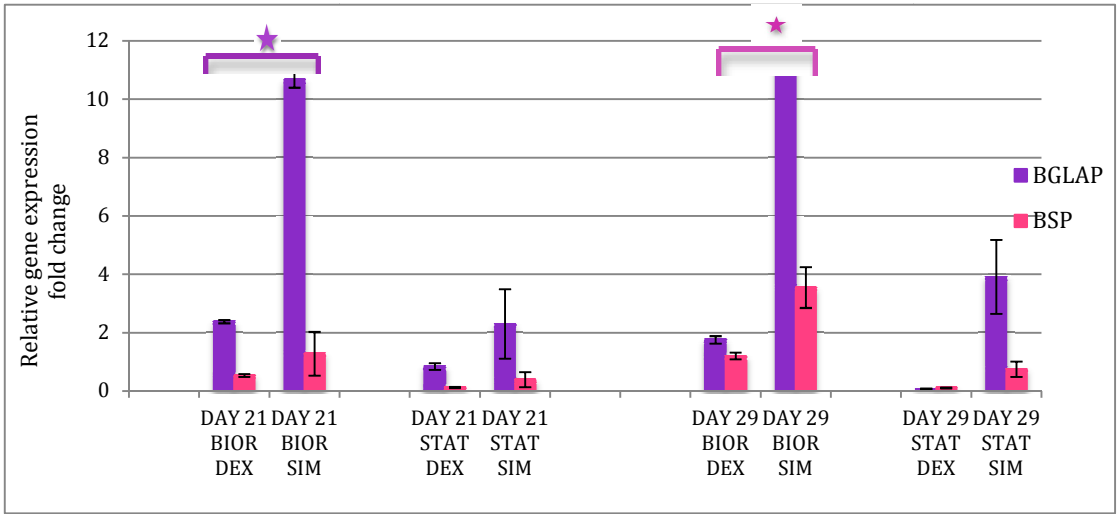


Figure 7.9: Gene expression of Bglap and BSP. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in BSP expression between chemical at the same time point and within the same platform while asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in Bglap expression between all the tested samples. Values are mean ± SD of N=3.

The above results indicated a higher level of expression for both tested late stage markers in bioreactor platform in comparison to static suggesting a better osteogenic differentiation process followed in that configuration. Moreover, Sim treatment significantly induced the expression of late stage markers, in particular of OCN in comparison to the low expression observed after Dex treatment. This outcome suggested an induction of late stage markers after Sim treatment and could also explain the low level of early stage markers.

OPN is a non-collagenous protein of bone matrix, which is characterised as an inhibitor of mineralisation. It is usually expressed early during the proliferative phase, it is then downregulated and it is again upregulated during mineralisation (Neve et al., 2011). In the graph on Figure 7.10, OPN expression was low and on the same level on day 21 with the use of Dex at both culture configurations. Then, on day 29, its expression marked a 4.5 fold increase in the bioreactor and an almost 3 fold increase in static culture. In the protocol where Sim was used, the expression profile was different and in lower levels of expression. To be more specific, OPN expression was low on day 21 in the bioreactor culture and increased by day 29. In static configuration, it was higher than perfusion on day 21 and decreased significantly by day 29.

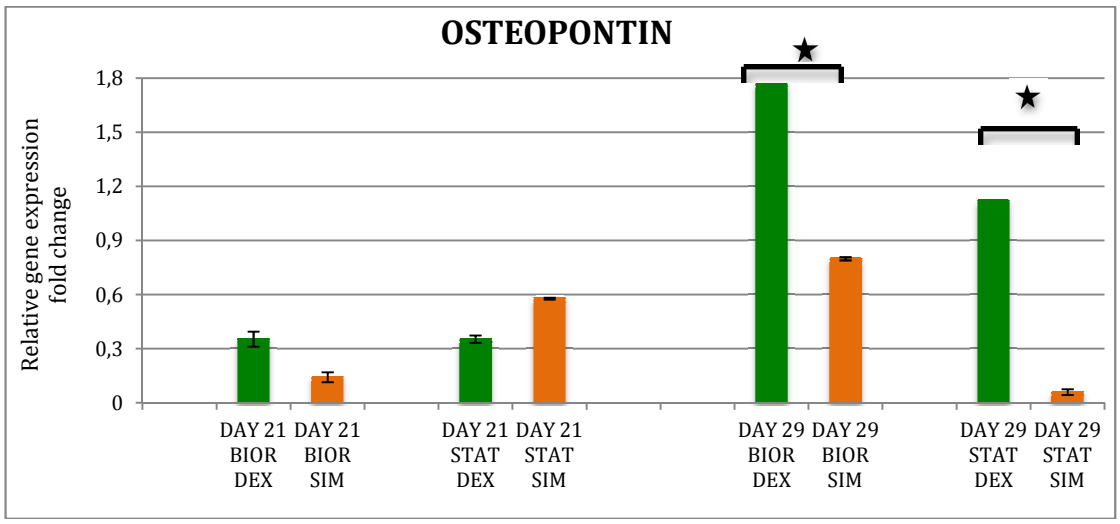


Figure 7.10: Gene expression of OPN. Comparison was performed between Sim and Dex protocol and among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference between chemical at the same time point and within the same platform for all tested samples. Values are mean ± SD of N=3.

The above results showed higher expression level of OPN on day 29, as it was expected during mineralisation and in the protocol employing Dex.

Another important bone matrix protein tested was ON (Figure 7.11). The interesting observation was that ON expression in both culture configurations and with both chemicals used for differentiation was in relatively high levels of expression in comparison to all the other tested genes.

ON exhibited in general very high expression after Dex treatment in comparison to Sim treatment. To be more specific, ON expression was high on day 21 in the Dex group, higher in the bioreactor compared to static. By day 29, the expression was significantly reduced to the same low level for both culture platforms. In the Sim group, ON expression started very low on day 21, lower in the bioreactor compared to static and then by day 29, decreased to the same very low level for both platforms tested.

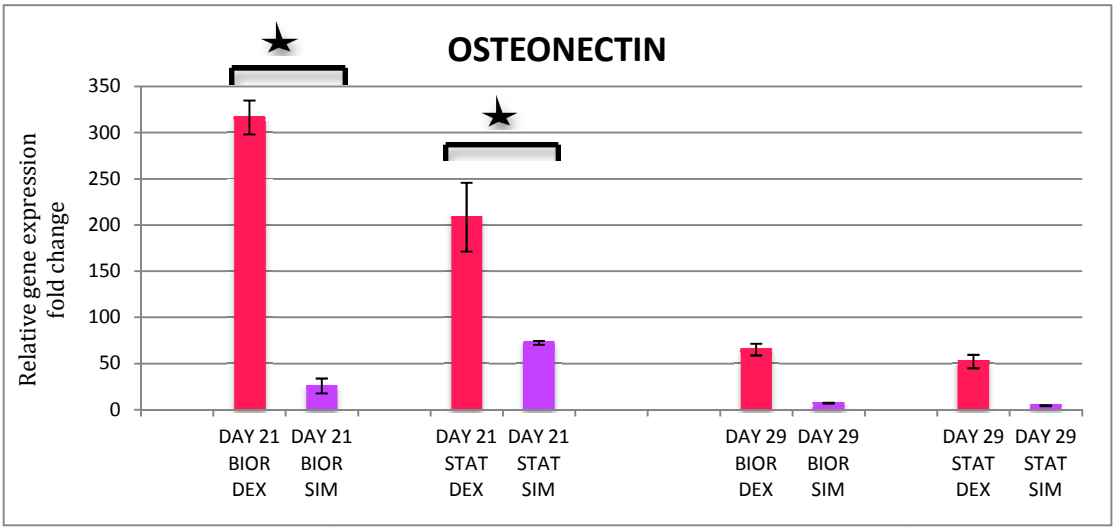


Figure 7.11: Gene expression of ON. Comparison was performed between Sim and Dex protocol and also among static and dynamic culture configuration for day 21 and day 29. Two-way ANOVA was used for statistical analysis. Asterisk ★ indicates $p < 0.05$ and shows that there is statistically significant difference in ON expression between all the tested samples. Values are mean ± SD of N=3.

The ON results indicated that cells cultured with Dex were in a more mature level regarding matrix synthesis in comparison to cells cultured with Sim. The gene expression indicated very high expression level on day 21 for both culture platforms using the Dex protocol while the expression level for the Sim group was significantly low at both time points and platforms tested. This result reinforced the idea that cells cultured with Dex matured faster compared to the cells cultured with Sim.

Finally, in order to get some information on metabolic activity, culture media samples were obtained from static cultures on day 29, the last day of the experiment and were analyzed with Bioprofiler. Bioprofiler provides information on various important molecules significantly affecting cell metabolism, especially during long-term culture. As part of this project, three crucial molecules, glucose, glutamine and lactate were analyzed and results are presented in Table 7.1. Samples included and compared the two tested chemicals, Sim and Dex. As a control,

glucose and glutamine levels from the employed culture media were analyzed with Biofrofiler and confirmed the expected levels. A-MEM media was used as a basal media with initial glucose level 5.55 mmol/L and glutamine level 2 mmol/L.

Table 7.1: Metabolic analysis was performed for glucose, glutamine and lactate using Bioprofiler. Comparison was performed between Sim and Dex protocol within 3D static configuration, on day 29.

	GLUCOSE	GLUTAMINE	LACTATE
A-MEM MEDIA	5.55 mmol/L	2 mmol/L	-
STAT DAY 29 SIM	5.6 mmol/L	0.39 mmol/L	4.2 mmol/L
STAT DAY 29 DEX	1.8 mmol/L	0.22 mmol/L	11 mmol/L

The acquired values indicated that samples obtained from Sim treated cultures maintained high glucose, in the same level as the initial a-MEM media whereas samples from Dex treated cultures indicated much lower glucose level by day 29. Glutamine level was reduced for both tested chemicals, with Sim sample maintaining a higher level in comparison to Dex. Finally, following the glucose consumption rate, lactate production was significantly higher in the Dex treated cultures compared to the Sim ones.

Glucose and lactate concentrations are related with metabolic stress affecting cell viability and gene expression. The above results suggested that Sim treatment at the appropriate time during culture maintained the system in low metabolic rate with high glucose level and low lactate and could be better for cell viability compared to Dex culture. However, Bioprofiler analysis would need to be repeated to further confirm these preliminary indications and probably be also combined with a more developed metabolic method in order to get more insight information regarding the metabolism and how it affects osteogenic differentiation and cell death.

7.5 Discussion and conclusions

In this final chapter, the aim was to compare the new protocol for osteogenic with the use of Sim to the old and widely used protocol with the use of Dex in 3D static and perfusion culture configuration. The aim was to identify similarities and differences regarding cell growth capacity, osteogenic differentiation, mineralisation and gene expression profile.

DNA quantification was performed to evaluate cell numbers (Figure 7.2). The obtained results indicated that perfusion culture enhanced cell proliferation and supported significantly higher cell numbers compared to static culture. This outcome was valid for both tested protocols and confirmed results from other groups which supported that the environment created by the perfusion culture, with continuous supplementation of fresh media and removal of metabolites, was favorable for cell growth and could support high cell numbers (Grayson et al., 2008; Yeo et al., 2013) with high viability. Moreover, it could be observed that the Sim-containing protocol allowed higher cell proliferation resulting in significantly higher cell numbers by the end of the experiment compared to the Dex-containing protocol, for both tested platforms. This finding follows previous observations supporting the idea that Dex is such a potent osteoinductive agent that when supplied does not permit the expansion of the cells, which start differentiating before completing proliferation (Bielby et al., 2004)

ALP activity (Figure 7.3) and ARS (Figure 7.5) were tested to evaluate osteogenic differentiation and mineralisation. Perfusion bioreactor increased and maintained high ALP activity throughout the whole experiment whereas in static configuration, ALP activity indicated the expected profile with initial increase, subsequent decrease and finally, increase again to support mineralization. ARS showed augmented calcium deposition in the bioreactor compared to static culture. Obtained results were the same for both tested chemicals. The above findings suggested that the combination of one of these osteoinductive factors together with the perfusion culture configuration possessed the superior capacity to promote osteogenesis and mineralisation of mESCs as proved by the increased ALP and ARS levels.

Comparison of the gene expression level of important bone markers between the two tested protocols was then performed. Results revealed different pattern of osteogenesis between the two tested chemicals. Early stage markers were initially tested. Starting with ALP (Figure 7.6), results indicated that perfusion culture supported higher ALP expression compared to static for both tested chemicals thus confirming previous results from other groups, which indicated that the favorable environment of perfusion bioreactor supported high ALP activity and high viability of the cells (Bjerre et al., 2008; Botchwey et al., 2001; Meinel et al., 2004). Moreover, ALP expression was significantly induced after Dex treatment compared to Sim treatment.

The transcription factors Runx2 and OSX, the master regulators of osteogenic differentiation, were then tested (Figure 7.7). Earlier expression of these two genes was observed after Sim treatment whereas treatment with Dex induced a later expression.

The expression of COL 1, the most abundant bone matrix protein constituting the majority of the organic content and of BMP2, the strongest osteoinductive agent, was then measured (Figure

7.8). Both genes were significantly upregulated after Dex treatment and their level was higher in static configuration compared to the bioreactor but at different time points, earlier for BMP2 and later for COL 1. It has been shown previously that rotating wall bioreactors decrease the expression of COL 1. In particular, Bjerre and his colleagues tested two sizes of the same scaffold and noticed that collagen expression in both construct types showed a statistically significant preference for static culture at all time points (Bjerre et al., 2011). For further support, Plunkett and his colleagues noticed that all groups cultured in the bioreactor showed significant decreases in COL 1 expression compared to the static group. Based on their findings, they also suggested that the mechanism of action for this could be either that culture in the bioreactor downregulated COL 1 expression or accelerated osteoblast maturation (Plunkett et al., 2010). BMP-2 expression in the bioreactor maintained in relatively high expression level for both tested time points.

To summarize, the higher gene expression of early bone markers supported the idea that bioreactor platform could support better osteogenic differentiation compared to static culture.

Gene expression of late stage bone markers was then performed. Bglap is one of the few osteoblast-specific genes and is considered a marker of mature osteoblasts. BSP is another bone matrix protein indicating late stage bone differentiation (Figure 7.9). They were both highly expressed in the perfusion bioreactor after Sim treatment while their level was very low after Dex treatment. This finding provided an indication on the role of Sim in promoting earlier matrix maturation and late stage bone formation.

Finally, the expression of two bone matrix non-collagenous proteins OPN (Figure 7.10) and ON (Figure 7.11) was calculated. OPN is expressed early during cell proliferation, then is downregulated and finally is upregulated again to inhibit mineralisation. Both genes were significantly expressed after Dex treatment compared to the low expression level after Sim treatment with ON get upregulated at an early time point whereas OPN at a later stage. They both indicated higher level in the bioreactor compared to static culture.

It has been suggested previously that increased OPN expression coupled with decreased COL-1 expression may indicate that bioreactor culture has enhanced expression of post-proliferative genes at the expense of those found during proliferation (Partap et al., 2010a).

Gene expression results suggested that Dex treatment provided better support and induction of osteogenic differentiation compared to Sim treatment, as indicated by the high gene expression of important bone markers. Similar to that, it has been indicated that cells retain a greater osteogenic capacity when Dex is present in the medium immediately after harvest (Holtorf et al., 2005) suggesting that Dex acted as a stronger osteoinductive compound. Sim seemed to better

support high cell numbers of more progenitor type of cells. Longer differentiation protocol is probably needed with the use of Sim in order to acquire a more mature cell phenotype. Moreover, perfusion bioreactor platform indicated superior properties to support osteogenic differentiation in comparison to static culture.

In the Tables 7.2 and 7.3, gene expression results of major bone markers tested between Sim and Dex and also between static and perfusion configuration are presented for day 21 and day 29.

Table 7.2: Summary of the gene expression level of bone markers on day 21 is presented. Comparison was performed between Siim and Dex protocol and also among bioreactor and static platform.

	ALP	BGLAP	BSP	RUNX2	OSTERIX	COLLAGEN 1	BMP2	OSTEOPONTIN	OSTEONECTIN
BIOR DEX	+++	++	+	+	+	+++ +++	+++ ++++	++	++++++++++ ++++++++++
BIOR SIM	+	++++	+	+	++	++	++	+	+++++++ +++++++
STAT DEX	+	+	-	+	+	++++ ++++	+++++ +++	++	+++++++++ +++++++++
STAT SIM	+	++	+	+	++++	+++++	+++	+++	++++++++ ++++++++

Table 7.3: Summary of the gene expression level of bone markers on day 29 is presented. Comparison was performed between Sim and Dex protocol and also among bioreactor and static platform.

	ALP	BGLAP	BSP	RUNX2	OSTERIX	COLLAGEN 1	BMP2	OSTEOPONTIN	OSTEONECTIN
BIOR DEX	+++ +	+	+	++	++++	+++ +++	+++ +++	+++++	++++++++ ++++++++
BIOR SIM	++	++++	++	++	+++	+++++	+++	+++	++++++ ++++++
STAT DEX	+	+	_	++	+++	+++++ +++++	+++	++++	++++++++ ++++++++
STAT SIM	-	+++	+	+	+	++++	++	+	++++++ ++++++

Finally, there were some metabolic data collected from media from static configuration samples on day 29 comparing and analyzing Sim-containing with Dex-containing differentiation media (Table 7.1). Acquired results using Bioprofiler indicated that Sim-containing media preserved higher glucose and lower lactate concentration compared to Dex-containing media. The glucose level measured was similar to the initial amount provided with fresh media suggesting there was no consumption and a less metabolically stressful environment for the cells.

Similar outcome was observed by another research group. In particular, Dominika Nowis and her collegues incubated human skeletal muscle cells and human hepatocellular carcinoma cells with lovastatin and they observed decreased glucose uptake as shown by increased glucose concentration in culture medium as compared with controls indicating that glucose is not efficiently transported into cells treated with lovastatin. They attributed this outcome to the induction of conformational changes in the glucose transporters (Nowis et al., 2014).

Reduction of glucose metabolism in tumor cells and adipocytes has been also indicated (Takaguri et al., 2008). Similar findings on cancer cells indicating higher glucose concentration in the conditioned cell medium have been obtained (Ganapathy-Kanniappan et al., 2013; Malenda et al., 2012). Smith and his colleagues observed a dose-dependent decrease in glucose uptake in human myotubes after exposure to Sim in concentrations not influencing cell viability. They suggested that the observed reduction in glucose uptake could be an indirect effect of impaired oxidation of glucose. Knowing more about this glucose dysregulation could help understanding the myotoxicity observed by statins (Smith et al., 2014).

Metabolic analysis results supported the previous findings indicating the high differentiation activity of the cells cultured with Dex and their fast maturation compared to cells cultured with Sim, which were maintained in a low differentiation rate system with gradual and slower transition to more mature phenotype and acquisition of a progenitor stage for longer. However, more analysis should be performed on metabolism to get more clear data and view of what exactly is going on with the metabolism through the osteogenic differentiation of mESCs and how that may affect important parameters like the expression of genes and proteins.

To summarize, two culture platforms and two osteogenic differentiation protocols were compared and the important observations included the fact that gene expression was significantly higher in the majority of the tested bone markers in the bioreactor culture compared to static. Moreover, Dex treatment induced significant upregulation and high gene expression of bone markers compared to Sim suggesting that Dex acted as a stronger osteoinductive compound. Gene expression of bone markers was in general terms in low expression levels, with few exceptions, something that has been observed previously by other groups as well (Evans et al., 2012). The important contribution of this work was the fact that significant novel information were provided regarding osteogenic differentiation in 3D configuration using different osteoinductive substances.

The conclusions that could be drawn from this chapter are the following:

- 3D bioprocess in dynamic culture indicated superior properties in matters of cell expansion, proliferation and osteogenic differentiation compared to static configuration
- Sim treatment resulted in the production of high numbers of more osteoprogenitor cells while Dex treatment generated fewer cells of a more mature phenotype
- Gene expression analysis of important bone markers demonstrated higher expression level after Dex treatment in comparison to Sim

8th CHAPTER

8. GENERAL DISCUSSION, CONCLUSIONS AND FUTURE DIRECTIONS

8.1 The need for TE strategies

Nowadays, due to the increased life expectancy and the augmenting aging population, there is a prevalence of musculoskeletal diseases around the world. People live with pain for many years and this fact deteriorates the quality of their everyday life. The loss of skeletal tissue that can accompany trauma, injury, disease or advancing years can result in significant morbidity and significant socio-economic cost. Currently employed grafting solutions are inefficient and emphasise the need for new, more reliable skeletal regeneration strategies with low cost.

To address the unmet need for bone augmentation, TE has come to the fore in recent years with new approaches for de novo skeletal tissue formation, which seek to harness stem cells, innovative scaffolds and biological factors that promise enhanced and more reliable bone formation strategies to improve the quality of people's lives (Black et al., 2015).

To be more specific, TE products and their applications are widely investigated as a potentialy effective treatment for large-scale bone defects. The challenge presented with the repair of these injuries is the need for high cell numbers in order to fill the created defect. TE is offering a solution to this problem by combining the use of high proliferative cell populations, such as ESCs with the 3D environment provided by the scaffold that better mimics *in vivo* conditions and the scale up of the culture with the employment of the appropriate bioreactor platform. By understanding the principles of tissue growth, TE allows the production of functional tissue for clinical use, without the need for grafting, in order to promote tissue regeneration (Roberts et al., 2008).

8.2 Important components of the study

In this project, mESCs were used as a cell source due to their unlimited self-renewal capacity and pluripotency, meaning the ability to differentiate into all different cell types of the three germ layers of an embryo, apart from the placenta. This feature renders these cells a promising source capable of generating the high cell numbers needed for cell therapies.

Recreation of the 3D environment was based on the employment of a hydrogel made from alginate as a supportive scaffold. Alginate is a water-based biocompatible material that can be easily created through cross-linking strategies and can be also manipulated to incorporate adhesion molecules.

The important element to scale up cell production is the use of the necessary platform, meaning a bioreactor system. A custom made RWV perfusion bioreactor was employed for the experiments of the osteogenic differentiation. This novel, scalable perfusion bioreactor has been shown to create an ideal environment for the cells to thrive due to the continuous supply of fresh media and removal of metabolites, which provided a robust and controlled metabolic environment that is conducive to high cell growth and quality ESC bioprocessing (Yeo et al., 2013).

Finally, two different osteoinductive molecules were employed and compared for their efficiency in osteodifferentiation and mineralisation in these experiments. The steroid Dex was used as a control and the small molecule Sim as a novel tested compound that has indicated bone anabolic properties. Dex is a common osteogenic additive currently widely employed in various protocols to enhance osteogenic differentiation. However, Dex is a synthetic glucocorticoid (GC), which can have some uncontrolled effects on osteogenic differentiation, such as induction of osteoporosis (Eastell, 1995; Patschan et al., 2001) by inhibition of osteoblast differentiation (Canalis and Delany, 2002) and for this reason, an alternative molecule that could replace it has been extensively researched. In 1999, Mundy and his group examined a number of small molecules and identified only Sim to be able to increase the expression of BMP2 promoter. Previous people in the lab supported the idea that Sim enhanced osteogenic differentiation of mESCs (Pagkalos et al., 2010).

8.3 Necessity and importance of 3D culture

The necessity and importance of 3D culture configuration for the proper and efficient cell expansion and differentiation in order to obtain high cell numbers needed for cellular therapies has been highlighted by many different groups (Handa et al., 2014; Lee et al., 2008).

3D platforms better mimic the *in vivo* environment and allow cells to settle and start growing. Cell-cell communication and also secretion of osteoinductive molecules is better achieved leading to the faster and better expansion and subsequent induction of the differentiation of the employed cell population.

The choice of the appropriate bioreactor is crucial depending on the required application. Examples from people with experience on utilising specific platforms are very useful and can support the decisions and the choices made by the researcher.

8.4 Osteogenic differentiation protocols

The aim of this project was to perform osteogenic differentiation of mESCs. Osteogenic differentiation is a complex procedure that is widely performed *in vitro*. A number of different cell types have been used and differentiated to osteoblastic cells, but the exact mechanism has not been elucidated yet. Most of the research groups employ ASCs, which are easy to direct towards osteoblasts. These cells, however, have restricted renewal capacity and increased senescence, a fact that means they cannot support high cell numbers of good quality. ESCs are a good candidate source with the capacity to generate high cell numbers of good quality homogenous populations. The issue with these cells is that they need to go through more stages to finally achieve osteogenic differentiation. In contrast to the adult cells, they first need to commit to a germ layer before proceeding to the desired cell type (Sui et al., 2013).

The majority of the currently used protocols for osteogenic differentiation go through the stage of EB formation. EB recapitulates aspects of early embryo development and contains cells from the three germ layers. EB formation poses the major disadvantage of spontaneous differentiation and difficulty to guide the cells towards only one specific cell type.

Alternative methodologies were explored and tested to bypass EB stage and enhance differentiation towards the required germ layer and cell type. It has been previously indicated that by omitting the EB step, a significantly greater number of osteogenic cells can be generated (Karp et al., 2006). Moreover, it has been shown previously in the lab that conditioned media derived from HepG2 cells could enhance mesoderm formation and lead to the creation of a restricted repertoire of mesodermal cells (Hwang et al., 2006).

A very common protocol used for osteogenic differentiation employs b-GP as a phosphate source, AA, which is responsible for secretion of Col I in the ECM and Dex, which has been shown to induce the expression of bone markers and the mineralisation of the bone matrix (Langenbach and Handschel, 2013). This is known as the DAG protocol and it has been validated with different cell types.

8.5 The two tested protocols and the two employed platforms in the experiments

Osteogenic differentiation was performed in 3D configuration and comparison was made between static and perfusion bioreactor platform. Two different osteoinductive molecules were also compared for their efficiency in osteodifferentiation and mineralisation. The steroid Dex was used as a control and the small molecule Sim as a novel tested compound that has indicated bone anabolic properties.

The two protocols lasted for 29 days, starting both with 3 days incubation in HepG2-CM to enhance mesoderm formation, followed by osteogenic media containing b-GP and ascorbate-2-phosphate, to end up with further supplementation of Dex or Sim at different time points, to induce mineralization (Figure 8.1).

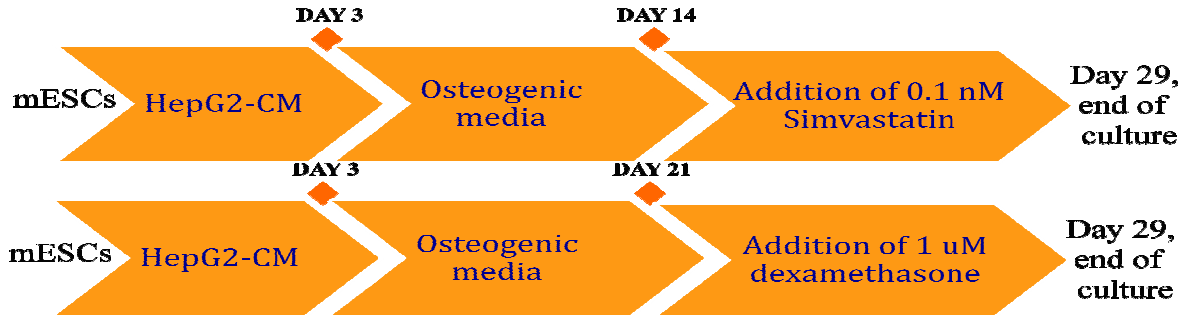


Figure 8.1: The two tested protocols for osteogenic differentiation of mESCs

8.6 Experiments, analysis and major findings

8.6.1 Biochemical analysis

Analysis was performed to evaluate the final product and results were compared between the two tested protocols. DNA quantification was used to evaluate cell proliferation throughout the experiment. Obtained results indicated that perfusion bioreactor enhanced cell growth and supported significantly higher cell numbers compared to static platform. This outcome was valid for both tested protocols.

Perfusion culture offers an ideal environment for the cells to thrive by continuously supplying fresh media and removing metabolic by-products. It has been shown previously that this type of culture enhanced cell proliferation (Bjerre et al., 2008; Grayson et al., 2010; Marolt et al., 2012a)

and supported high cell numbers (Cartmell et al., 2003; Gomes et al., 2003; Hosseinkhani et al., 2005b; Papantoniou et al., 2014; Sonnaert et al., 2014; Wang et al., 2013) compared to static configuration (Sonnaert et al., 2014; Yeo et al., 2013). The advantage of this platform is that nutrient and oxygen transport are performed not only by simple diffusion but also with convection, allowing the efficient transport even in the centre core of the hydrogel whereas in static culture, diffusion is the only available mechanism. When cells proliferate and increase in numbers, diffusion is not capable to support cells in the interior of the hydrogel resulting in the creation of a necrotic core, thus explaining the reduction in cell numbers (Anada et al., 2012; Carpenedo et al., 2007; Keogh et al., 2011; Lyons et al., 2008; Partap et al., 2010a).

Between the two tested chemicals, treatment with Sim protocol resulted in significantly higher cell densities and generated about double the amount of cells by the end of the experiment compared to Dex protocol and this was valid for both culture platforms.

One important element to point out was the fact that experiments with Sim were performed in a stage of the PhD where bioreactor operation was relatively optimized and the possible problems that could appear were minimized. This is the possible reason for better performance and higher cell numbers achieved in comparison to the Dex protocol, the first couple of weeks of the experiment, where common media was employed in both experimental conditions. Due to the better culture configuration and the more efficient operation of the bioreactor, higher proliferation rate was induced suggesting the possible reason for the reduction appeared on day 14 in static culture which cannot support high cell numbers due to diffusion limitations in the transport of nutrients and oxygen.

ALP was tested biochemically to evaluate osteogenic differentiation and ARS was used to indicate mineralisation. ALP is considered an important marker characteristic of bone formation. It is expressed early during bone formation and it has a siginificant role to support mineralisation. In the performed experiments, the obtained profile was the same for both chemical compounds and for both platforms tested with ALP maintaining similar and high level throughout the whole experiment in the perfusion bioreactor whereas in static, ALP followed the expected pattern for osteogenic differentiation with initial increase, followed by reduction and subsequent upregulation to support mineralisation. Due to continuous perfusion, ALP activity maintained in the same high level throughout the whole experiment in the bioreactor suggesting a possible maintenance of a more progenitor phenotype for longer before committing and beginning to differentiate into a specialized cell type while in static configuration, cells acquired a more mature phenotype as indicated by the acquired profile. There were no major differences exhibited between the two protocols and within the same platform.

ARS analysis indicated significantly higher calcium deposition in the perfusion bioreactor in comparison to the static configuration for both tested time points, thus confirming previous findings (Fassina et al., 2005; Gomes et al., 2006; Gomes et al., 2003; Sikavitsas et al., 2003; Yeatts and Fisher, 2011). Mineralisation levels were similar between the two protocols and within the same platform. Results suggested that treatment with Sim or with Dex induced the same level of calcium deposition at each platform tested.

To summarize, ALP and ARS analysis indicated similar outcome with no major differences observed between the Sim-containing and the Dex-containing protocols.

To further interrogate that, ATR-FTIR analysis verified the distinct presence of phosphate in the mineralised constructs and confirmed HA deposition, through the presentation of characteristic peaks of phosphate and carbonyl, which indicated the presence of mineralised calcium/phosphate as a major component of bone mineral (Hunter and Goldberg, 1993). However, the obtained images presented a relatively diffuse mineral accumulation in mESC cultures, something that has been previously observed by another group, which suggested the possibility of non-specific accumulationof mineral in ESC cultures (Evans et al., 2012). In another paper, they attributed this outcome to a different mechanism suggesting probably dystrophic mineral deposition, which can be caused when inorganic phosphates from locally high levels of ALP activity precipitate with calcium ions in cell culture media (Gentleman et al., 2009).

8.6.2 Gene expression analysis of bone markers

Gene expression analysis of important bone markers was performed on samples obtained from the last week of culture, day 21 and day 29, using qRT–PCR in order to evaluate osteogenic differentiation and to perform a comparison between Dex and Sim protocols and also among 3D static and dynamic culture platforms. Starting with the early stage bone markers, ALP, Runx2 and OSX, they all indicated higher expression level in the bioreactor platform compared to static configuration, with higher induction after Dex treatment compared to Sim.

Cells treated with Dex have been shown previously to exhibit enhanced expression of mRNA for ALPase (Wong et al., 1990; Yamanouchi et al., 2001). Increased ALP expression in the bioreactor and low in static culture has been noticed previously by other research groups (Bancroft et al., 2002; Barron et al., 2012; Cartmell et al., 2003; Datta et al., 2006; Gomes et al., 2003; Sikavitsas et al., 2003) which supported the idea that the favorable culture conditions created by the perfusion bioreactor enhanced ALP gene expression.

The transcription factors Runx2 and the downstream OSX are considered master regulators of osteogenic differentiation. OSX exhibited a distinct time frame of expression based on the different substances, indicating an earlier induction after Sim treatment and a later induction after Dex treatment.

Runx2 is characterised as an osteoblast-specific transcription factor. At the early stage of embryogenesis, it directs pluripotent mesenchymal stem cells to the osteoblast lineage and supports immature osteoblast to form immature bone (Ziros et al., 2008). The level of Runx2 determines the maturational stage of the osteoblast and its expression has to be downregulated in order to proceed to terminal differentiation to mature osteoblasts. Runx2 also triggers the expression of major bone matrix protein genes (Komori, 2010a; Komori, 2010b). Forced expression of Runx2/Cbfa1 in non-osteoblastic cells induces the expression of the principal osteoblast-specific genes (Ducy et al., 1997).

OSX is an important transcription factor required for bone formation (Nakashima et al., 2002). During bone development, osteoblast precursor cells, which express OSX, are the ones, which will generate mature osteolineage cells (Karsenty and Wagner, 2002; Maes et al., 2010). OSX-expressing cells provide a transient source of osteoblasts (Park et al., 2012a), implying the presence of a more primitive source sustaining osteolineage cells throughout the lifetime. It has been shown recently that OSX expression is correlated with successive waves of progenitors with the capacity to generate mature osteolineage cells (Mizoguchi et al., 2014).

The expression of another two early stage bone markers, Col I, the most abundant bone matrix protein and BMP2, the strongest osteoinductive agent, was also evaluated. Col I indicated significant upregulation after Dex treatment compared to Sim and higher level in static compared to dynamic configuration. Increased Col I expression in static platform has been indicated previously by other research groups (Chang and Hughes-Fulford, 2009; Cho et al., 2010; Dahlin et al., 2013; Wang et al., 2009). BMP2, as well, was also expressed after induction with Dex compared to Sim and the effect was more profound at an early time point for both tested platforms. The favorable environment created by the perfusion culture maintained the high expression of BMP2 for longer time compared to static.

The above results suggested better support of early osteogenic differentiation in the perfusion bioreactor platform compared to static configuration, as indicated by the higher and timely ordered expression of the tested early bone markers starting with Col I, BMP2 and ALP by day 21 followed by Runx2 and OSX by day 29. Moreover, treatment with Dex induced higher expression of early stage markers of osteogenic differentiation in comparison to Sim treatment.

In the next step, gene expression of late stage bone markers was also calculated. Bglap and BSP are two characteristic bone matrix proteins starting to be expressed when cells begin to acquire and achieve a more mature phenotype. BSP is considered a middle-to-late-stage marker of osteogenic differentiation (Hatakeyama et al., 2013), which is expressed in developing bones during endochondral ossification and during mineralisation (Holm et al., 2014) while Bglap is a marker of the more mature phenotype of osteoblastic cells. Analysis of osteoprogenitor development indicated that BSP is expressed at two distinct times: the first during the proliferative immature osteoprogenitor phase and the second during maturation from preosteoblasts to osteoblasts (Aubin, 2001). Both genes were highly upregulated after Sim treatment and the induction was more pronounced in the perfusion bioreactor configuration. Results suggested that Sim treatment significantly induced the expression of these important late stage bone markers compared to Dex treatment.

The expression of two bone matrix proteins, OPN and ON, was then evaluated. OPN is expressed late after mineralization has been initiated (Sodek et al., 2000) whereas ON is early expressed and decreases when cells go towards mineralisation. Similar to this information, OPN indicated late expression whereas ON was earlier expressed at both culture configurations with higher expression in the bioreactor compared to static. Moreover, the expression of both genes was significantly induced after Dex treatment compared to Sim treatment. It has been also shown that immature osteoblasts express OPN and BSP whereas mature osteoblasts strongly express OCN (Maruyama et al., 2007).

The above findings suggested a better support of late osteogenic differentiation by the perfusion bioreactor platform as indicated by the higher expression level of the tested markers, which also followed the expected time ordered expression profile starting with Bglap and followed by BSP expression. To further support that, ON was initially expressed followed by OPN during mineralisation.

To summarize, treatment with Dex induced the expression of early bone markers while treatment with Sim increased significantly the expression of late bone markers. Gene expression was better supported by perfusion culture as indicated by the higher level achieved for both early and late stage bone markers tested and by the more appropriate differentiation pathway followed together with the expected time line profile of gene expression patterns, compared to static configuration, which exhibited low gene expression, suggesting not proper induction towards the osteoblastic phenotype. To further support this outcome, it has been previously indicated that Dex enhanced the differentiation and promoted the expression of osteoblastic phenotype in human osteoblastic cells *in vitro* through gene expression analysis (Yamanouchi et al., 2001).

In conclusion, higher gene expression levels of bone markers in combination with higher calcium deposition and increased cell numbers suggested the superior capacity of perfusion bioreactor to properly support osteogenic differentiation.

One important observation from the gene expression results is the low level achieved by the majority of bone markers. There is further literature support for this outcome. Evans and his colleagues observed in their experiments that important bone markers were all expressed, but their expression level was relatively low, something that was also observed in the obtained results from these experiments. They strongly supported however that it is possible to produce osteoblastic cells from ESCs without the characteristic upregulation of bone markers (Evans et al., 2012). Another group observed that ESCs treated with common osteogenic medium mineralised considerably, but their differentiation was incomplete and the ECM, which formed late during the experiment, didn't exhibit the expression of characteristic bone markers. They supported that mineralisation observed was not conventional osteogenesis (Shimko et al., 2004).

8.6.3 Evaluation of ossification and germ layer formation through gene expression

For the first time to our knowledge, the ossification pathway followed was tested through the examination of a couple of chondrocyte and hypertrophic chondrocyte markers and also the progress in the gene expression of the three germ layers after treatment with Sim in order to get more insight information regarding the whole differentiation process.

The transcription factor Sox9 has been shown to express in mesenchymal condensations and common progenitors of osteoblasts and chondroblasts have been indicated to derive from Sox9 expressing precursors (Akiyama et al., 2005). Sox9 has been also proved to regulate the expression of major proteins of cartilage matrix, such as Aggrecan. It has been suggested that early mesenchymal progenitors cells with the capacity to differentiate to various cell types, for example osteoblasts or chondrocytes, contain promoter elements for the expression of Sox9, Aggrecan and Collagen 2 (Ono et al., 2014b). In these experiments, Sox9 and Aggrecan indicated similar expression profile with higher level achieved in the bioreactor culture compared to static after Sim treatment.

The marker of hypertrophic chondrocytes, Collagen 10, was also evaluated and its expression pattern followed that of Aggrecan. Examination of these three markers supported the idea that endochondral ossification was followed after treatment of mESCs with Sim and once more, the perfusion bioreactor better supported this process, as indicated from the higher expression levels.

Finally, one more thing performed for the first time was extended gene expression analysis of markers from all the three germ layers.

Evaluation started with two ectoderm markers, the transcription factor Pax6 and the Nestin protein. Pax6 has been shown to regulate neuroectoderm formation both in humans (Zhang et al., 2010a) and in mice (Quinn et al., 2010; Suter et al., 2009) and Nestin is a well-known and widely tested marker of ectoderm that has been shown to express in ES-derived progenitor cells that have the potential to develop into neuroectodermal, endodermal and mesodermal lineages (Wiese et al., 2004). Both genes indicated high expression level in the bioreactor compared to the low level in static culture.

Although Nestin is a marker of ectoderm, it has been shown recently that nestin-expressing cells are related with vasculature and contain early cells of the osteoblast and other lineages. In particular, it has been indicated that osteoblasts and bone lining cells expressed Nestin. Based on that, it has been suggested that Nestin acts downstream of Runx2 in the mesenchymal lineages and Runx2 is needed in order to direct mesenchymal precursors to become either cells of the osteoblast lineage positive for Nestin expression or preosteoblasts expressing OSX (Ono et al., 2014a). For further confirmation, it has been indicated that nestin-expressing MSCS contribute normally to skeletal formation (Mendez-Ferrer et al., 2010).

Next analysis involved the examination of two endoderm markers, the transcription factors Gata 4 and Sox 17. These factors have been shown to express during the differentiation of ESCs into the extraembryonic endoderm (ExE) and to be essential for its development (Niakan et al., 2010). ExE has been suggested to play crucial roles in mammalian development. Gata factors contribute to the early-stage differentiation of ESCs while Sox factors support and enhance the late-stage differentiation (Shimoda et al., 2007). Both endoderm markers indicated a better induction of their expression in static culture configuration.

Finally, the expression of three markers of the germ layer of interest, mesoderm in this case, was evaluated. Tested genes included the transcription factors Hand1 and BMP4 and the Desmin protein. In general, all the three genes exhibited higher expression in the bioreactor platform compared to static, at both tested time points. Gene expression of the above markers suggested a better induction of mesoderm formation in the perfusion bioreactor compared to static culture, as indicated by the higher gene expression at crucial time points. Results will need to be repeated for further validation.

To summarize, gene expression results from markers of the three germ layers suggested that the protocol used for osteogenic differentiation of mESCs employing AA, b-GP and Sim generated a cell population that retained in a very low level the expression of markers from all the three germ

layers suggesting the more progenitor status of the cells and the possibility of having also other cell types before committing to fully mature osteogenic phenotype. In particular, cells cultured within the perfusion bioreactor maintained a more progenitor like phenotype while cells in static seemed to acquire a more mature phenotype but without achieving the high gene expression level needed in order to properly support osteogenic differentiation.

It is well established that, the process of osteogenesis from ESCs is usually triggered, by supplementing the medium with AA, b-GP and Dex. These factors, however, are not specifically osteoinductive and it has been previously indicated from several research groups that cells from other lineages can be also generated (Sato et al., 2006; Shin et al., 2004; Srivastava et al., 2006; Tsuneto et al., 2005). It has been shown specifically that such osteogenically treated cultures retain a potentially undifferentiated population of cells (Karner et al., 2009). Similar to that and based on the acquired results, it could be suggested that cultures treated with Sim preserved a cell population with the ability to generate cells from all three germ layers of the embryo.

Last analysis was comparison of the metabolic profile between Sim and Dex in static configuration from samples obtained from day 29. Preliminary results indicated higher glucose and lower lactate in the Sim-containing media compared to the Dex-containing media suggesting better culture environment with less metabolic constraints in the Sim-containing media.

8.7 Side experiment

It has been observed that ECM protein components regulate not only cell adhesion but also differentiation (Reilly and Engler, 2010). Matrix composition has been proven to influence ESC differentiation (Battista et al., 2005).

Following that idea, a recently published paper from the lab supported that fibronectin enhanced osteogenic differentiation of mESCS (Kang et al., 2015). Moreover, fibronectin has been previously identified (Kang et al., 2009b) as one of the main components secreted in HepG2-CM. In order to obtain additional information, fibronectin coating was tested in combination with Sim treatment. Comparison was performed with gelatin coating as a control. ARS and ALP analysis suggested better performance with fibronectin, but more experiments would need to be performed for further validation.

To further support that idea, the important role of adhesion molecules in osteogenic differentiation has been recognized. In particular, several studies from different groups suggested and proved the idea that fibronectin supports and regulates osteoblast differentiation (Moursi et al., 1996; Moursi et al., 1997; Weiss and Reddi, 1980; Weiss and Reddi, 1981a; Weiss and

Reddi, 1981b). It also has an essential role during the early stages of osteoblast differentiation before initiation of matrix formation and subsequent mineralisation (Cowles et al., 1998).
Fibronectin has been shown to influence osteogenic differentiation both in 2D and 3D culture configurations (Martino et al., 2009). In particular, enhancement of osteoblast differentiation and mineralisation has been indicated on fibronectin treated plates (Mathews et al., 2012; Singh and Schwarzbauer, 2012)

8.8 Outcome of the project

The aim of this project was the creation of 3D mineralised cellular constructs made from mESCs encapsulated in alginate hydrogels after chemical induction with Sim and cultured in the custom made RWV perfusion bioreactor, which can support high cell numbers needed for potential use in cellular therapies. The obtained results suggested the generation of a progenitor cell type with the capacity to further differentiate into osteoblast cell.
Previous results, by another group, implied the generation of greater amount of stem and progenitor cell types from the Synthecon bioreactor compared to the static system (Fridley et al., 2010). It was not desired to obtain a fully mature cell phenotype. For *in vivo* applications, it is better to implant not a final and fully completed product, but something that will be in a stage that could activate mechanisms inside the body to participate more effectively in the healing process by incorporating body's own regenerative capacity.
The above suggestion can be further supported by recent findings, which demonstrated that progenitor cells derived from pluripotent stem cell sources and cultured within the dynamic environment of a bioreactor, may represent an interesting cell source for the fabrication of bone substitutes for the repair of large skeletal defects in humans, as indicated by their superior performance in ECM production and bone development, compared to MSCs derived from adult sources (de Peppo et al., 2013a; de Peppo et al., 2013b).
Last but not least, it has been demonstrated recently the principle idea of using dynamic culture for ES cell differentiation into therapeutic cell lineages. Bioreactor platforms could provide clinically relevant numbers of homogenous cell populations in order to be applied for various therapeutic applications. It has been suggested that bioreactor type and culture parameters significantly influence the differentiation of ESCs and lead to the generation of unique population of progenitor cells from all three germ layers of embryonic development (Fridley et al., 2010).

8.9 Challenges of the study

One more thing that should be taken into account for the development of an efficient protocol for osteogenic differentiation is the ability to supply the necessary factors at the appropriate time point in order to induce different functions. To be more specific, cells initially need to proliferate and expand their numbers; they will then gradually start producing the ECM that will support the final stage of mineralisation.

Challenges associated with the scaling up of TE constructs to clinically relevant dimensions, such as limited oxygen and nutrient supply may begin to look for novel approaches that seek to mimic developmental processes. The possibility to combine developmental and engineering processes together with molecular, biochemical and clinical techniques for skeletal TE can help in the improvement in healthcare costs and quality of life of the increasing aging population (Dawson and Oreffo, 2008).

8.10 Accomplishments of the study

One significant feature of this study was the employment of a novel custom-made bioreactor that combined the advantages of two widely employed platforms: the RWV and the flow perfusion. Experiments with this system provided novel information regarding osteogenic differentiation that could also contribute to the development of protocols that would potentially improve the process of cell differentiation towards the osteoblast lineage.

As mentioned previously, the generation of novel, reproducible and efficient protocols is an urgent requirement for osteodifferentiation. These protocols should be based on the developmental growth of the desired tissue to be formed. In this project, the employment of Sim, a widely used molecule as a drug to lower cholesterol, was tested. The evaluation of a small molecule, previously used as a drug, indicates great safety and faster approval time with less *in vivo* validation in order to be tested in humans. It also indicates potentially less side effects from its application. Moreover, as mentioned before, the need for low cost therapeutic solutions is urgent. One of the problems with the use of growth factors for osteogenic differentiation is their high cost due to the recombinant technology utilized for their production.

To summarize, it is important to consider that this study provided information regarding the use of a bioreactor platform combined with a small molecule osteoinductive agent in order to achieve the generation of cellular mineralised constructs that could be applied for treatment of

critical-size bone defects in humans. The positive element of this study was the fact that differentiation was performed without the use of any expensive growth factors, something that can contribute to the reduction of the cost for cell culture in the future.

This study also reinforced a recent idea, supported by other groups as well, that the generation of high number of osteoprogenitor cells is required by a bioreactor platform in order to achieve proper regeneration by exploiting also the advantages provided by the body's own regenerative capacity. Mature cells will be effective but probably with limited lifetime and without the ability to interact with the local niche and integrate with the environment to fully restore tissue function. One significant outcome from this study was the necessity to analyze and examine various aspects of the desired tissue and employ different methods and various techniques for analysis of the final derived product when the aim is to form new strategy for clinical applications. Obtained results indicated that many times, relying only on biochemical or only on genetic tests could have some false-positive or false-negative results. In order to have the complete image and be able to make the right decisions, it is necessary to analyse several different aspects and be confident after that to reach a safe conclusion.

8.11 Important observations and implications of the study

The tissue of interest in this project was bone and based on that, genetic analysis usually focus on the valuation of the expression of various bone markers in order to confirm the phenotype of the end product. One additional thing observed from this project was the fact that evaluation of the whole followed pathway, from the initial undifferentiated cell to the mature osteoblast should be performed in order to elucidate the precise differentiation process. It is important to understand all the different stages the cell goes through in order to achieve the final desired cell type. In the case of pluripotent stem cells, it has been shown that they first need to commit to a specific germ layer before proceeding to the differentiation to the desired cell type (Sui et al., 2013). Osteogenic differentiation is enhanced and better supported by the RWV perfusion culture configuration compared to the 3D static culture in tissue culture flasks. High cell numbers of good quality differentiated cells needed for cellular therapies can be also obtained from culture in the favourable environment generated by continuous supply of fresh media and removal of metabolic by-products in that platform configuration. The importance of 3D cultures, as a valuable tool for the development of novel strategies for clinical applications, was highlighted through this study. 3D cultures can bridge the gap between 2D cultures and animal models, thus providing significant information regarding both in vitro and in vivo experiments.

8.12 Contribution

To summarize, this study contributed to the acquisition of significant and novel information regarding 3D culture and osteogenic differentiation of pluripotent stem cells in a bioreactor platform. Details of the performed analysis were provided together with their significance and the respective outcome. All these elements could be employed by people in future studies on bone formation, regeneration and osteogenic differentiation. They would provide a useful guideline for the experiments they need to perform and details on the parts they need to be aware of. The significant cost reduction of the generated cellular constructs, by using small molecules instead of growth factors for the osteogenic differentiation, was also highlighted.

To summarize, the conclusions analyzed and presented on this chapter are the following ones:

- 3D bioprocess of mESCs using the favourable environment created by the perfusion bioreactor generated and supported high cell numbers
- Treatment with Dex induced osteogenic differentiation as indicated by high gene expression of important bone markers
- Sim supplementation in the culture media should be performed in the appropriate time point to avoid cell cycle inhibition
- Treatment with Sim in 3D culture dynamic configuration can support the generation of high cell number of osteoprogenitor cells
- Superior capacity of perfusion bioreactor to properly support osteogenic differentiation as indicated by the high gene expression of bone markers and increased calcium deposition

8.13 Future directions

The results obtained from this PhD project indicated and suggested an important advantage and potential use of the custom made RWV perfusion bioreactor in generating clinically relevant cells numbers of good quality for therapeutic applications related with bone defects and osteogenic malformations. *In vitro* studies should be further extended with the analysis not only of the gene expression but also of the protein expression profile. Moreover, metabolic analysis should be performed in a more detailed and high-throughput way to investigate the effects of metabolic stress on gene expression and other significant culture parameters. Finally, it is crucial to evaluate the osteogenic capacity of the cells generated from the bioreactor configuration and to proceed for *in vivo* confirmation through animal experiments.

The necessity for *in vivo* evaluation of osteogenesis through appropriate animal experiments has been recognized from the beginning of this project. Based on this idea, set up of the future animal experiment, in order to optimize the created fracture, identify and solve all the possible problems that could appear during surgery, has been already performed by my other supervisor, Mr Eleftherios Tsiridis, who is an expert Orthopaedic Surgeon. Rabbits were used as a good animal model being closer to the human status and mimicking in a higher lever human critical size bone defects compared to the widely used nowadays mice models.

mESCS were the first group of pluripotent stem cells widely employed and tested for various research purposes, mainly related with regenerative applications. These cells are easy to obtain, culture *in vitro* and expand their numbers compared to hESCs. These features rendered them a good cell source for TE applications. They also don't have problems regarding ethical issues, as it is the case with human cells. Fundamental research has been performed for years, by many groups around the world, employing these cells and significant conclusions have been draw (Duplomb et al., 2007; Garreta et al., 2006; Kirkham et al., 2012). ESCs from other animals have been isolated but there are limited information regarding their use and restricted *in vitro* capacity for proliferation and differentiation and rapid decline in pluripotency after several passages compared to mouse cells (Honda et al., 2008).

For these reasons, mESCS were used and tested in this PhD research project as a good and previously tested source with the capacity to provide high cell numbers needed for potential application in future cell therapies. Moreover, culture protocols developed in mouse cells have been employed successfully in human cells indicating their transferable capacity (Bielby et al., 2004). The existence of similar signaling pathways for the regulation of both mouse and human ESCs has been indicated (Keller, 2005) (Murry and Keller, 2008).

In vitro investigation needs to be followed and validated by *in vivo* experiment in the appropriate animal model. Mouse models are widely used. However, in view of developing cell therapies for humans, the necessity to employ a better animal model to perform experiments is urgent. Bigger animals, such as rabbits, constitute a better animal model being closer to the human status and mimicking in a higher lever human critical size bone defects compared to the widely used nowadays mice models. Rabbit is one of the most commonly used animals for musculoskeletal research studies (Pearce et al., 2007) and for this reason, it was used in this PhD research project for *in vivo* validation of the generated construct.

Three, 16-week-old male, New Zealand white rabbits with a mean weight of 3.6kg were used in this study to be tested for a metaphyseal fracture healing capacity. All sterility precautions were followed as per human surgery. Anaesthesia was performed using different injections of

antibiotics in concentrations calculated according to each animal's weight. Through surgical process, the creation of a non-critical size defect was performed at this stage. There were two groups of animals. The experimental group was filled with 2.5 ml of alginate beads, without cells at this point while the control group was left unfilled. In Figure 8.2, several representative photos from various stages during the process of the animal preparation are presented.

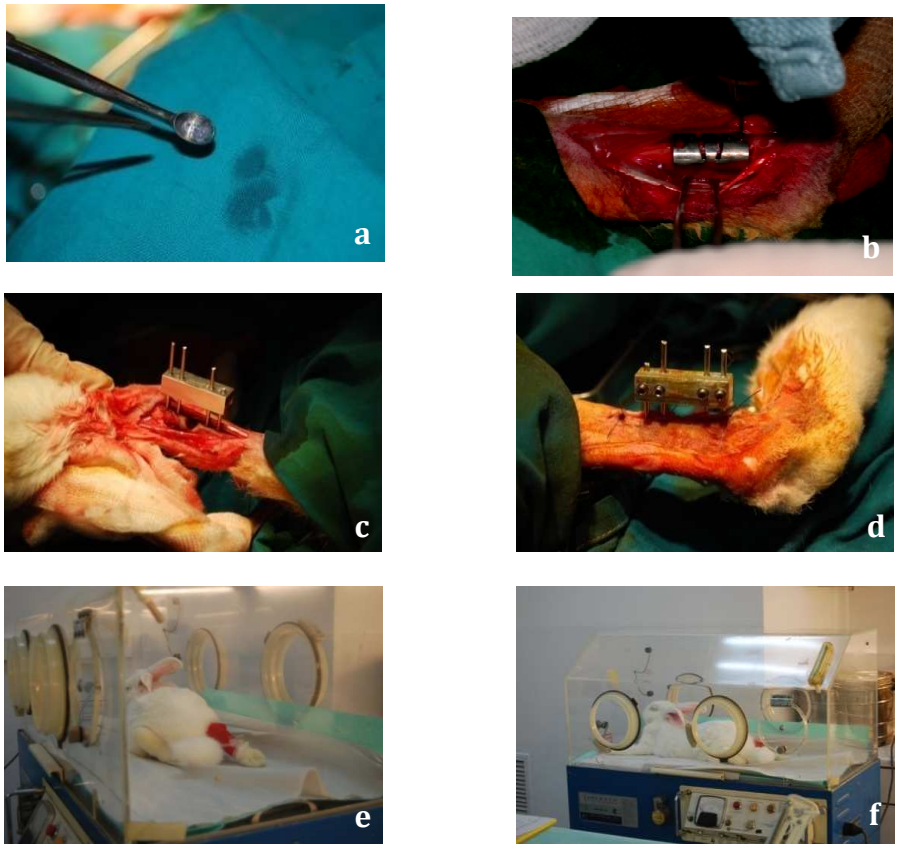


Figure 8.2: Animal experiment set-up. Alginate hydrogels implanted in the defect (a). The created defect wrapped around with the osteotomy jig (b) and stabilized with the external fixator (c) and (d). The animal incubator where experimental animals were placed under control environmental conditions (e) and (f). Animal surgery was performed by Mr Tsiridis in 2010.

In the surgery, two important tools have been used: an external fixator and an osteotomy jig (Figure 8.3), which were both manufactured in the School of Engineering at the National Technical University of Athens.

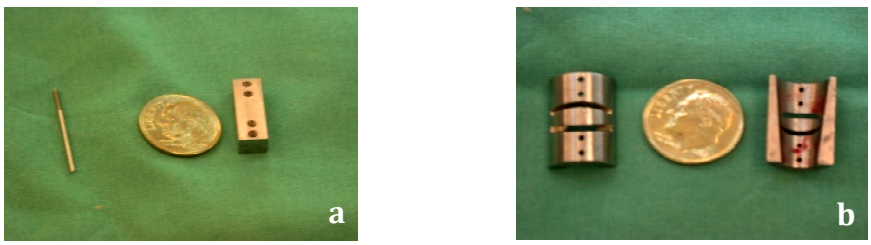


Figure 8.3: The external fixator (a) and the osteotomy jig (b)

The experiment was running for 3 months. After this period, the animal was sacrificed and it could be observed that the defect has been completed repaired. The obtained results indicated and strongly suggested that a period of less than three months would have been more appropriate in order to test and evaluate the regenerative capacity of the implanted hydrogels in comparison to the negative control. Three months was a long time and even the control group defect has been completely repaired, thus there was no possible comparison to be made between the control and the experimental group. X-ray photos were also acquired in order to better observe and evaluate the final outcome (Figure 8.4).

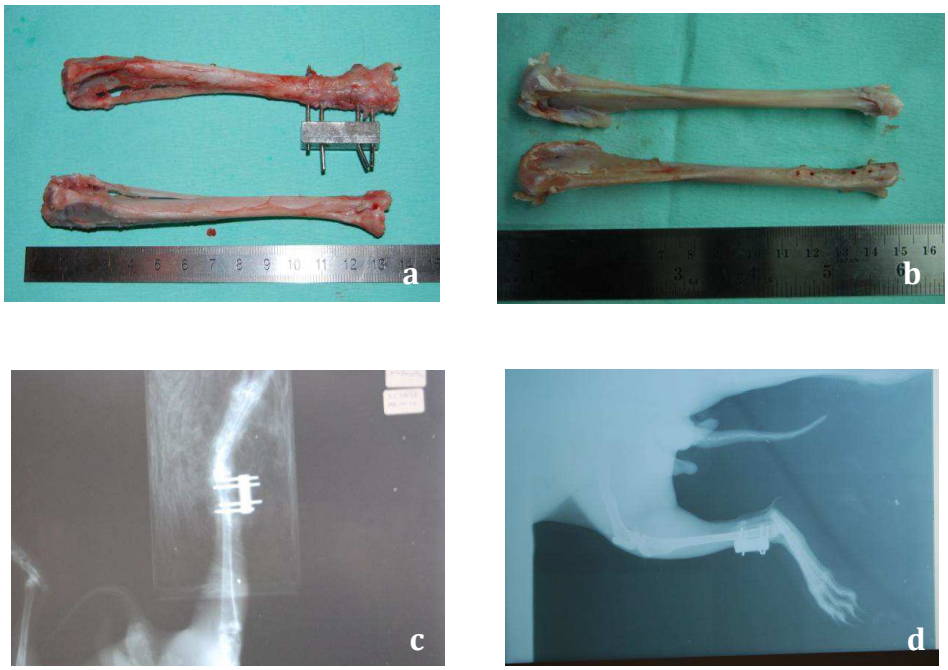
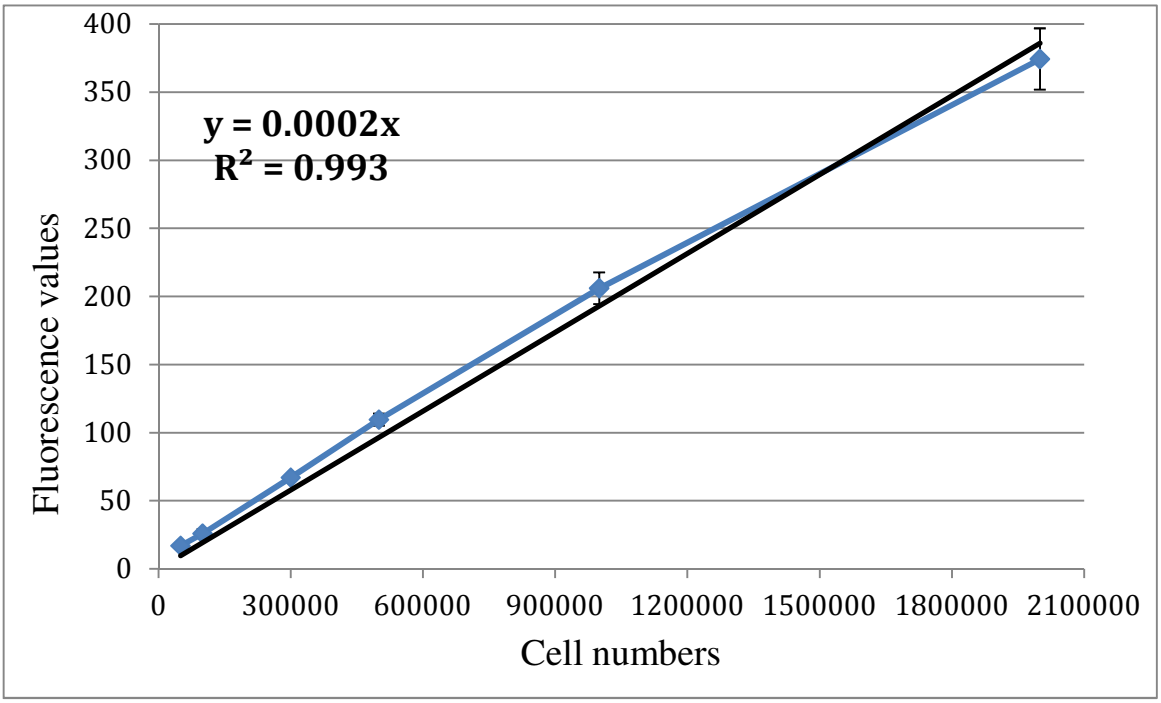


Figure 8.4: Animal experiment analysis. Bone from the experimental group (a) compared to bone from the negative control (b) obtained after three months of the implantation. X-rays were obtained on the beginning of the experiment (c) and also in the end, after 3 months (d). Photos were provided by Mr Tsiridis in 2010.

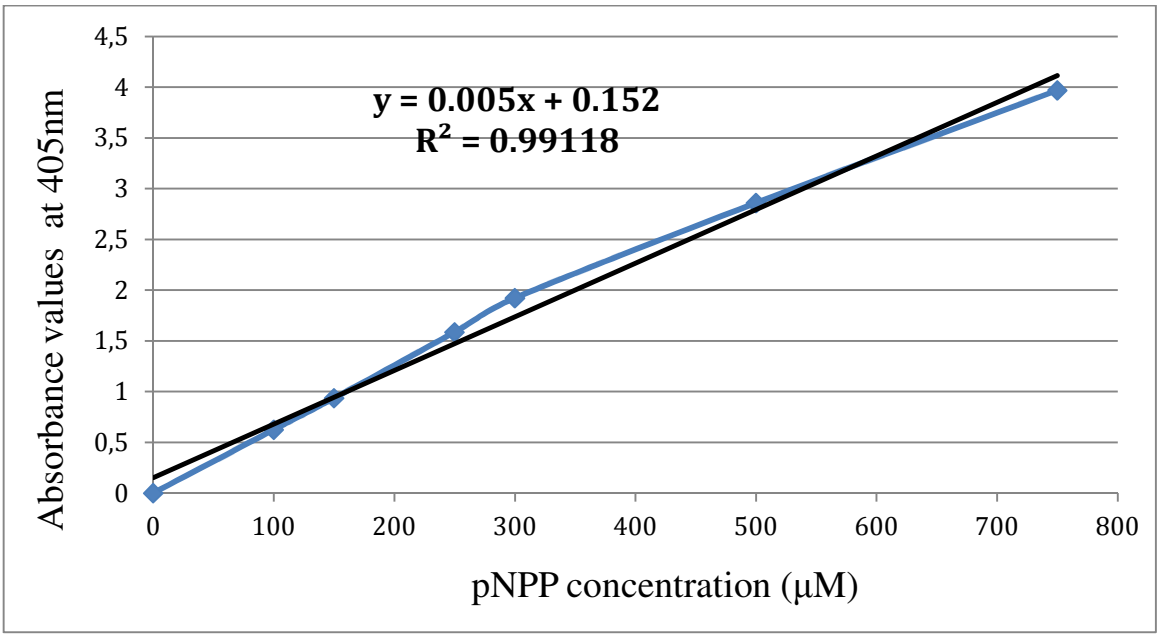
In this project, mESCs were used but it has been indicated the transferable capacity of the tested protocols for osteogenic differentiation in the hESCs as well. It has been previously suggested that some aspects of the regulation of hESCs may be similar to that observed in mESCs (Keller, 2005). In particular, the same research group after a couple of years recommended that similar signaling pathways regulate not only human ESC differentiation but also mouse ESC and culture from other organisms as well (Murry and Keller, 2008). To further support this idea, another research group recently indicated successful cardiac differentiation of both mESCs and hESCs using the same substance, indicating the similar mechanism employed by both cell types (Wang et al., 2012).

APPENDIX

1. DNA QUANTIFICATION STANDARD CURVE



2. ALKALINE PHOSPHATASE STANDARD CURVE



3. Mechanical strength analysis of bone-like construct

3.1. Results obtained from Dr Yushik Hwang thesis (Hwang, 2007)

Particle	Calculated Young's modulus (kPa)
Alginate	35.2
Alginate + Cell	29.3
Bone Construct	666.2

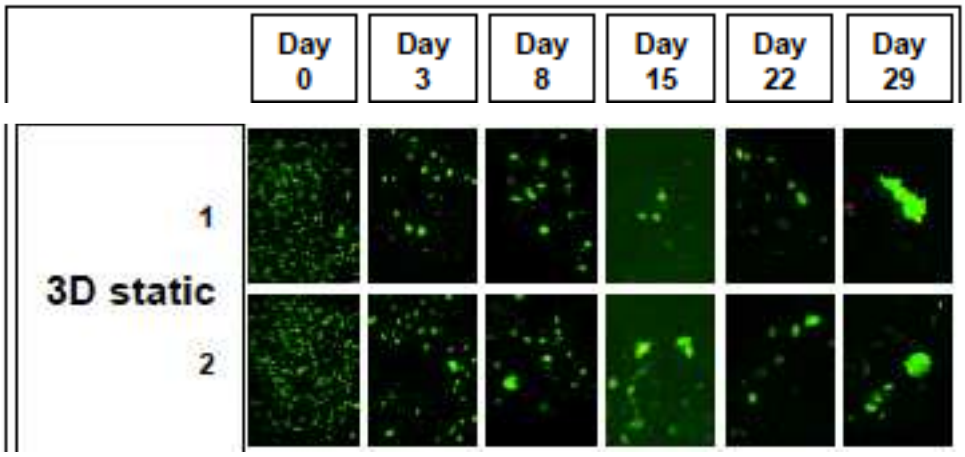
Alginate: pure alginate bead without cells.
Alginate + Cells: Alginate bead encapsulating undifferentiated mESCs with same cell density as Bone construct

3.2. Results obtained from Dr Jae Min Cha thesis (Cha, 2010)

Young's Modulus (kPa)	
Alginate bead	35.23
Control	13.32
BSEL	572.1
HARV	219.2
STATIC	93.05

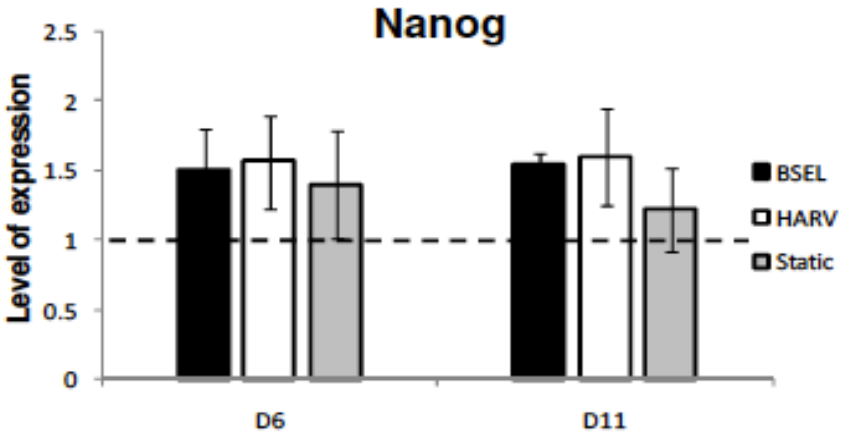
Alginate bead: alginate hydrogel without cells
Control: hydrogel composed of undifferentiated ESCs with a similar cell density as the bone-tissue like construct (about 3.5 - 4 x 10^5 cells/bead)

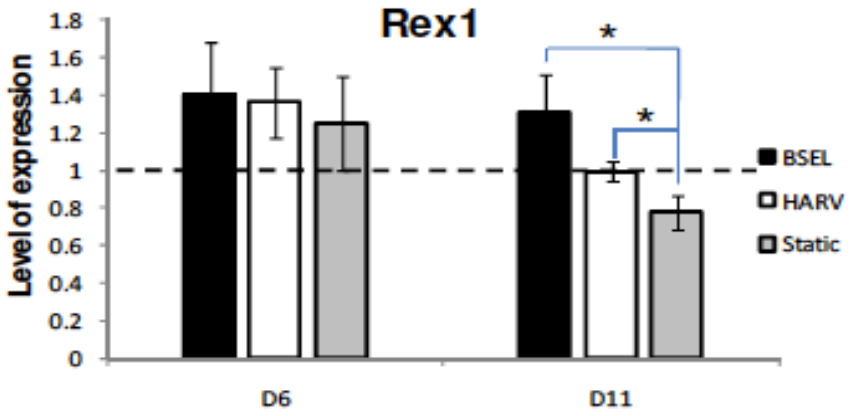
4. Live-dead results in static culture obtained from Dr Wesley Liam Randle thesis (Randle, 2006)

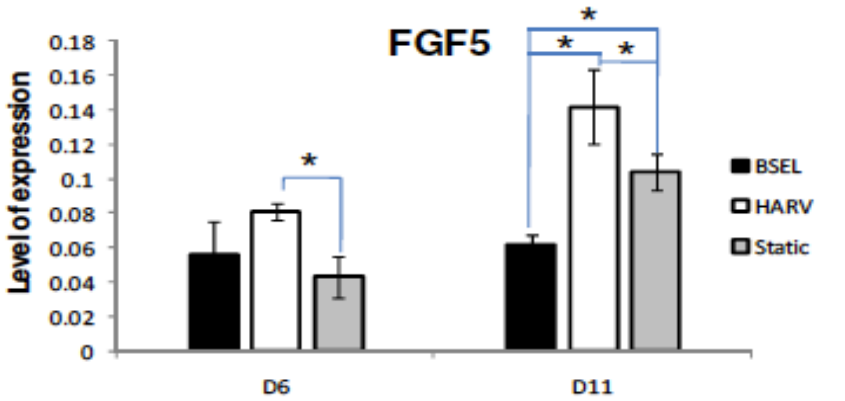


Live / Dead staining images of 3D static cultured for 29 days.
Image dimensions = 12367 x 845 µm. x10 objective.
Green fluorescence indicates viable cells

5. Real time PCR data from Dr Jae Min Cha examining the expression of plurirotent markers Nanog and Rex1 and primitive ectoderm Fgf5







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