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IMPACT OF BMP-2 RELEASING SCAFFOLDS ON HUMAN ADIPOSE STEM CELL DIFFERENTIATION

Master of Science thesis

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ABSTRACT

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Human adipose tissue is a good source of mesenchymal (adipose) stem cells which can be used in bone tissue engineering both in studying differentiation and for treating patients. Bone morphogenetic protein 2 (BMP-2) is often used in culture medium to differentiate mesenchymal stem cells *in vitro* and *in vivo*, even thiough its efficacy has been questioned. The mechanical properties of the substrate the cells grow on have been found important to the outcome of differentiation. The aim of this study was to investigate the effects of an electrospun BMP-2 releasing polymer scaffold which enables controlled and targeted drug-release on the adipogenic and osteogenic differentiation of human adipose stem cells *in vitro*.

Human adipose stem cells (hASCs) from four different donors were cultured on the electrospun polymer material, loaded with BMP-2. The properties of BMP-2 scaffolds and control scaffolds (CTRL) were compared in basal medium (BM) or osteogenic medium (OM) with or without added BMP-2. The osteogenicity of the materials was evaluated using quantitative alkaline phosphatase activity assay (qALP), as well as Alizarin red staining and gene and protein expression of marker genes using quantitative real time polymerase chain reaction (qPCR) and Western blotting (WB). The proliferation and morphology of hASCs were studied using total DNA quantification and immunofluorescence respectively. The adipogenicity of the material was evaluated using qPCR as well as Oil Red O staining and fluorescence imaging.

It was found that hASCs seemed to grow in aggregates on the BMP-2 scaffolds when compared to CTRL scaffolds. The BMP-2 released from scaffolds did not significantly increase the cells' ALP activity and mineralization of ECM. Instead the BMP-2 releasing scaffolds and CTRL scaffolds seemed to bolster adipogenic differentiation based on the shape of the cells and increased intracellular fat as well as the qPCR results. However, no statistically significant differences were observed between cells grown in different conditions when the results from different cell lines were pooled. It seemed however from the morphological data that the cells grown on BMP-2 releasing scaffolds were more similar to adipocytes than osteoblasts.

It was determined that BMP-2 releasing scaffold material is more beneficial for adipogenic than osteogenic differentiation. This is probably a result of the material not being stiff enough and being too hydrophobic. It is likely that the amount of loaded BMP-2 is too low, as it was significantly lower than the concentrations regularly used in differentiation protocols.

Key words: Adipose stem cells, mesenchymal stem cells, osteogenic differentiation, adipogenic differentiation, BMP-2, drug releasing scaffold, tissue engineering.

TIIVISTELMÄ

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Ihmisen rasvakudos on hyvä lähde mesenkymaalisille (rasvan) kantasoluille, joita voidaan käyttää luukudosteknologiassa, sekä solujen luuerilaistukseen tähtääviin kantasolututkimuksiin, että hoitoihin. BMP-2 on kasvutekijä, jota käytetään useissa luuerilaistusprotokollissa laboratorioissa sekä myös kliinisesti, vaikka sen tehokkuus on kyseenalaistettu useissa tutkimuksissa. Tämän tutkimuksen tarkoitus oli tutkia kohdennetun ja hallitun lääkeaineen vapautuksen mahdollistavan sähkökehrätyn polymeeriskaffoldin vaikutuksia rasvan kantasolujen luu- ja rasvaerilaistukseen.

Neljän eri luovuttajan rasvan kantasoluja (hASC) viljeltiin sähkökehrätyllä BMP-2:ta luovuttavalla polymeeriskaffoldilla. BMP-2 skaffoldien ja kontrolli–scaffoldien (CTRL) vaikutuksia vertailtiin basaali- ja ostoemediumesissa (BM ja OM) ilman lisättyä BMP-2:ta sekä sen kanssa. Materiaalien luuerilaistamisominaisuuksia arvioitiin alkaalifosfataasientsyymin aktiivisuuden perusteella (qALP) ja Alizarin Red -värjäyksellä, sekä tutkimalla kohdegeenien ja -proteiinien ilmentymistä kvantitatiivisen polymeraasiketjureaktion (qPCR), sekä Western Blot –tekniikan (WB) avulla. Solujen lisääntymistä ja morfologiaa tutkittiin kokonais-DNA:n määrän määrityksellä sekä immuno–fluoresenssimikroskopialla. Rasvaerilaistumista arvioitiin solujen sisäisen rasvan Oil Red O-värjäyksellä ja mikroskopoinnilla, sekä qPCR:n avulla.

hASC:t kasvoivat kasaantuneina BMP-2 skaffoldeilla kontolliskaffoldeihin verrattuna. Materiaalista vapautuva BMP-2 ei merkittävästi lisännyt solujen ALP aktiivisuutta tai mineralisaatiota. Sen sijaan BMP-2 skaffoldit vaikuttivat lisäävän rasvaerilaistumista solujen muodon, sekä lisääntuneen solujen sisäisen rasvan määrän, sekä qPCR-tulosten perusteella. Tilastollisesti merkittäviä eroja ei löydetty ryhmien väliltä, kun kaikkien neljän solulinjan tulokset yhdistettiin. Morfologisen datan perusteella solut muistuttivat kuitenkin enemmän rasva- kuin luusoluja.

BMP-2:ta luovuttavan skaffoldimateriaalin havaittiin olevan enemmän otollinen rasva- kuin luuerilaistukseen. Materiaalin hydrofobisuus, ja riittämätön jäykkyys lienevät pääsyyt nähtyihin tuloksiin sen ohella, että skaffoldeihin ladatut BMP-2 määrät olivat huomattavasti pienemmät kuin mitä normaalisti käytetään erilaistukseen.

Avainsanat: Rasvan kantasolut, mesenkymaaliset kantasolut, luuerilaistus, rasvaerilaistus, BMP-2, lääkeainetta luovuttava skaffoldi, kudosteknologia.

PREFACE

This study was carried out in Adult Stem Cell Group, BioMediTech, University of Tampere.

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CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

ALP	Alkaline phosphatase
BM	Basal culture medium
BMSC	Bone marrow stem cells
(rh)BMP-2	(Recombinant human) bone morphogenetic protein 2
BMPR	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
cDNA	Complementary DNA
C/EBP	*CCAAT-enhancer-binding protein*
CTRL	Control scaffold
DAPI	4, 6-Diamino-2-penylindole, Dihydrochloride
DLX-5	*Distal-less box 5*
DMEMF/12	Dulbecco's modified Eagle medium
DPBS	Dulbecco's phopsphate buffered saline pH 7.4
ECM	Extracellular matrix
ESC	Embryonic stem cells
(H)AP2	*(Human) adipocyte protein 2*
Hspaa	Human serum
FDA	Food and drug administration
(h)ASC	(Human) adipose stem cell
(H)FSC	(Human) fat stem cell line
iPS	Induced pluripotent stem cell
ISCT	International Society of Cellular Therapy
MSC	Mesenchymal stem cell
NTU	Nanyang Technological University
o/n	Overnight
OM	Osteogenic culture medium
ORO	Oil Red O
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCL	Poly(caprolactone)
PGA	Poly(glycolide)
PLA	Poly(lactide)
PLGA	Poly(lactide-co-glycolide)
PPARg	*Peroxisome proliferator-activated recepror-γ*
qALP	Quantitative ALP activity analysis
qPCR	Quantitative real time PCR
RPLPO	*Human acidic ribosomal phosphoprotein P0*
RT	Room temperature ~22°C
Runx2	*Runt related Transcription factor-2*
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SVF	Stromal vascular fraction
TGF-β	Transforming growth factor β
SOP	Standard operation protocol
VEGF	Vestigial endothelial growth factor
% V/V	Volume/volume percentage
% W/V	Weight/volume percentage
WB	Western blotting

1. INTRODUCTION

Bone tissue engineering covers a wide scope of different techniques which aim in healing defects in bone. To do this it is imperative to understand the biological and mechanical properties of bone as well as how bones form and heal in the human body.

The concept of tissue engineering was first properly described in Langer and Vacanti (1993) where they discussed that by combining cells, growth factors and proper materials it would be possible to engineer tissue (Langer & Vacanti. 1993). Since then tissue engineering has grown into its own cross-scientific field of study, combining the engineering approach to knowledge of biology and medicine. Great advancements have been made in the field to gain insight into the mechanisms by which tissues are formed and how their healing can be aided.

Autologous grafts and synthetic cell free metal implants are the golden standard in treating large defects in bones at the moment, as they are osteoinductive, osteogenic and non-immunogenic, but autologous bone cannot always be harvested in large quantities for all the defects (Thesleff, et al. 2011). Furthermore, there are other disadvantages to autografts than availability: The need to operate on two sites and chronic pain. Allografts and even xenografts have been used for the same purposes, but they are immunogenic and not as effective or as osteoinductive (Thesleff, et al. 2011). Other techniques, such as distraction osteogenesis have been used, but they can be very painful or non-effective. A more novel approach is the use of cells, scaffolds and growth factors that are implanted at the site of defect and that are to be assimilated to the native structure (Padalhin AR, et al. 2014). Novel treatment ideas for bone defects have been surfacing rapidly since the conceptualization of tissue engineering by Langer and Vacanti (Langer & Vacanti. 1993). Clinical trials have been increasingly run on treatments based on cell- or biomaterial implants and the combination of autologous stem cells and biodegradable polymers have gained more interest lately, due to problems with the conventional treatments based on autologous bone implants.

Adipose stem cells (ASCs) have proven to be a good source of stem cells that can be used in bone treatments. ASCs can be harvested in great amounts in an easier procedure than bone marrow stem cells and have been shown to differentiate into osteoblasts when properly

stimulated with correct growth factors with or without mechanical stimulation. (Thesleff, et al. 2011)

There are many ways to stimulate the differentiation of stem cells, but the combination of suitable materials with the right growth factors is a strategy that has been thought to be simple and effective (Tirkkonen, et al. 2013a). The problem is finding the correct materials and growth factors. Stiff extracellular matrix (ECM), which gives support for the osteocytes, is characteristic of a bone tissue. The importance of scaffold with proper mechanical and topographical properties cannot be overstated, as mechanotransduction is a very important factor in stem cell differentiation, especially in bone (D'Angelo F, et al. 2011; Mathieu PS & Loboa EG. 2012). An equally important requirement is for the material to mimic the topographical environment of the target tissue (Kilian, et al. 2010). The electrospun polymeric scaffolds in this study are flexible and topographically variable due to the choice of materials and the processing method. Many studies have been conducted to find good combinations, but improvements can still be made.

The differentiation of cells is often directed by growth factors alone: growth factors such as bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) have been shown in many studies to affect the differentiation of mesenchymal stem cells (MSC) (Solheim. 1998). In the case of osteogenic differentiation, bone morphogenetic proteins are thought to be especially important. Therefore, in this study we have decided to culture cells on electrospun polymer scaffolds that slowly release BMP-2, which has been shown to have both osteogenic and adipogenic effects on mesenchymal stem cells (Zuk, et al. 2011; Vanhatupa, et al. 2015). It is an interesting growth factor to study as it has already been approved for clinical use in bone applications by the USA food and drug administration (FDA).

The material used in this study is a blend of biodegrable and -compatible polymers poly(caprolactone) (PCL) and copolymers poly(lactic-co-glycolic acid) (PLGA) (PLGA-PCL). These materials have been used extensively in biodegradable bone fixation devices and controlled drug-release applications (Garvin & Feschuk. 2005) and the electrospun loose structure is considered to be similar to native bone ECM in texture (Rohner, et al. 2003; Lee, et al. 2012).

The aim of this study was to study the effect of BMP-2 releasing electrospun scaffolds on hASC morphology, proliferation and differentiation towards osteoblasts and adipocytes in order to see if this combination of material and growth factor is suitable for osteogenic differentiation.

2. LITERATURE REVIEW

2.1 Stem cells

The discovery of stem cells is often accredited to McCulloch and Till in 1963, although the term ‘stem cell’ and the concept dates back to as early as the 19th century. Nonetheless, after the paper (Becker Aj, et al. 1963) stem cells have been extensively studied and much progress has been made in the medical field thanks to their work.

Stem cells are an effective, yet controversial treatment method in the eyes of the general public. Many people outside the field associate stem cells with embryonic stem cells and falsely think that all stem cells are derived from sources that could become a complete human. This is untrue as new sources for stem cells are being discovered all the time. As a result, the ethical controversies become less prominent when the stem cells can be harvested from adult donors with little to no harm to their health (Thesleff, et al. 2011).

Stem cells are unspecialized cells that retain both their ability to proliferate and their ability to differentiate into different cell types and they can be categorized according to their origin, or their potency (Alvarez, et al. 2012). Totipotent stem cells can form an entire embryo, including the extra embryonic placental cells; pluripotent stem cells can differentiate into all the cell types of an organism, but cannot form a new individual. Multipotent stem cells can turn into multiple cell types, that are generally limited to cell types from a specific germ layer (endoderm, mesoderm or ectoderm) and finally unipotent into a single cell type. The ability to differentiate depends greatly on the cell source: as a general rule, the earlier the stage of development, when the stem cells are isolated, the greater their potency (Alvarez, et al. 2012). Stem cells can be derived from many sources such as embryos, bone marrow and fat, and nowadays even created from somatic epidermal cells in the form of induced pluripotent stem cells (iPS)(Takahashi, et al. 2008). Figure **1** summarizes some of the properties of different stem cell types.

Embryonic stem cells (ESC) are pluripotent stem cells that have been studied most extensively of all human stem cells. As such they are often the cells to which other stem cell types are compared. ESCs have enormous potential to be used in regenerative medicine, but as they are derived from embryos they entail equally great ethical issues (Lovell-Badge. 2007). Due to the ethical controversies, legislature concerning harvesting and use of ESCs can cause difficulties

for both research and clinical treatments. Pluripotent stem cells need to meet a set of strict criteria. They need to be able to form structures that have characteristics of endoderm, mesoderm and ectoderm. In practice, they need to express pluripotency genes, which show their ability to differentiate into all the aforementioned germ layers as well as form embryoid bodies in vitro and teratomas and hybrids in vivo(Lovell-Badge. 2007; Stadtfeld & Hochedlinger. 2010). The tendency to form teratomas is also dangerous for patients.

iPS have similar potential as embryonic stem cells, impose an increased risk of cancer, due to the method their pluripotency is induced: iPS can contain mutations that have accumulated during the donor's life time and as the pluripotency genes are inserted into the genome of the cell at random, additional mutations occur and the expression of genes can be altered (Angelos & Kaufman. 2015). As a result, iPS cells have seen less clinical use. The first clinical trials in tissue engineering using iPS was in treatment of age related macular degeneration but had to be discontinued due to safety concerns (https://www.ipscell.com/2015/07/firstipscstop/ (04.04.2015)). Instead, iPSs can be used as patient specific disease models, bringing us one step closer to personalized medicine (Mou, et al. 2015; Wiley, et al. 2015).

Mesenchymal stem cells (MSCs) are multipotent stem cells of mesodermal origin which can differentiate into many different cell types, such as osteoblasts, chondrocytes and adipocytes (Horwitz, et al. 2005; Gir, et al. 2012). They can be isolated from different sources including bone marrow or adipose tissue and have been used in a number of clinical applications (Thesleff, et al. 2011; Angelos & Kaufman. 2015). MSCs and iPSs have been the center of attention for tissue engineering since their discoveries because of two reasons: they can be used to regenerate different types of tissues and they do not entail as many ethical issues as embryonic stem cells.

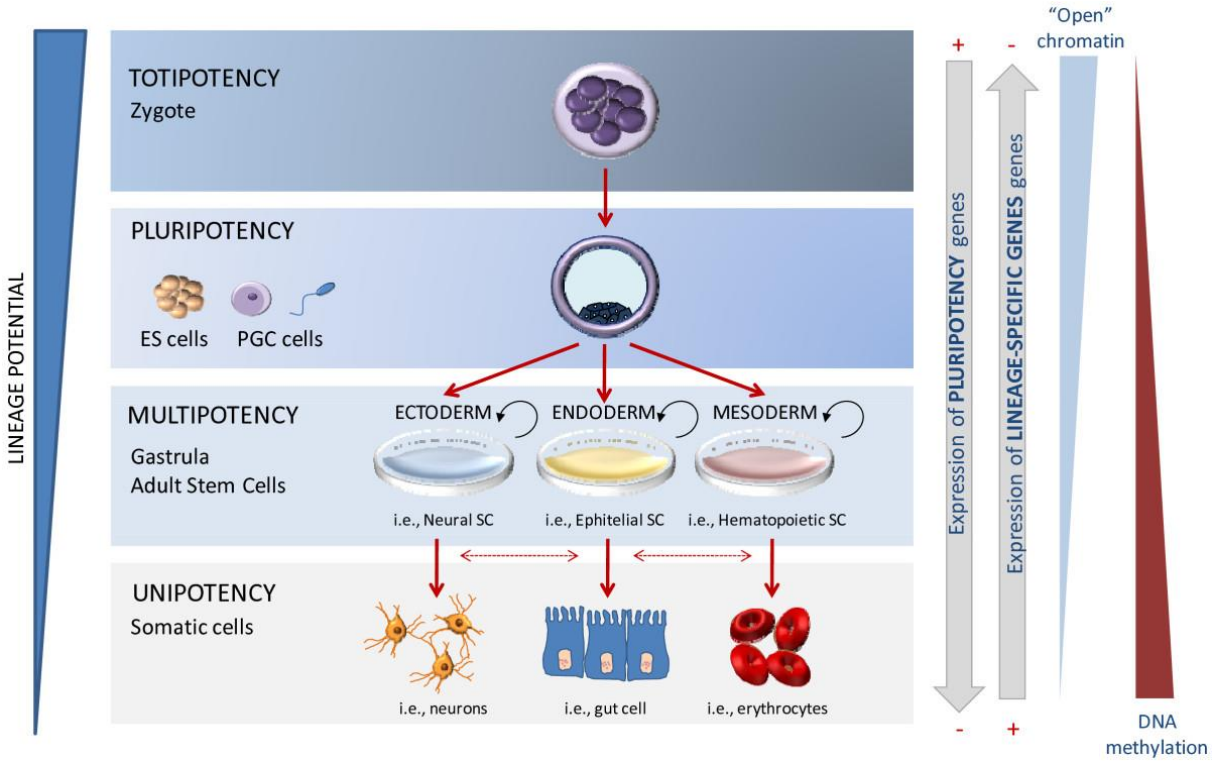

Figure 1. The relatioships of lineage potential, the expression of pluripotency genes and lineage specific genes as well as epigenetic changes (DNA methylation) and the amount of open chromatin to stem cells derived at different developmental stages, sites and their potencies. The image is adapted from (Berdasco & Esteller. 2011).

2.2 Adipose stem cells

Human adipose stem cells are MSCs derived from adipose tissue and they have been shown to be multipotent (Gimble & Guilak. 2003), (Zuk, et al. 2001a; Dominici, et al. 2006). They can be harvested in greater amounts than bone marrow stem cells (BMSCs) and the procedure is easier and less painful to the patient (Thesleff, et al. 2011). A downside for adult stem cells (including both BMSCs and ASCs), is that often the derived cell lines have a tendency to differentiate into the cell types present in the tissues they were derived from, probably due to epigenetic changes (Alvarez, et al. 2012; Lee, et al. 2014b).

MSCs have the ability to prevent the proliferation of immune cells and can thus be injected at inflammation sites to control the immune reaction (Djouad F, et al. 2009). ASCs have been shown to be better at immunosuppression than BMSCs (Gir, et al. 2012). Regrettably, this property along with the fact that MSCs seem to have the ability to home on cancer cells can be harmful, so great care must be taken if a cancer patient is being treated with MSCs (Gir, et al. 2012; Luo, et al. 2014; Ridge, et al. 2014). However this same homing ability has been

suggested as a means for treating cancer, by using the cells to carry drugs, or to destroy cancer cells with the aid of suicide genes and the bystander effect (Hu, et al. 2010). Recently, it has been shown that human ASCs (hASC) have potential as a means to fight melanoma (Ahn JO, et al. 2015). Being immunoprivileged also makes the use of allologous ASCs plausible, which means that we may have off the shelves ASC products in the future. Adult stem cells in general have one more major advantage over embryonic stem cells and iPSs: The later the developmental stage is when the stem cells are isolated, the less likely they are to differentiate into cancerous cells (Alvarez, et al. 2012).

Just a decade ago the existence of adipose stem cells was a controversial issue. It was relatively recently in 2001, that the multipotency of these cells was proven (Zuk, et al. 2001b; Zuk, et al. 2002). Since then the number of tissues they have been successfully differentiated into has expanded dramatically from just adipocytes to chondroblasts, osteoblasts, tenocytes, myoblasts and even neuron-like cells as well as insulin secreting cells (James. 2013) and many other cell types. One of the most interesting properties of hASCs, along with the abundance and accessibility of these cells (Strioga M, et al. 2012), is this ability to trans-differentiate into cell types normally originating from other germ layers, however reported cases have proven to be hard to replicate and the differentiation efficiency has been low. This transdifferentiation ability has been questioned as some evidence indicates that the transdifferentiation of adult stem cells are actually a result of cellular fusion(Wurmser AE & Gage FH. 2002).

Whether it is the result of differentiation, transdifferentiation, cell-fusion, growth factor production, immune system suppression or a combination of these properties, MSCs and lately especially ASCs have shown potential from treatments for diabetes (Gimble, et al. 2007; Gir, et al. 2012; Wu H & Mahato RI. 2014) to treating strokes (Gutierrez-Fernandez M, et al. 2013). For now, they have been studied and used most extensively clinically in bone tissue engineering applications. Autologous hASCs isolated from donor tissues at Tampere University have been used in multiple regenerative treatments (Thesleff, et al. 2011; Sandor, et al. 2014). The most famous one of these is a case, where a large mandibular defect was repaired using autologous hASCs that were expanded *ex vivo,* differentiated *in vivo* in the patient's abdomen and implanted to the defect site (Sandor GK, et al. 2013). Although such treatments have not become a standard of care yet, they showcase the possibilities.

2.2.1 Isolation and donor variability

Adipose stem cells, as discussed before are isolated from adipose tissue. The source of fat can however vary. Mostly the cells are from liposuction or tissue samples from abdominal fat or breast reduction surgeries (Zuk, et al. 2001b).

While cells from different sources show the same ability to differentiate, they can have different preferred direction of differentiation. Toyoda and coworkers showed that ASCs isolated from subcutaneous fat are better suited for osteogenic and adipogenic differentiation than those derived from omental fat (Toyoda, et al. 2009). Studies have shown that while omental and sub-cutaneous derived cell lines may have different preferences for differentiation, sub-cutaneous ones from different regions seem to have relatively similar differentiation profiles. There are still a number of unanswered questions and controversies when it comes to the differentiation ability of adipose stem cells. Strioga and co-workers showed that the gender of the donor was linked to the cells' ability to differentiate, male cells having a greater tendency for osteogenic differentiation (Strioga M, et al. 2012), while in other studies no such connection has been found (Toyoda, et al. 2009). The ability to differentiate into osteoblasts has however been shown to reduce with growing body mass index and age of the patient (Dechat T, et al. 2008; Choudhery MS, et al. 2014).

Adipose stem cells, when compared to other stem cell types have certain advantages, the most obvious ones being that they can be isolated from the patients with little ethical issues and low risks or discomfort for the patients and their ability to trans-differentiate. One major benefit is their abundance. After isolation, the number of cells is relatively high compared to e.g. BMSCs (Strioga M, et al. 2012). Fat grafting operations with fat enriched with ASCs in order to enhance the outward appearance of target areas of patients have also been conducted with the idea that the added ASCs make the operations more permanent by proliferating in the target area (Gir, et al. 2012; Zielins ER, et al. 2015). MSCs derived from different locations have differing immunoprivilege properties. Small differences in the expression of surface markers such as CD106 make BMSCs more adept at controlling cell-mediated immune responses than e.g. ASCs, but no significant adverse effects have been noticed in patients with either cell types (Strioga M, et al. 2012). It has been thought that as there are more than 400 000 liposuction operations done in the US alone every year, isolating hASCs from the lipoaspirates could prove a good source for transplantable cells in the future if hASC treatments become more common.

2.2.2 Characterization and classification

Dominici and coworkers defined the ISCT's (International Society of Cellular Therapy) minimal criteria for mesenchymal stem cells used today (Dominici, et al. 2006). The minimal criteria include plastic adherence, certain profile for cell surface markers and the ability to differentiate into osteoblasts, adipocytes and chondrocytes. The criteria are summarized in Table **1**.

Table 1. Minimum criteria for mesenchymal stem cells (Dominici, et al. 2006).

1.	Adherence to plastic in standard culture conditions		
2.	Phenotype	Positive(≥95% +)	Negative(≤2%+)
		CD105	CD45
		CD73	CD34
		CD90	CD14 OR CD11b
			CD79α OR CD19
			HLA-DR
3.	*In vitro* differentiation: osteoblasts, adipocytes, chondroblasts		

CD105, or endoglin is a glycoprotein associated with TGF-β receptors and angiogenesis. CD73 is a 5'-ectonucleotidase associated with adenosine dephosphorylation and its activity is important for osteoblast differentiation (Takedachi M, et al. 2012). CD90, or Thy-1 is a stem cell marker present in many kinds of stem cells. All the markers that the cells should be negative for according to ISCT are associated with hematopoiesis and the immune system.

2.2.3 Osteogenic differentiation

There are several approaches for differentiating stem cells. They can be categorized roughly to differentiation via genetic engineering, chemical factors or physical factors. Often they are applied at the same time (Zhang R, et al. 2013; Hamam, et al. 2015). Osteogenic and adipogenic differentiation have special relationship: One usually inhibits the other (Lin GL & Hankenson KD. 2011; James. 2013). This relationship can be pictured as a seesaw, with adipocytes on one end and osteoblasts at the other (Figure **2**).

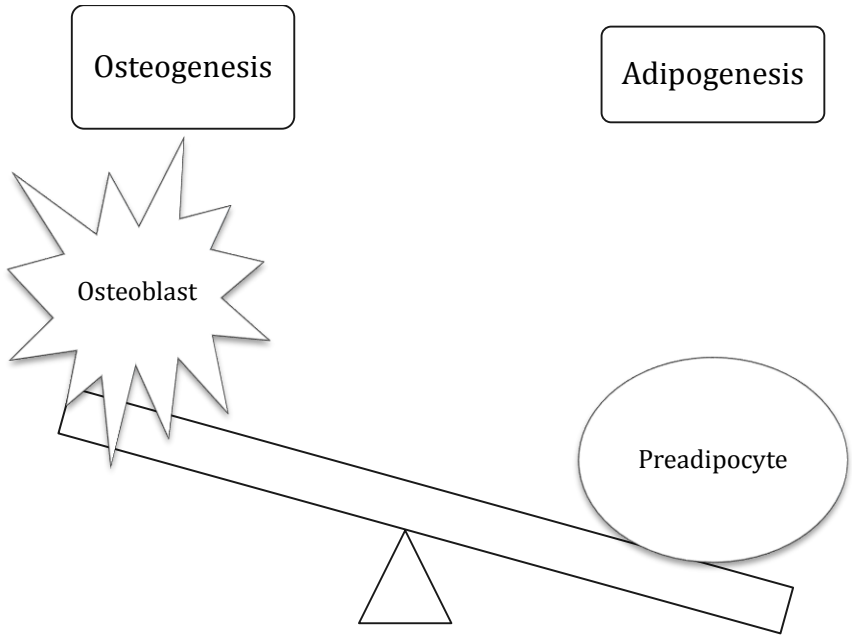

Figure 2. An illustration of the inverse relationship between adipogenesis and osteogenesis modified from(Gimble & Guilak. 2003).

Osteogenic differentiation occurs in two major steps: commitment into osteoprogenitor cell and differentiation into osteoblast. Osteoblasts mature into osteocytes *in vivo* once they become surrounded by the ECM they produce (Solheim. 1998).

The use of soluble factors to direct the differentiation of adipose stem cells is the most used approach. The wanted differentiation outcome can be reached either by applying activators to signaling pathways important to wanted cell type, applying inhibitors to other pathways, or both. (Osyczka AM, et al. 2004; Wan DC, et al. 2007).

Osteogenesis in MSCs is usually orchestrated through the activation of *Runt related transcription factor-2 (Runx2)*, which is considered the master regulator of osteogenic differentiation. Several factors and cell signaling routes are associated with osteogenic differentiation, but most of them converge at *Runx2* (Chen, et al. 1998).

Bone morphogenetic proteins are involved in many pathways leading to differentiation of stem cells. BMPs 2, 4, 6 are involved in osteogenesis and BMP-7 in chondrogenesis as well as in the differentiation of brown adipose tissue (Tseng, et al. 2008). BMPs bind to BMP receptors (BMPR) that phosphorylate downstream signal molecules. There are more than 20 known BMPs and several BMPRs, but not all of them are involved with osteogenesis (Hoffman, et al. 2006). BMP-2 signaling is discussed in detail in section 2.3.

Wnt-signaling pathways have been shown to be important for both osteogenic and adipogenic differentiation. Canonical Wnt-signaling (Wnt/b-cat) pathway promotes the expression of *peroxisome proliferator-activated receptor-γ (PPARg)*, whereas the less well known noncanonical pathways have been shown to inhibit it (Lin GL & Hankenson KD. 2011; van Amerongen. 2012; James. 2013). Wnt-signaling has also been connected to osteogenesis: depending on the initiator of the pathway, the expression *Runx2* can also be activated, but the general consensus is that Wnt signaling is more relevant for osteoblast differentiation (Lin GL & Hankenson KD. 2011). It is also worth keeping in mind that there is evidence that non-canonical wnt-signaling modulates the osteogenic differentiation of MSC derived from different sources differently (Liu, et al. 2014).

Many growth factors, including a number of bone morphogenetic proteins, fibroblast growth factors and vestigial endothelial growth factor 1 (VEGF-1) are associated with increased osteogenesis (Tirkkonen, et al. 2013b). For VEGF the mechanism has been shown to be intracellular in recent studies: VEGF added to medium has little or no effect (Liu et al. 2015). VEGF controls the amount of Lamin A/C in the nuclear envelope. Lamin A/C is associated with mechanotransduction as well as increased osteogenesis and suppressed adipogenesis.(Li W, et al. 2011; Vidal C, et al. 2012).

Genetic engineering can encompass many techniques from RNA-interference to adding genes to the cells, either transiently via plasmids, or long term with integration to the genome using integrative viruses (Zhang R, et al. 2013; Hamam, et al. 2015). Genetic engineering is made use of when inducing pluripotency into somatic cells by turning them into iPS (Takahashi, et al. 2008). The process involves inducing the expression of a set of pluripotency genes (usually *Oct3/4, Sox2, Klf4, c-Myc*)(Alvarez, et al. 2012; Schlaeger TM, et al. 2015). Similar approach can also be used to induce the expression of osteogenic transcription factors, or using RNA-interference to inhibit the expression of other transcription factors or proteins that suppress osteogenesis (Peterson B, et al. 2005; Lian, et al. 2012; Zhang R, et al. 2013).

It is general knowledge that the ECM surrounding a stem cell can greatly affect its differentiation. This is why different materials having different chemical and physical properties are used to direct the differentiation of cells (Wang YK & Chen CS. 2013). Several signaling pathways in a cell are activated or enhanced by the rigidity or elasticity of the material the cell grows on (D'Angelo F, et al. 2011; Cassidy JW, et al. 2014). These mechanical factors can also be induced with other means, such as vibration and changes in pressure. These kinds

of stimulations are usually induced by speaker systems and pressurized air that pushes and pulls a scaffold on which the cells are growing (Tirkkonen L, et al. 2011). Electric current and magnetic fields can also be used to guide the differentiation of cells either by manipulation of the material or directly affecting the cells (Parssinen J, et al. 2015).

The constituents of OM are optimized to promote osteogenic differentiation. The OM used in this study was optimized for adipose stem cells by (Tirkkonen, et al. 2013a). The composition is listed in Table 2. Ascorbic acid increases osteogenesis, by up regulating type I collagen secretion. L-glut acts as a source of energy and has been shown to greatly improve osteogenesis. Type 1 collagen is a major constituent in the ECM of bones and glutamine, which is prevalent in the protein structure, has been shown to induce collagen synthesis in cell cultures (Langenbach F & Handschel J. 2013). Dexamethasone, a synthetic glucocorticoid is a drug which is used to treat a number of diseases in humans. Its relevant effects for osteogenesis include the activation of glucose metabolism and lipolysis (Mikami Y, et al. 2011). Less dexamethasone is used in the OM optimized for ASCs than in the traditional OM used for BMSCs (Tirkkonen, et al. 2013a). β-glycerophosphate acts as a source of phosphate that is needed for the mineralization of ECM (Halvorsen YD, et al. 2001; Langenbach F & Handschel J. 2013).

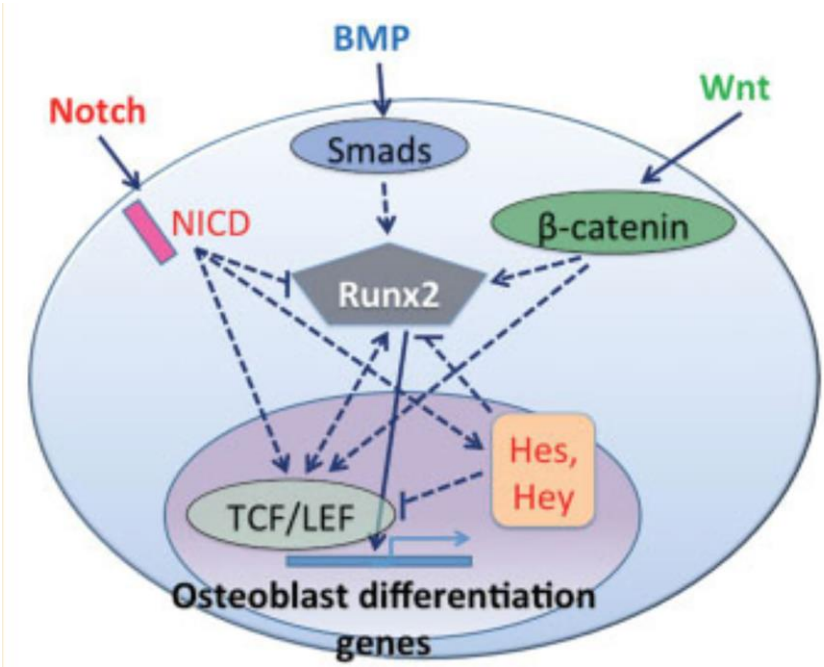

Figure 3. Showing interactions between well-known signaling pathways regulating Runx2 activation. Arrows indicate activation and lines inhibition. TCF/LEF is a transcription factors that can activate Runx2. Adapted from (Lin GL & Hankenson KD. 2011).

2.2.4 Adipogenic differentiation

As adipocytes act as the body's energy reservoir, insulin, glucocorticoids and other factors involved in metabolism, such as insulin like growth factors are important factors in determination of adipogenic differentiation (Ali, et al. 2013). Activating, or preventing the inactivation of pathways that increase the intake of fatty acids and other high-energy molecules such as carbohydrates in general leads to adipogenic differentiation rather than osteogenic differentiation (James. 2013).

Many of the same signaling pathways that are involved in osteogenesis are also associated directly or indirectly with adipogenesis, which makes it difficult to control the differentiation process towards one or the other outcome. Adipogenic differentiation, just like osteogenic differentiation occurs in two steps: Commitment into preadipocyte and differentiation into adipocyte. While preadipocytes are committed to become adipocytes under normal circumstances, recently it has been shown that treating them with all trans retinoic acid, which is an activator of lamin expression, can turn the cells into osteoblasts (Liu Y, et al. 2014).

Adipogenic differentiation is controlled by many factors, but *PPARg* is considered to be the master regulator (Mikami Y, et al. 2011; James. 2013). *CCAAT-enhancer-binding proteins (C/EBPs)* are activators of *PPARg*, which in turn when activated, can upregulate the expression of *C/EBPs* in a positive feedback loop. Correspondingly, inhibiting this signaling pathway downregulates adipogenesis (Mikami Y, et al. 2011). *Adipocyte protein 2 (AP2)* is another protein that is controlled by *PPARg* and has been found to control the adipose differentiation (Darlington, et al. 1998). It is a carrier molecule needed in fatty acid uptake, transport and metabolism and its expression level rises especially in the preadipocyte to adipocyte transition. This gene alone is not a specific adipocyte marker as it is also expressed in leucocytes and stromal vascular fraction (SVF) cells, but it has been shown to be important for adipogenic differentiation (Shan, et al. 2013).

Adipogenic differentiation of MSCs in vitro requires higher cell concentrations than osteogenic differentiation (McBeath R, et al. 2004). While osteogenesis is not prevented by multiple cell-cell contacts, they are a requirement for adipogenic differentiation, as cells are scarce in mature bone and plentiful in adipose tissue. Similarly the amount of focal adhesion complexes between the ECM and the cells increases osteogenic and decreases adipogenic differentiation (Mathieu PS & Loboa EG. 2012). Moreover, several studies have shown that morphology is important

to hASC differentiation outcome. Whereas osteogenic differentiation needs the cells to be in spread out morphology, adipogenic differentiation requires a round shape for the cells (McBeath R, et al. 2004; Kilian, et al. 2010; Mathieu PS & Loboa EG. 2012).

Just as osteogenic differentiation is encouraged in OM, there is also an adipogenic medium. Its individual components each have an effect, which aims at adipogenesis. Dexamethasone as a glucocorticoid has also several effects that benefit adipogenesis, including the uptake of glucose and activation of metabolism in general (Rubin, et al. 2009). Insulin increases the uptake of glucose, needed for the typical action of adipocytes: storage and synthesis of fat. Biotin is needed for the first steps in fatty acid synthesis and has been shown to act as an activator of *PPARg*. Isobutyl-methyl-xanthine has several effects, but its main effects are caused by its action as a phosphodiesterase inhibitor. In this role, it helps to prolong the effects of insulin, by increasing the intracellular lifetime of secondary messenger cyclic adenosine monophosphate, which has been shown to be important in the early phases of adipocyte differentiation. The combination of the above mentioned substances and their synergistic effects have been shown to be good for adipose differentiation of hASCs. (Ali, et al. 2013)

To make things interesting the control of osteogenic and adipogenic differentiation is controlled by many known signal pathways which overlap at several points: Wnt-signaling, Hedgehog-signaling, Notch-signaling, BMP-signaling and insulin like growth factor-signaling all share common mediators (Lin GL & Hankenson KD. 2011). As a result, some substances, e.g. dexamethasone seem to induce both osteogenesis and adipogenesis. This can in part be explained by line specific differences and timing of administering the substances: for example, Mikami and coworkers showed that early exposure to dexamethasone contributes to adipogenic differentiation and inhibits the action of BMP-2, but cells treated with BMP-2 gain a synergetic advantage for osteogenesis, if dexamethasone is added later on (Mikami Y, et al. 2011). Lee and co-workers studied a similar set-up and concluded that TNF-α in turn inhibits the action of dexamethasone and increases the potency of BMP-2's osteogenic properties (Lee, et al. 2014b).

Contradictions in studies involving the balance between these two differentiation outcomes are numerous and the subject needs further studying, before a clearer picture of the process is gained. In general, it can be said that the sum of different stimuli results in the balance of adipogenesis and osteogenesis tipping one way or the other. Proteomic studies that specifically look at the effects of BMP-2 or other growth factors while certainly expensive, could prove invaluable.

2.3 BMP-2

Bone morphogenetic proteins belong to TGF-β family of growth factors and are associated with differentiation pathways in many stem cells (Solheim. 1998; Lin GL & Hankenson KD. 2011). BMP-2 and BMP-7 are both used for chondrogenic and osteogenic differentiation of mesenchymal stem cells, and the latter has been used for brown adipocyte differentiation (Gir, et al. 2012). Bone morphogenetic proteins are involved in skeletogenesis, organogenesis and in fracture healing. Many BMPs have osteogenic effects, but not all of them are essential for the formation of bone. While BMP-4 has been use for both chondrogenic and osteogenic differentiation, BMP-2 and not BMP-4 has been shown to be essential in endochondral ossification, one of the two major mechanisms by which bone is formed in the body (Shu B, et al. 2011; Lin GL & Hankenson KD. 2011). Bone morphogenetic proteins, including BMP-2 are produced in fracture sites and BMP-2 has been found to be essential for the initiation of fracture healing (Tsuji K, et al. 2006).

ASCs have been used in multiple bone regeneration applications with or without additional BMP-2 (Thesleff, et al. 2011; Seppanen R & Miettinen S. 2014). The growth factor has been shown to be beneficial for the healing of bone defects in mice (Peterson B, et al. 2005). However, in these studies the inclusion of ASCs seem to have little or no effect on the healing process (Peterson B, et al. 2005), which suggests that BMP-2 might not affect the osteogenic differentiation of ASCs significantly, but instead work on preosteoblasts already present in an *in vivo* situation. Said studies have shown that in ASCs downstream transcription factors such a *Distal –less box 3* are not activated by BMP-2 induced signaling, which they believe to suggest that the signaling route itself does not function as expected. Another important point to keep in mind when comparing studies is that most of the studies that our current understanding of MSC differentiation are based on, are conducted in murine and rat models, where the results are usually more clear. Applying this understanding to humans has proven difficult and the results have been more varied (Osyczka AM, et al. 2004). Vanhatupa and coworkers came to the conclusion in their extensive in vitro study that the effects of BMP-2 are donor dependent (Vanhatupa, et al. 2015).

2.3.1 BMP-2 signaling pathway

BMP-2 pathway begins with BMP-2 binding with BMP-2 receptors, which dimerize to gain the ability to phosphorylate SMADs. Two or more phosphorylated receptor regulated SMADs

combine with co-SMADs and enter the nucleus, unless inhibitory SMADs combine with the complex. Once the SMAD-complex enters the nucleus, it acts as a transcription factor. The access to nucleus can be inhibited by nuclear envelope proteins such as MAN1 (Dechat T, et al. 2008), which are in turn controlled by Lamin A/C and intracellular VEGF (Berendsen AD & Olsen BR. 2015).

There are a total of 9 different SMAD proteins in humans that can be classified into three categories: Receptor regulated SMADs (SMADs 1, 2, 3, 5 and 8/9), which are phosphorylated by the receptors, the common mediator (co-SMAD) SMAD 4 and inhibitory SMADs (SMADs 6 and 7) (Heldin CH, et al. 1997). BMP-2 induces the formation of SMAD1/5/8 complex, which can be used as a marker for activated signaling pathway (Lin GL & Hankenson KD. 2011).

BMP-2 has been shown to induce both osteogenesis and adipogenesis depending on the dosage and the BMP-receptor it binds to: Binding to BMPRI-A induces adipogenesis and binding to BMPRI-B osteogenesis (Chen, et al. 1998; Kang, et al. 2009). BMP-2 pathway, when activated by BMPRI-B induces the expression of *Runx2* –expression factor, which is the master regulator of osteogenesis and antagonist of *PPARg*, the master regulator of adipogenesis, the expression of which is increased by the same pathway activated through BMPR1-A. High doses of BMP-2 have been associated with osteogenesis, but exceedingly high doses can cause unwanted phenotypes (Zara JN, et al. 2011). On the other hand, low doses of BMP-2 seem to be beneficial for adipogenesis (Chen, et al. 2016)

The pathway and its regulators are still not fully understood so interfering with one factor of the pathway can yield unpredictable results. Some studies suggest that the effects of BMP-2 on ASC differentiation are not significant at all (Peterson B, et al. 2005; Zuk, et al. 2011). Vanhatupa and coworkers came to the conclusion that the effects of BMP-2 are donor specific (Vanhatupa, et al. 2015).

Noggin is an extracellular protein that acts as an antagonist for many bone morphogenetic proteins and thus inhibits the pathway (Chen, et al. 2012). There is evidence that noggin is excreted as a regulator for BMP- signaling through a feedback loop and its inhibition has thus been recently studied as a possible means to enhance osteogenic differentiation (Wan DC, et al. 2007). The results have varied: While some early research was interpreted so that noggin is required for osteogenesis, the current consensus is that its inhibition is more beneficial. The

noggin feedback loop has been thought to be one reason, why the effects of BMPs are so varied on different cell lines (Osyczka AM, et al. 2004).

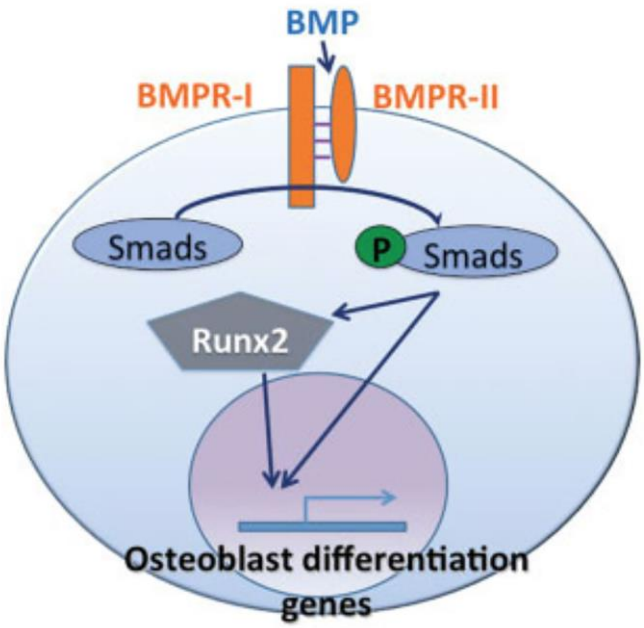

Figure 4. BMP-signaling pathway leading to Runx2 activation. Adapted from (Lin GL & Hankenson KD. 2011).

2.3.2 BMP-2 as a drug

BMP-2, along with BMP-7, have been approved by FDA to be used in treatment of bone defects. However, BMPs have several other roles in the human body and high doses in the wrong places can lead to apoptosis, unwanted differentiation and even cancer (Zara JN, et al. 2011; Kim, et al. 2013). Furthermore, the production of BMPs for medical use is expensive. Therefore, new ways to administer the growth factor locally need to be studied.

Osteopenia and osteoporosis are sometimes treated with BMP-2, but it is used especially in spinal fusion surgeries (Carragee EJ, et al. 2011). Safety issues have led to lawsuits against companies using recombinant human BMP-2 (rhBMP2). Spinal fusion constructs such as INFUSE bone grafts have been shown to increase the risk of certain types of cancer and male sterility (Zara JN, et al. 2011; Carragee EJ, et al. 2011); http://www.drugwatch.com/infuse/(13.02.15)) among other serious and less serious complications. Nonetheless the improved quality of life after a vertebrae fusion in patients and the ease of the operation when using BMP-2, have kept the products on the market. BMP-2 has also been used in combination with biomaterials in clinical bone tissue engineering applications

at the University of Tampere (Sandor, et al. 2014). In one case BMP-2 was adsorbed on β tricalcium phosphate (TCP) scaffolds, by incubating the biomaterial for 48h in a solution that contained 12mg BMP-2 (Sandor GK, et al. 2013). Later these scaffolds, with autologous hASCs were implanted in the patient.

2.4 Polymer scaffolds in bone tissue engineering

Materials can be categorized into metals, ceramics, polymers and composites (Piskin. 1995). Biodegradable polymers are the most used in bone tissue engineering, followed by ceramics and composites.

Metals have been used for bone fixing devices for a long time, but have certain disadvantages compared to the other material groups. Metals have superior mechanical properties compared to bone so when metallic implants are used in load bearing bones, the bone surrounding the implants degrades slowly due to the micromovements of the metal and due to weakening caused by the metal bearing most of the weight. This effect is called stress shielding (Engh CA, et al. 1987). Due to this, other materials which more closely match the properties of bones are desired for long time implantation. Ceramics have mechanical properties comparable to bones and certain ceramics, such as bioactive glasses and hydroxyapatite have osteoinductive properties (Waselau M, et al. 2012; Ojansivu M, et al. 2015). The major problems associated with ceramics are that they are brittle, their tensile strength is weak and they are hard to process.

Biodegradable polymers are the most widely used scaffold materials for bone tissue engineering. This is a result of multiple factors: Very little stress shielding, surface topography can be easily processed and drug release can be controlled (Garvin & Feschuk. 2005; Li, et al. 2006). A number of polymers, especially biodegradable ones, have been studied for scaffold purposes in bone applications. These polymers can roughly be categorized as natural or synthetic biodegradable polymers (Li, et al. 2006).

Natural biodegradable polymers, such as chitosan, silk and collagens, are derived from organisms and generally have excellent biocompatibility, but may contain contaminants due to their origins and processing (Lee, et al. 2012). They are generally hard to process, as they are sensitive to changes in heat, pH and other conditions. Furthermore, as they are usually degraded enzymatically *in vivo*, the degradation rate is hard to control as people have varying expression levels of said enzymes. (Li, et al. 2006). In contrast, synthetic biodegradable polymers are more easily processed and their degradation rates are easier to control. They are, as a result of being

synthetic, less likely to have contaminants and are generally more easily sterilized. Some of the most common synthetic polymers used in bone applications include polylactide, polyglycolide, polycaprolactone and polytrimethylene carbonate (Garvin & Feschuk. 2005; Vo TN, et al. 2012). As a down side the degradation products can be acidic depending on the polymer.

Both natural and synthetic polymers as well as their combinations have been used in bone tissue engineering both *in vitro* and *in vivo* (Piskin. 1995; Rohner, et al. 2003). The intended use of materials determines which materials and processing methods are best suited for the task. The needed processing methods can also affect the choice, as natural polymers are sensitive to variations in temperature and chemical surroundings (Lee, et al. 2012). If the material needs to give mechanical support to broken bones, fix ligaments, or needs to have a controlled drug release rate, synthetic polymers are generally good. If maximum biocompatibility in the form of cell attachment is required to assist in healing, natural polymers are often better suited for the task (Porter, et al. 2009).

2.4.1 Poly(lactic-co-glycolic acid) and poly(caprolactone)

Poly(glycolide) is a polymer derived from glycolide and has been widely used in biodegradable sutures (Sill TJ & von Recum HA. 2008). It has a glass transition temperature around the physiological temperature, which makes it rather flexible in *in vivo* applications. Its degradation in a biological environment occurs in two phases: First random hydrolysis of the polymer and second, entering the formed glycolic acid into the citric acid cycle. The polymer is ultimately broken down to carbon dioxide and water.

Lactide is a chiral molecule, meaning that it has two optical isotopes referred to as enantiomers. The two enantiomers have different degradation rates. Poly(lactide) can consist of one or both of the enantiomers in varying ratios and can thus have varying degradation rates. It has a glass transition temperature of around 70 °C, making it rather rigid *in vivo* (Madhavan Nampoothiri K, et al. 2010). By varying the ratios of the enantiomers, the degradation rate of the polymer can be controlled. The degradation of PLA results in the formation of lactic acid lowering the pH. *In vivo* lactic acid is transported to the liver and converted to glucose in gluconeogenesis.

Poly(lactide-co-glycolide) (PLGA) is a copolymer of PLA and PGA, which is used extensively in biomedical applications. It is bulk eroding, as are the homopolymers. PLGA can have varying ratios of PLA or PGA monomers and this ratio is responsible for the rate of degradation *in vitro*

and *in vivo*. Varying the ratio makes it possible to control the rigidity and the drug release rate of the material (Garvin & Feschuk. 2005; Madhavan Nampoothiri K, et al. 2010).

Polycaprolactone is a polymer, whose degradation rate is much slower than those of PLA and PGA (Woodruff & Hutmacher. 2010). PCL is typically used in applications where the needed degradation time for the material is more than a year. By adding PCL to the polymer blend, the interval of controlled drug release lengthens and longer lasting mechanical support for the cells is achieved. Owing to its low glass transition temperature, (-60°C)(Kurniawan D, et al. 2011) PCL is also less rigid than PLA, PGA or PLGA and can be copolymerized or blended with the material to make the total construct more flexible. Both PLGA and PCL degrade by bulk erosion, meaning that the scission of bonds occurs throughout the structure, and water can enter the material bulk. PCL like PLA, PGA and their copolymers have been extensively used in sutures, and fixation devices (Woodruff & Hutmacher. 2010; Madhavan Nampoothiri K, et al. 2010).

2.4.2 Electrospinning

Electrospinning is a process in which polymer melt or a solution containing polymers dissolved in it, is ejected through a nozzle and the emerging string is spun by a magnetic field and collected on to a charged collector(Bhardwaj N & Kundu SC. 2010). Figure 5 illustrates the electrospinning process. By varying different parameters such as the size, or sometimes the number, of nozzles, the state of the polymer, the voltage of the nozzle, different kinds of polymer fibers and patterns can be gained.

Electrospun materials are typically very sparse, thin and mesh-like sheets (Bhardwaj N & Kundu SC. 2010). The polymer strings can move with respect to one another. This makes the material flexible, which can be good or a bad thing depending on the intended use of the material.

Very different polymers can be electrospun: Both natural polymers such as chitosan and synthetic ones such as polyurethanes can be used to form sheets (Lee, et al. 2012). Because solution electrospinning does not require the temperature to be raised above melting temperature of the polymers, growth factors and other medicinal molecules that are heat sensitive can be incorporated into the fibers with their biological activity intact, during the spinning process (Sill TJ & von Recum HA. 2008; Bhardwaj N & Kundu SC. 2010).

Alternatively the drug can be loaded after the material has been spun, making it possible to use either melt or solution spinning for drug-releasing purposes.

Melt spinning and solution spinning give different sized strings: Melt spinning can be used to give strings with thickness in the micron range, while solution spinning can yield fibers in even in 100-500nm thickness range (Bhardwaj N & Kundu SC. 2010). Porogens can be incorporated into the melt or the solution to give the resulting fibers even more varying porosity (Woodruff & Hutmacher. 2010). All in all, electrospinning can be used to make fibers and scaffolds with very different topographical profiles. Scaffolds that are biodegradable and release drugs are hard to design. Both diffusion of the drug through the material as well as the degradation rate of the polymer are important factors: the slower the degradation rate, the more accurately the rate of release can be controlled (Bhardwaj N & Kundu SC. 2010). One method to control the drug-releasing properties of an electrospun material is to make a layered polymer with a technique called core-shell spinning. Here the inner and outer layers have differing degradation rates, making it possible to have a time delayed release of the drug. Alternatively the drug can diffuse through the two layers at different rates (Sill TJ & von Recum HA. 2008).

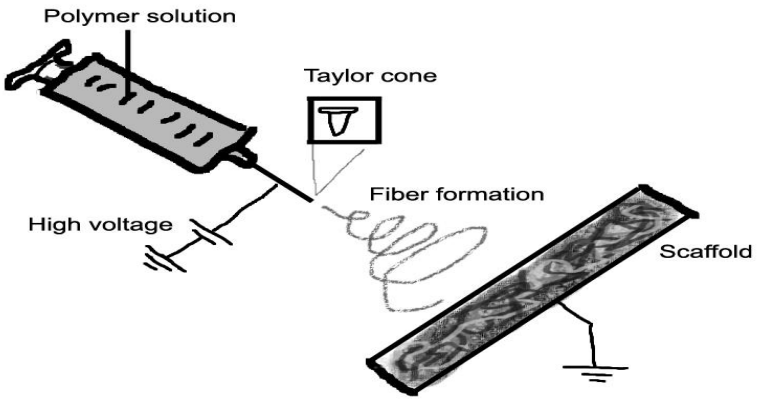

Figure 5.A Schematic drawing of solution electrospinning: A polymer solution is pushed through a charged taylor cone and the formed fiber, spun by an electromagnetic field is gathered on a collector.

2.4.3 Surface topography and chemistry

Surface topology and chemistry play a major role in cell attachment and differentiation just as the mechanical properties of the material. The best results are obtained, when the topology and

chemistry are similar to the ECM of the target tissue (Haimi S, et al. 2009b; Padalhin AR, et al. 2014).

While stiffness of the scaffold has been considered to be a more important factor in inducing osteogenic differentiation, if the cells cannot properly adhere to a surface, there is little possibility for mechanotransduction occurring. This brings us to surface chemistry: the addition of charged proteins, oligopeptides or salts can greatly increase the adherence of cells to surfaces. (Haimi S, et al. 2009a; Padalhin AR, et al. 2014; Ojansivu M, et al. 2015). The ECM in bones is highly polar due to the presence of the hydroxyapatite, the mineral part of bones. Thus hydrophilicity should increase osteogenicity.

Viswanathan and coworkers studied the effects 3D surface topology on the adhesion and differentiation of mesenchymal stem cells and found that porosity had a significant effect on stem cell differentiation (Viswanathan P, et al. 2015). Pärssinen and coworkers studied how the number and size of focal adhesion complexes are related to osteogenic differentiation of hASCs and found there to be a positive correlation between them (Parssinen J, et al. 2015).

As discussed in earlier sections, cell morphology, or cell shape is a key factor in differentiation. What affects the cell shape is the cytoskeleton, which is directly affected by the surface on which the cells grow on. Actin is a cytoskeletal component that affects the cell morphology the most: it connects to both the protein on the nuclear lamina and to proteins on the plasma membrane and thus mediates mechanical signals inside the cell.(Vidal C, et al. 2012). Intracellular fat is an indicator to the direction an adipose stem cell will differentiate (Vanhatupa, et al. 2015). The more there is fat inside the cells the closer it is to adipogenesis. The mechanical and chemical properties of the surroundings of the cells greatly affect amount of intracellular fat as a result of activating signaling pathways that either lead to the activation or inhibition of genes involved in fat metabolism (Shan, et al. 2013).

3. AIM OF THE STUDY

The aim of this study was to quantify the effects of BMP-2 releasing electrospun PLGA-PCL scaffolds on the adipogenic and osteogenic differentiation of hASCs and to evaluate the potential of this combination of material and growth factor as a means for increasing osteogenic differentiation. The evaluation was based on on hASC morphology as well as proliferation and differentiation towards osteoblasts and adipocytes. The main hypothesis in this study wast that the BMP-2 releasing scaffolds would increase osteogenic differentiation.

4. MATERIALS AND METHODS

4.1 Isolation and culture of hASCs

In this study, four different patient cell lines of hASCs were studied. All the hASC lines were isolated from adipose tissue samples obtained from patients undergoing elective surgeries at Tampere University Hospital, Tampere, Finland, with the patients' written consents. The isolation and use of hASCs were carried out in accordance with the Ethics Committee of Pirkanmaa Hospital District, Tampere Finland (ethical approval R03058).

4.1.1 Isolation

The hASC lines used in this study were isolated from subcutaneous fat; either from breast (human fat stem cell lines (HFSC) 5/13, 5/12 and 38/12) or from abdomen (HFSC 9/13). All the donors were women of age 60±5 years. The fat tissue samples were mechanically chopped into small fragments with scissors and the ECM further digested with a solution containing collagenase type I (1.5mg/mL; Invitrogen, CA, USA) in shaking conditions in a water bath at +37°C for 60min. The SVF was isolated by centrifugation at 600g for 10 min and discarding all the other fractions. The red blood cells that might remain in the SVF were lysed by incubating the fraction in sterile water for 2 min. Osmotic conditions were returned with DPBS with added NaCl. Another centrifugation step was performed with the same conditions as above and the pellet resuspended in basal culture medium (BM, see Table 2), consisting of Dulbecco's modified Eagle medium Ham's nutrient mixture F-12 (DMEM/F-12) 1:1 (Gibco®, Thermo Fisher Scientific), supplemented with 1 % L-alanyl-L-glutamine (GlutaMAX; Gibco, Thermo Fisher Scientific), 1% antibiotics/ (100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza, Basel, Switzerland) and 5 % human serum (Hspaa) (PAA Laboratories GmbH, Pasching, Austria) then filtered through 100µm filter and washed with BM prior to plating. The adipose stem cells in the SVF will grow due to their plastic adherence, while other cells wash out during passaging. Figure 6 summarizes the isolation procedure. The cells were expanded in T75 cm^2 flasks made of polystyrene (Nunclon™ Delta treated flask; Thermo Fischer Scientific™) to get rid of the non-plastic adherent cells. All the media containing Hspaa in this study were sterile filtered using Vacucap® 90 PF Filter unit with w/0.8/0.2um Supor® membrane.

Table 2. The compositions of BM and OM.

Components	Quantity(BM)	Quantity(OM)
DMEM/F-12 1:1 1x		
Human serum	5 %	5 %
GlutaMAX	1 %	1 %
Pen/Strep	1 %	1 %
L-ascorbic acid 2-phosphate	200 µM	-
β-Glyserophosphate	10 mM	-
Dexamethasone	5 nM	-
BMP-2	(100 nM)	(100 nM)
Insulin	-	-
Biotin	-	-
Isobutyl-methylxanthine	-	-
Pantothenate	-	-

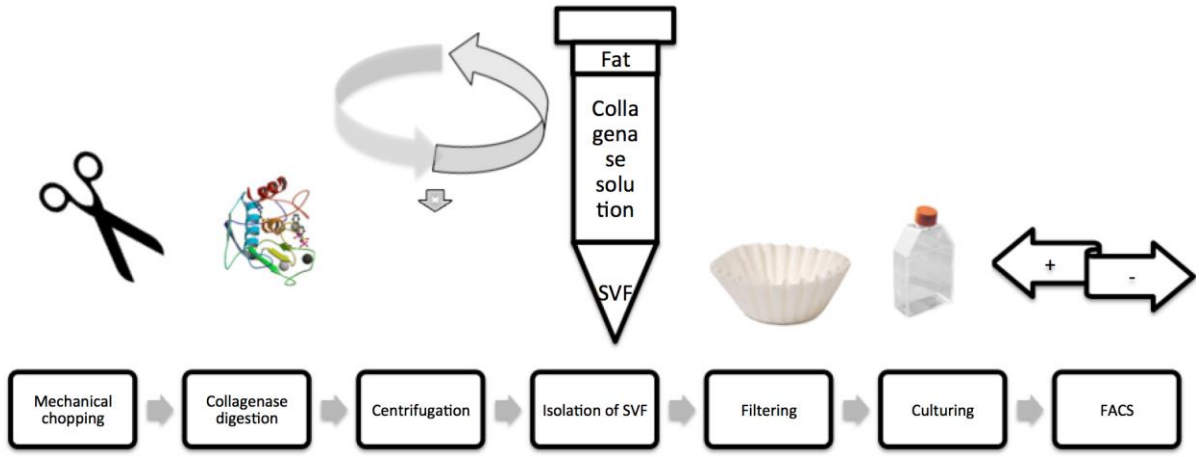

Figure 6. A schematic diagram of the primary culture isolation process.

4.1.2 Characterization

To verify their mesenchymal origin, all the cell lines used in this study have been characterized by showing that they adhere to plastic during isolation and have a pattern of surface marker expression in accordance with the guidelines of ISCT see Table **1**). The relevant surface marker expressions were routinely measured using flow cytometry and the results are listed below.

Table 3. Expression levels of relevant surface markers for the cell lines used in the study. Information for generalized hASC gene expression combined from (Tsuji, et al. 2014; Satish, et al. 2015).

Surface protein	Antigen explanation	HFSC 5/13	HFSC 9/13	HFSC 5/12	HFSC 38/12	Generalized hASC
		Expression level				
3	T-cell co-receptor	-	-	-	-	-
11a	Integrin αL	-	-	-	-	-
14	Serum lipopolusaccharide binding protein	-	-	-	-	-
19	B-lymphocyte-lineage differentiation antigen	-	-	-	-	-
34	sialomucin-like adhesion molecule	±	-	±	-	±
45	Leukocyte common antigen	-	-	-	-	-
54	I-CAM1	±	-	±	-	-
73	Ecto-5'-nucleotidase	++	+	++	+	++
80	Human B7-1 cell surface receptor	-	-	-	-	-
86	Human B2-1 cell surface receptor	-	-	-	-	-
90	Thy-1	++	++	++	++	++
105	Endoglin	++	++	++	++	++
HLA-DR	MHC-II antigens	-	-	-	-	-

Legend: ++= Strong expression >85%, + =expression ≤ 85% ≥ 50% ± =Moderate expression ≤ 50% > 10%, - = weak or no expression ≤ 6%

4.2 Primary cell culture

Cells were cultured in standard cell culture conditions: 5 % CO_2 and 20 % O_2 partial pressure at 37°C in the humidified atmosphere. The cells were expanded in BM and the medium was changed twice a week until they were passaged, seeded or frozen under sub-confluency. TrypLE™ Select (1x) (Gibco®, Thermo Fisher Scientific™), a highly purified animal origin-free replacement for trypsin, was used to detach the cells from the flasks, after which they were centrifuged, supernatant removed, resuspended in BM and counted using a hemocytometer. If extra cells remained after the previous procedure, they were frozen. To store the cells, they were centrifuged and the pellet resuspended in 1,5ml freezing solution consisting of 10 % volume per volume (v/v) dimethyl sulphoxide (DMSO HybriMax™; Sigma-Aldrich, St. Louis Missouri, USA) and 90% (v/v) sterile filtered Hspaa. The cell suspension was placed in a cryotube and frozen in a freezing chamber filled by isopropanol at -80°C over night (o/n) to avoid damage to the cells from quick freezing. The cryotube was moved into gas phase nitrogen cryostorage for long time storage.

When needed for the experiments, cells were taken from the cryostorage, thawed quickly in a +37ºC water bath, resuspended in BM and centrifuged to get rid of the DMSO in the freezing solution by resuspending the pellet again in BM before moving the cells into T75 flasks for further expansion and culturing. The growth and morphology of the cultures were monitored routinely using a phase contrast light microscope and hASCs at passage 2-4 were used for the experiments in order to ensure that there was very little spontaneous differentiation before the experiment.

4.3 Biomaterials

The biomaterial scaffolds have been produced in associate professor Joachim Loo's research group at Nanyang Technological University (NTU) in Singapore using solution electrospinning on a blend of PLGA and PCL (PLGA-PCL). Two types of scaffolds were produced: ones loaded with BMP-2, where BMP-2 was present in the spinning solution and diffused into the polymer bulk before the structure of the material was formed, and ones with no BMP-2, referred to as control scaffolds from now on.

The scaffolds were stored at -20°C air tightly to avoid degradation of the polymer chains and the BMP-2. The scaffolds were weighed on an analytical scale, then placed on 24 well plates and UV-sterilized for 20 min in a laminar hood. Autoclaved cell crowns (Scaffdex, Tampere, Finland) were used to pin the materials to the bottoms of the wells, and the scaffolds were pre-wetted in 1ml of BM for 24 h inside an incubator to get rid of the effects of initial burst of BMP-2 and possible soluble monomers as well as to wash the material after the UV sterilization.

According to information from the NTU, after the initial burst, the material releases BMP-2 for up to 15d at a steady rate of 0,45ng/10mg/day. The masses of scaffolds varied between 1-10 mg.

4.4 Experiment setup, plating and culture

There were two major setups for the study. Experimental setup **I** for the main experiment to quantify the effects of BMP-2 releasing scaffold material on a cellular level, which was repeated with hASCs from four different donors, and setup **II** to gain information about the effects of BMP-2 releasing scaffold material on a RNA and protein levels.

The experimental setup **I** consisted of six different hASC culturing conditions (Table 4): Cells growing on the control scaffolds (groups 1 and 4), cells growing on the BMP-2 releasing scaffolds (groups 2 and 5) and cells growing on control scaffolds with BMP-2 (Sigma) added in the culture medium in 100 ng/ml concentration (groups 3 and 6). Groups 1-3 were cultured in BM and groups 4-6 in osteogenic medium (OM; see Table 4 on page 28). Four different hASC lines, HFSC 5/12, HFSC 38/12, HFSC 9/12 and HFSC 9/13 were cultured under these conditions for up to 21d, during which time relative cell number, alkaline phosphatase (ALP) activity, mineralization, cell morphology morphology and intracellular fat vacuoles were studied as described in more detail below.

In the experimental set up **II**, the HFSC 5/12 cells were cultured in five different conditions (Table 4): Cells growing on polystyrene culture plates with and without BMP-2 added to the medium (groups 7 and 8, respectively), cells growing on control scaffolds with and without added BMP-2 in the medium (groups 9 and 11, respectively) and cells growing on the BMP-2 releasing scaffolds (group 10). The medium used was starvation medium: BM with only 1% (v/v) Hspaa with or without added BMP-2 (50 ng/ml). Western blot (WB) analysis as well as qPCR analysis were run for samples taken at 7 and 14 day time points to detect the activation of BMP-2 signaling pathway as described in more detail below.

Table 4. *Different culture conditions and groups used in this study.*

Experimental setup	Group	Scaffold	Medium	BMP-2 added to the medium	Cells seeded/ 1.9cm^2
I	1	CTRL	BM	-	8000
I	2	BMP-2	BM	-	8000
I	3	CTRL	BM	100ng/mL	8000
I	4	CTRL	OM	-	8000
I	5	BMP-2	OM	-	8000
I	6	CTRL	OM	100ng/mL	8000
II	7	None	1%BM	-	10000
II	8	None	1%BM	50ng/mL	10000
II	9	CTRL	1%BM	-	10000
II	10	BMP-2	1%BM	-	10000
II	11	CTRL	1%BM	50ng/mL	10000

The pre-expanded hASCs were plated onto the pre-wetted scaffolds as follows: The pre-wetting medium was removed and 900ul of BM, BM+BMP-2, OM or OM+BMP-2 medium was added on the scaffolds. Then, the hASCs were seeded onto the scaffolds in 100ul of BM. The number of cells seeded/well (1.9 cm^2) was 8000 or 10000 for the experimental set ups **I** and **II,** respectively (See Table 4).

During the experiments the medium was changed precisely every three days. In the medium exchange, 480 µl of medium was removed from each well using a pipette and 500 µl of fresh pre-warmed medium was added to make up for medium lost through evaporation. Three parallel samples of each group and of the four cell lines were used in all analytical methods, resulting in the total number of parallel samples 12, except for qPCR and immunofluorescent microscopy, for which only two parallel samples were used. In addition, a cell-free blank

sample was used for each group and for each cell line. The overall work flow and the timing of analyses can be seen in Figure 7.

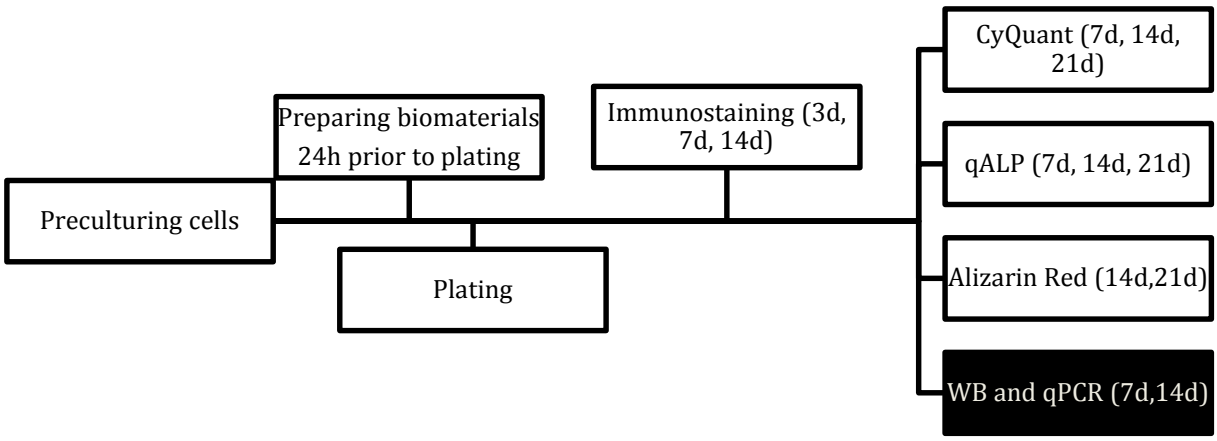

Figure 7. Showing the workflow of the experiments and the analyses run at different time points. Experimental set up II analyses in reversed colors.

4.5 Analyses

4.5.1 Proliferation

The relative cell number of the samples was analyzed using CyQUANT Proliferation Assay Kit (LifeTechnologies, Thermo Fisher Scientific) based on the total deoxyribonucleic acid (DNA) amount in the samples. In the method, the total DNA of the cells is stained, and the absorbance is measured, giving a relative amount of cells per sample.

At time points 7d, 14d and 21d the cells cultured on the biomaterials were lysed by pipetting them with 300 µl of 0.1% (V/V) Triton-X 100 in PBS, while scratching the surface of the biomaterial. The plates were frozen at -70C to further lyse the cells and wait for the analyses. After thawing, the samples were collected into separate micro centrifuge tubes by repeating the pipetting and scratching as above and centrifuged to separate the debris from DNA and to get rid of the foam that formed during the collection. The cell lysates that were used for CyQuant were also used later for quantitative ALP analysis (qALP).

Three parallel 20 µl samples were pipetted onto a flat-bottom 96-well plate and 180 µl working solution (consisting of cell lysis buffer and GR-Dye (CyQUANT® GR-Dye) that was produced according to the manufacturer's instructions was pipetted on the samples. The fluorescence of the samples at 480/520 nm was measured using Wallac Victor 1420 Multilabel Counter; Perkin Elmer; Waltham, Massachussetts, USA) and the relative cell numbers were considered as

directly proportional to fluorescein counts. Fluorescence from Triton-X-100 was taken into account in the final analysis, by subtracting its emission from the samples.

4.5.2 Fluorescence imaging

Immunostaining along with fluorescence imaging was used to study the morphology and growth patterns of the hASCs as well the amount of lipid droplets inside the cells at 3d, 7d and 14d. AlexaFluor® 488 Phalloidin (Pha) (ThermoFisher Scientific) was used to visualize the cytoskeleton of the cells. Phalloidin works by binding to the fibrous form of actin and preventing it from depolymerizing. This allows for the visualization of the cytoskeleton and cell shape. The samples were counter-stained with 4, 6-diamino-2-phenylindole-dihydrochoride (DAPI) to visualize the nuclei of the cells and Oil red O (ORO) to detect the fat vacuoles in the cells and the scaffold material.

The medium was removed and the cells were washed twice with 1ml Dulbecco's phosphate buffered saline (DPBS). The cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) for 60min and washed thrice with 1ml DPBS. The cells were then permeabilized with 0,1% Triton X-100 for 12 min and washed again thrice with 1ml DPBS. The cells were then blocked with 1% weight per volume (w/v) bovine serum albumin (BSA) overnight at +4°C. AlexaFluor® 488 Phalloidin (ThermoFisher Scientific) was diluted 1:300 into the blocking solution and the cells were incubated in the 250µl of phalloidin solution for 60min in dark. Prior to ORO staining, the cells were washed thrice with 1ml DPBS, once with water and the samples were incubated in 400µl 60% isopropanol (2-propanol; EMD Millipore) for 2min. The Oil Red O stock solution (Sigma-Aldrich) was diluted 3:2 in milli-Q water, then filtered twice through a filter paper and incubated with the samples for 15min. The cells were thoroughly washed four times with 1ml water and mounted on slides using Vectashield H-1200 mounting agent that included DAPI (Vector Laboratories). All images were taken within one week's time after the staining. OLYMPUS IX51 microscope and DP manager and controller software were used to take gray scale images that were modified with Adobe Photoshop CS4 to give color and merge them.

4.5.3 Quantitative ALP activity analysis

ALP is an enzyme active in the early phases of bone differentiation (Halvorsen et al. 2001; Zuk et al. 2001). Its activity is thus an early marker for bone differentiation. The activity is

quantitated by providing the phosphatase substrate and the correct alkaline conditions for the enzyme to work for a fixed period of time. In the analysis, *p*-nitrophenyl phosphate is used as a substrate. ALP removes a phosphate group from the substrate at pH 10.4 to produce a yellow colored p-nitrophenol and a phosphate. ALP concentration in the sample is proportional to the rate of p-nitrophenol formation, which can be measured with colorimetry.

For the quantitative ALP activity analysis (qALP), the same lysate samples that were used for CyQuant and collected at 7d, 14d and 21d were used. 0.1% Triton-X-100 was used as a blank. The three parallel 20 µl samples were incubated with a working solution consisting of alkaline buffer solution (2-amino-2-methyl propanol; 1.5M; pH 10.3; Sigma-Aldrich) and substrate solution (p-nitrophenyl phosphate, disodium; Sigma-Aldrich) for 15min at 37 °C. 50 µl of 1M NaOH (Sigma- Aldrich) was added to stop the reaction after which the absorbance at 405 nm was measured using a microplate reader (Victor Wallac 1420 Multilabel Counter, Turku, Finland). The ALP activity and cell numbers were determined from the same cell lysates, which allowed for calculating the normalized ALP activity in proportion to cell number.

4.5.4 Alizarin red staining and quantification

Alizarin red dye binds to divalent cations such as magnesium and calcium, the latter of which is a major constituent of bone ECM. It is a late marker for bone differentiation. The dye can be extracted with cetylpyridum chloride solution to quantify the results.

At time points 14d and 21d the medium and the cell crowns were removed and the cells washed once with 1ml PBS. The cells were fixed onto the biomaterials by incubating them in 500 µl of 4 % PFA for 15-30min at room temperature (RT). The cells were washed twice with distilled water (dH_2O) and stained with 2 % Alizarin Red S (Sigma-Aldrich) solution (pH 4.1-4.3) for 10min. The excess dye was washed away several times with (dH_2O) and once with 1ml of 70% ethanol. The wells were photographed with digital pocket camera Panasonic Lumix DMC-TZ6 digital camera to gain a qualitative estimate of mineralization. The dye was then extracted by incubating the cells in 500 µl of 100 mM cetylpyridium chloride monohydrate (Sigma-Aldrich) for 3h at RT on a shaker. Three parallel 100 µl samples from each well were pipetted onto a 96-well plate and their absorbances were measured at 544nm with a microplate reader (Victor Wallac 1420 Multilabel Counter) to quantify the mineralization.

4.5.5 SDS-PAGE and Western Blotting

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an electroforetical method in which proteins are separated based on their molecular weight and charges with the help of electric current on a gel. Without the SDS in the gel, the charge of the protein affects how its movement on the gel, but as 1,4g of SDS binds to 1g of protein and masks its charge, the separation occurs primarily on the basis of protein mass. Once the proteins are separated, they can be transferred onto a nitrocellulose membrane using electric current and probed directly or indirectly with enzyme conjugated antibodies such as horseradish peroxidase antibodies to ascertain the presence of selected proteins in a process known as Western Blotting (WB). The workflow is summarized in Figure **8** below.

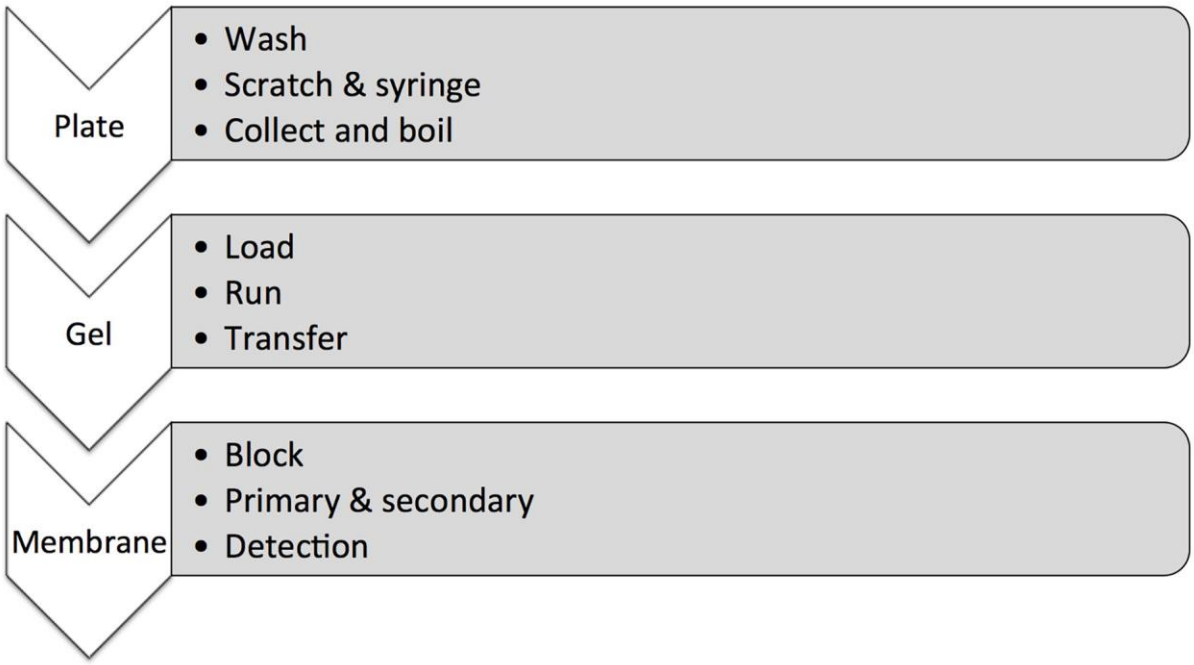

Figure 8. A diagram showing the workflow for sample preparation and western blotting.

The medium and the cell crowns were removed from the wells. The samples were washed once with 1ml of DPBS and the plates were placed on ice. Cells were collected from three parallel wells by scraping into a total of 150 µl of SDS-PAGE loading buffer. The samples were then repeatedly drawn and pushed through a 20G needle to aid the lysis of the cells. The samples were incubated for 5min at 95°C to denature the proteins and reduce the Cys-Cys sulphur bridges in them. The samples were then frozen at -20C°C.

The 5 µl samples for β–actin analysis and 30 µl for the other analysis and 5 µl of molecular weight marker (PageRuler™ prestained protein ladder, Thermo Fischer Scientific™) were

loaded onto gels and the gels were run using Mini-protean® Tetra system; BIO-RAD; Hercules; California, USA) with 120V voltage, until the lightest of prestained molecular markers reached the end of the gel.

The gels were moved to semidry transfer device (Trans-Blot® SD Semi dry transfer cell; Bio-Rad; Hercules; California; USA; Electrophoresis Power Supply EPS-601; GE Healthcare) and the proteins were transferred onto a methanol (EMD Millipore) activated PVDF membrane (Amersham™ Hybond™ 0.45 um PVDF Blotting membrane; GE Healthcare; Little Chalfont; UK) with 15 V and 300 mAh, 1h. The membranes were washed with Tris-buffered saline with 0,05 % Tween 20 (Sigma-Aldrich) (TBST). The non-specific binding sites on the proteins were blocked with 5 % (W/V) milk (Non-fat milk powder; Valio; Lapinlahti; Finland) TBST solution o/n. After 3 x 5 min TBST washes the membranes were incubated with primary (Mouse Anti-b-actin; Santa Cruz Biotechnology, Dallas, Texas, USA; Rabbit Anti-Noggin antibody; Abcam®, and P-SMAD-antibodies (Cell Signaling Technology, Danvers, MA, USA) (1:1000 dilutions in 5 % (W/V) milk in TBST) and secondary antibodies (Goat anti-rabbit IgG HRP conjugated antibody; Santa Cruz Biotechnology, Dallas, Texas, USA and Goat anti-mouse IgG HRP conjugated antibody; Santa Cruz Biotechnology, Dallas, Texas, USA) (1:10000 dilutions in 5% (W/V) milk in TBST) for 1h at RT with 3 x 5 min TBST washes in between. After the secondary antibody incubations the membranes were exposed to Amersham enhanced chemiluminescent substrate (GE Life Sciences) for 5 min to induce chemiluminescence. X-ray films (Amersham™ Hyperfilm™ ECL; GE Healthcare) were placed on the blots and developed at varying exposure times from 30 s to 15 min.

Semi quantification of PSMAD and b-actin levels was done using FIJI software package.

4.5.6 Real time quantitative polymerase chain reaction

Quantitative real time polymerase chain reaction qPCR was used to compare the relative or absolute gene expression levels in cell samples. Expression levels of osteogenic and adipogenic genes in different culturing conditions were compared in this study.

The total RNA was isolated from the samples using a nucleospin kit (Macherey-Nagel GmbH&Co.KG, Düren, Germany) at 7d and 14d time points under starvation culture conditions according to the instructions of the manufacturer and stored at -70C. A High-Capacity cDNA Reverse Transcriptase Kit (Applied biosystems, Foster City, CA, USA) was used to synthesize

the cDNA from the mRNA in the isolated total RNA. Reverse transcription was carried out as follows: 10 min at 25 °C, 2h at 37 °C, 0,05 sec 85 °C and incubation at 10 °C.

The expression levels of osteogenic marker genes *Osterix* (*OSX*) and *Distal-less homeobox 5* (*DLX5*) as well as the adipogenic marker genes *Human adipocyte protein 2* (HAP2) and *PPARg* were analyzed. The level of Noggin, an extra cellular antagonist of BMP-2 was also analyzed. *Human acidic ribosomal phosphoprotein P0* (*RPLP0*) is a gene that codes for a protein in the large sub unit of a human ribosome. Its expression levels are relatively constant in the cell so it is useful as a reference gene to which all the other expression levels were normalized.

The qPCR mixture contained 30 ng cDNA, 300 nM forward and reverse primers, and SYBR Green PCR master Mix (Applied biosystems). The reactions were carried out with an Applied Biosystems 7300 Real-Time PCR System machine using the protocol in Table 5.

Table 5*. The qPCR program.*

1. Activation	50°C 0min
2.Hot start	95°C 10min
3.Dissociation	95°C 15sec
4.Annealing and extension	60°C 1min 45xBack to 3.
5. Storage	4°C as long as needed

4.5.7 Statistical analysis

The amount of parallel samples was sufficient for statistical analysis, excluding WB and qPCR. Mann-Whitney test with Bonnferroni correction for the p-values was conducted for the CyQuant, qALP and Alizarin Red results. Corrected p-values of 0.05 or smaller were considered statistically significant, however no statistical significances in the data between different culturing conditions and time points were found in this study. The statistical analysis was conducted with RStudio R version 3.1.2 which is a non-commercial software.

5. RESULTS

5.1 Proliferation of cells

The proliferation of cells was measured using CyQuant cell proliferation assay, as described in section 4.5.1. The results of total DNA measurements are presented below in Figure 9.

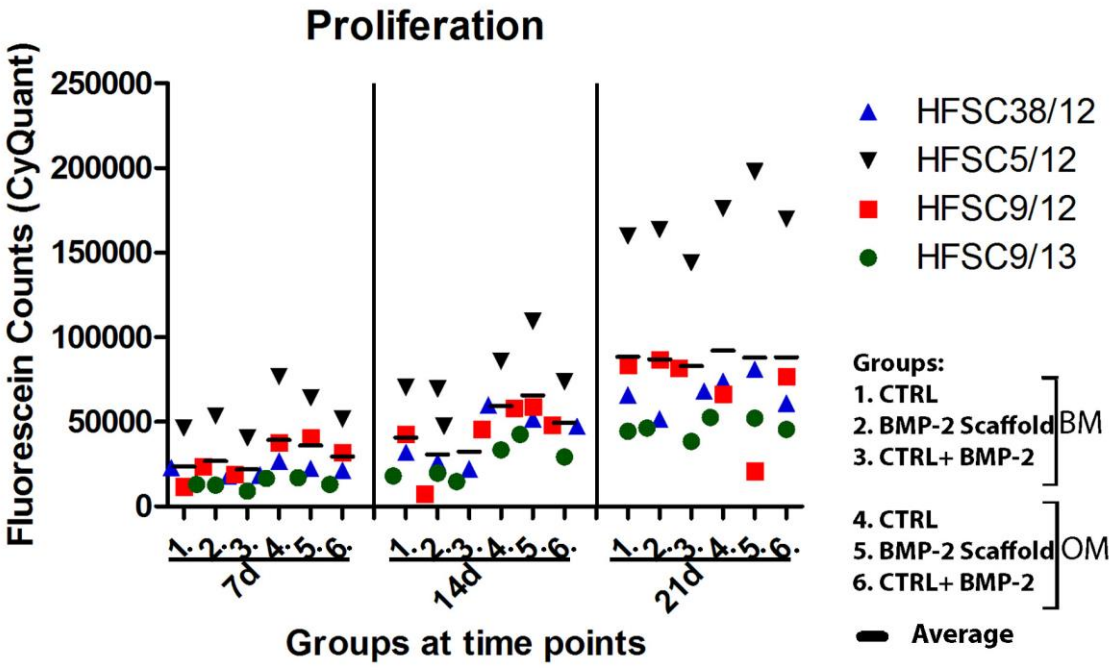

Figure 9. Relative cell numbers in groups of different cell lines at time points 7-21d. For further details in the culture conditions refer to Table 4 on page 28.

From Figure **9** it can be seen that on average the fluorescein counts, which are relative to cell numbers, are higher in OM groups than in BM groups. Similarly there seems to be an inverse relation with BMP-2 concentration to cell numbers, except at 14d, where the BMP-2 releasing scaffolds in OM have highest Fluorescein counts except for HFSC38/12. The difference in average cell numbers between BM and OM groups is most apparent at 14d, but at 21d the difference has diminished.

As can be seen from Figure **9**. HFSC 5/12 seems to have the most cells in any given group at any given time point, while HFSC 9/12 has the least cells at 14d group 2 and again at 21d group 5. Most variation in cell numbers between the groups can be seen at time 14d especially between groups 2 and 5 as well as 3 and 6. Least changes between groups can be observed with cell line

HFSC 9/13. The general trend is that OM samples have more cells and the cell numbers increase with time.

5.2 Morphology and intracellular fat results

The results of morphology studies are seen in the form of immunofluorescence images below. While only images of HFSC 9/13 are shown, images taken from HFSC 9/12 were virtually indistinguishable from those presented with respect to growth patterns, morphology and intracellular fat vacuoles.

From Figure **10** it is apparent that cells grow in tight clusters on the BMP-2 releasing scaffolds throughout the study. Figure **10** also shows that the cells proliferate faster in OM than in BM and that cells grow as clusters on the BMP-2 releasing scaffold material. The intracellular fat cannot be seen at this magnification, but in images with higher magnification such as in Figure 11, the fat is easily distinguishable.

Throughout the study from 3d to 14d, intracellular fat was present in all the groups. From Figure 11, it is apparent that there are fat vacuoles present in the groups and time points, where the least and most osteogenic differentiation is hypothesized to have occurred. There is however considerably less fat present in CTRL + BMP-2 in OM group at 14d than at group CTRL in BM group at 3d (Figures 11 A and D respectively).

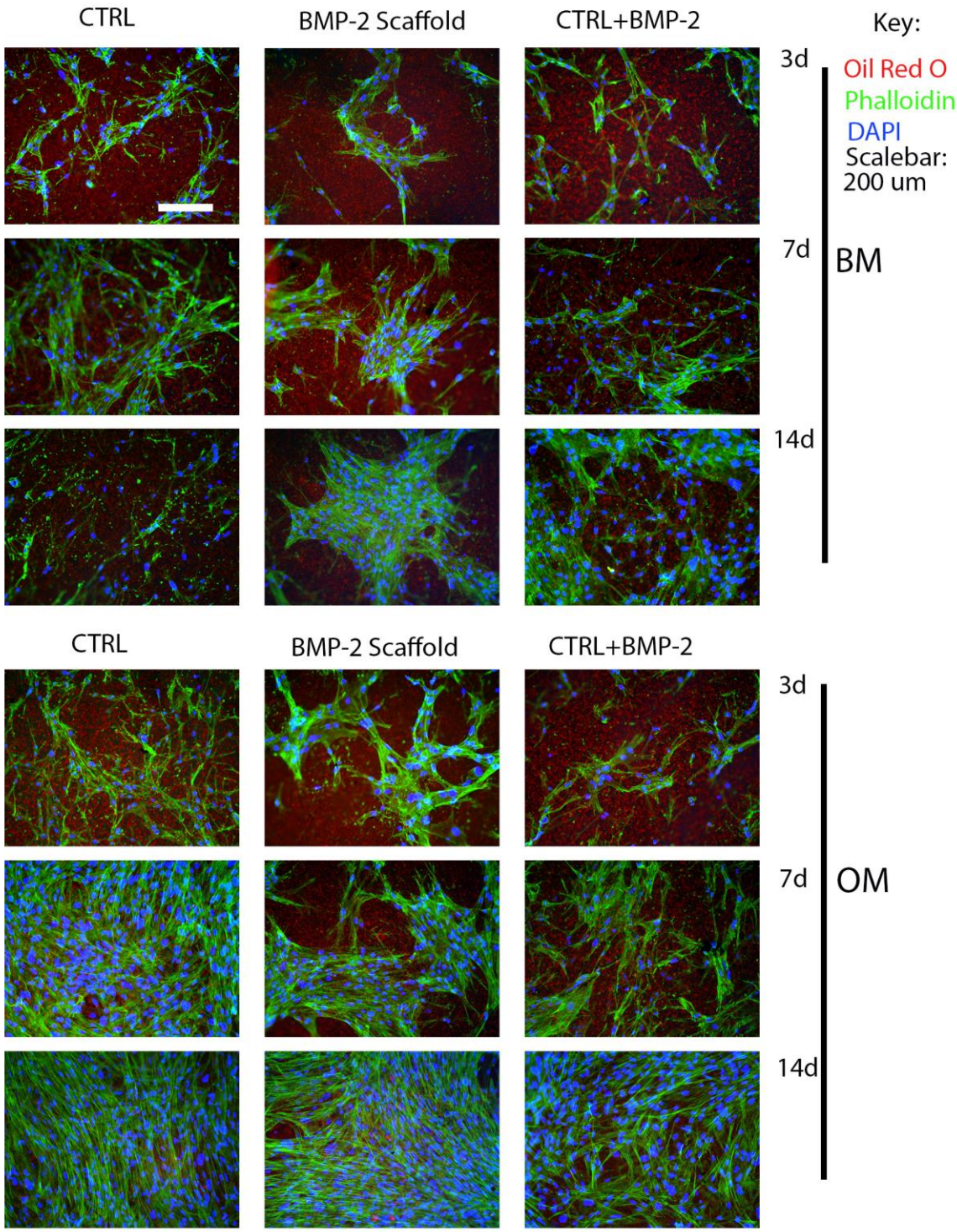

Figure 10. Comparing the growth pattern of HFSC 9/13 in different culturing conditions at time points 3d, 7d and 14d.

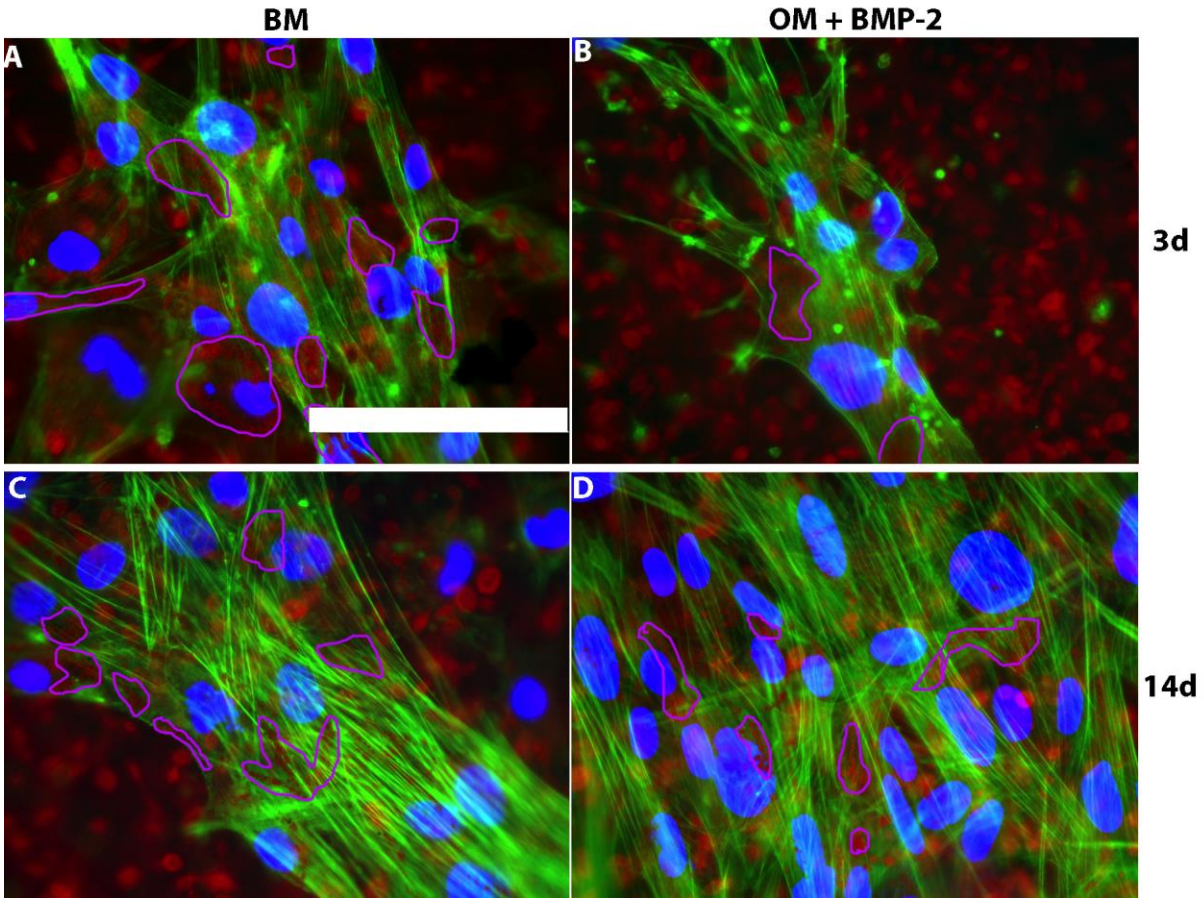

Figure 11. 40x magnified Pha-ORO-DAPI staining of HFSC 9/13 showing 40x magnified representative images of cells in groups where the least (A, C) and most (B, D) osteogenic differentiation was expected to take place BM at 3d and 14d (A, C) and cells cultured on control scaffolds in OM + BMP-2 at 3d and 14d (B, C). Scale bar= 200 µm. ***Green= Phalloidin/cytoskeleton, Red=ORO/material/fat vacuoles, Blue=DAPI/nucleus.***

5.3 Early differentiation results

The results of qALP related to the total DNA results are presented below in Figure 12 below. As discussed in section 4.5.3, ALP activity is an early marker for osteogenic differentiation.

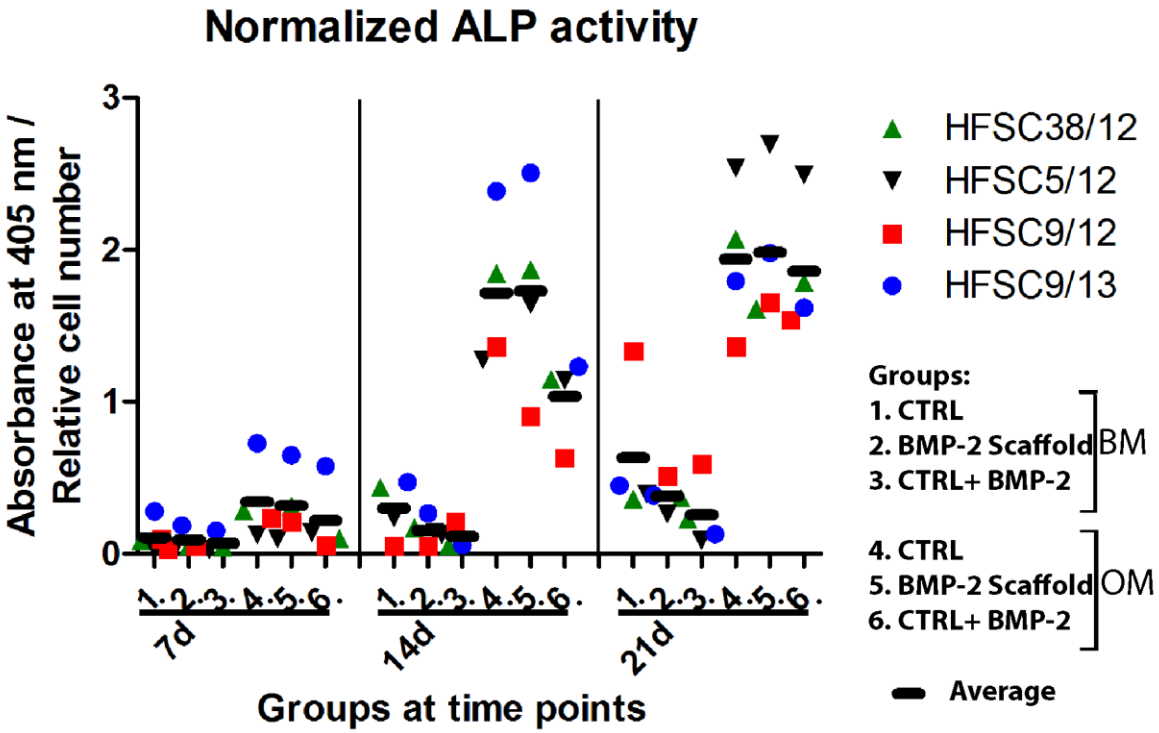

Figure 12. Results of normalized qALP. The cell line average absorbances measured at 405 nm and normalized to the total DNA represented as individual values. Black bars represent the average of all the cell line values.

As can be seen from Figure 12, the relative ALP activities vary greatly between cell lines and it is hard to see any similarities between them. For HFSC9/12 there seems to be an inverse relationship between BMP-2 concentration and activity at 14d, although it is not statistically significant. For the rest of the cells lines at 14d in OM, it seems that the BMP-2 scaffold groups have the highest ALP activity. Interestingly on average the lowest ALP activities, when comparing BM and OM groups at any time points, can be seen in groups where the BMP-2 concentrations are highest. At 21d, the BMP-2 scaffold groups have the highest ALP activities on average as well, although the results from HFSC38/12 differ from the other cell lines. The most obvious changes between ALP activities within time points are seen between BM and OM groups

5.4 Mineralization results

Images taken from the Alizarin red stained biomaterials at 14d and 21d can be seen in Figure 13 and Figure 14 respectively. The results of Alizarin red stain quantification related to cell

numbers are presented below in Figure 15. As discussed in section 4.5.4, mineralization is a late marker for osteogenic differentiation and therefore samples were not collected at 7d.

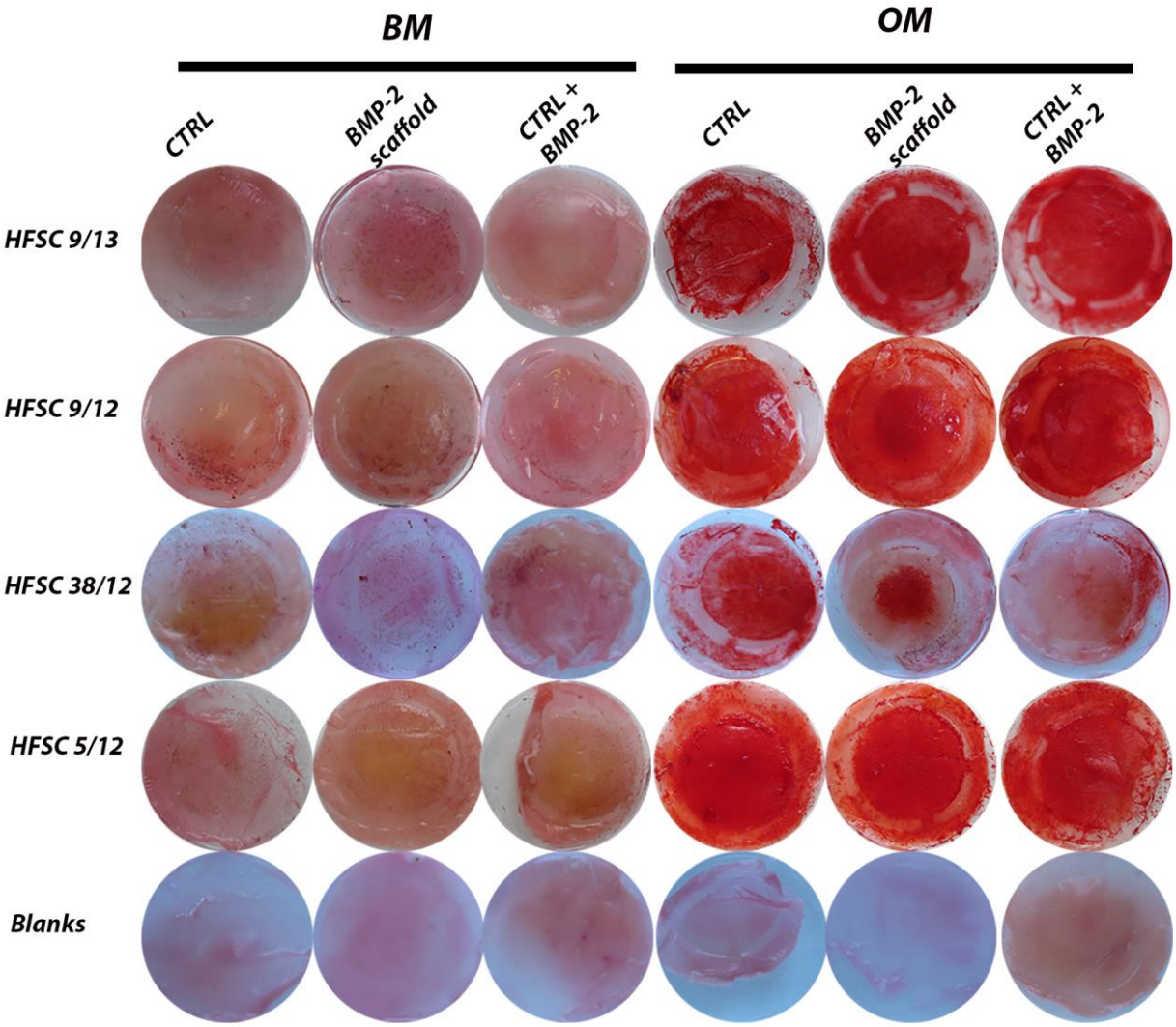

Figure 13. Digital images taken from individual scaffolds at 14d time point. The cells were cultured in set up condition I and stained with Alizarin red.

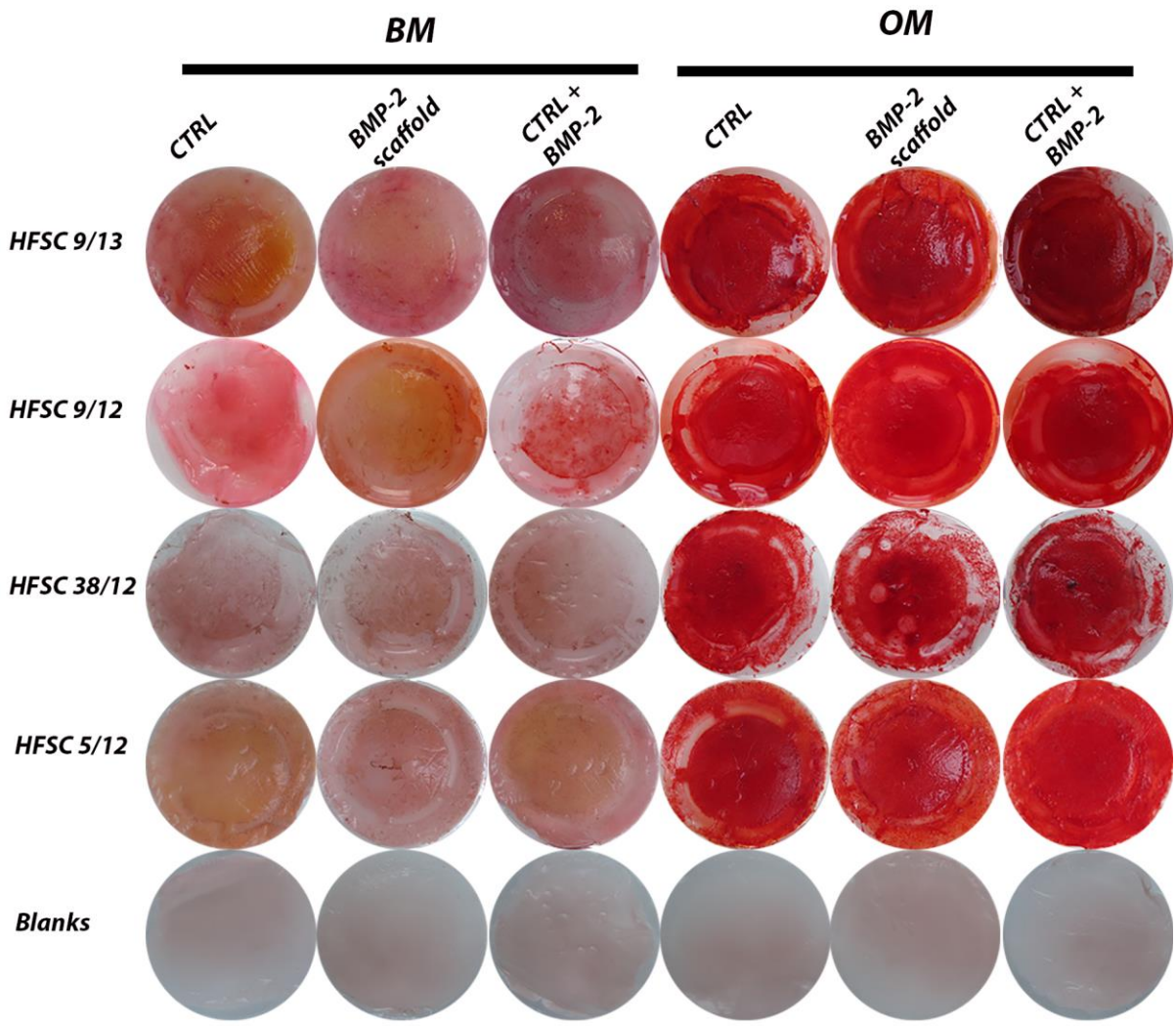

Figure 14. Digital images taken from individual scaffolds at 21d time point. The cells were cultured in set up condition I and stained with Alizarin red.

While Alizarin red analysis was made to analyze the mineralization of different groups, these images also show the material. The variance in the quality of the materials is made apparent, when comparing images taken from the individual wells. It can be seen how the thickness of the material varied and how the individual scaffolds were not even and straight, but rather had the tendency to curl up on their own without the cell crowns.

Differences in mineralization between 14d and 21d are clearly visible when comparing Figure 13 and Figure 14 respectively. The differences between BM and OM groups are also clear, but

no major differences can be seen between groups that differ only in the BMP-2 concentration. The quantified data can be seen in Figure 15.

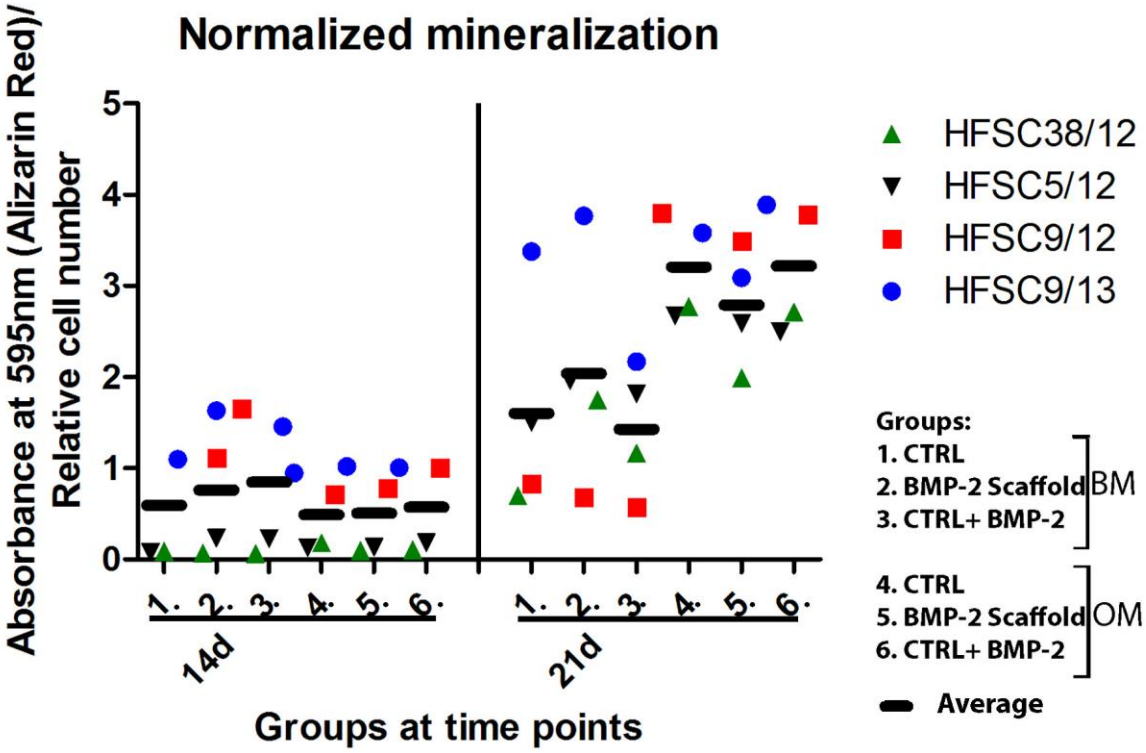

Figure 15. The results of Alizarin red Staining related to cell numbers. Cells were cultured in set up conditions I.

While there seems to be a lot of variation between the groups within time point 21d as well as when comparing the results between groups at both the time points, there are no statistically significant differences owing to the great variation between cell lines within groups. Interestingly at 14d OM groups have slightly lower mineralization than BM ones. For HFSC9/12 BMP-2 increasing BMP-2 concentration seems to increase mineralization at 14d, but lower it in BM at 21d. The differences are however minor. Excluding HFSC9/12, at 21d the BMP-2 scaffold group has highest mineralization for all the cell lines in BM. Interestingly excluding HFSC5/12 the reverse is true in OM. However, the greatest differences in mineralization can be seen between OM and BM at time points.

5.5 WB and Activation of SMAD signaling

The results of the WB and immunodetection are presented are presented below on the parts that are representable in Figure 16.

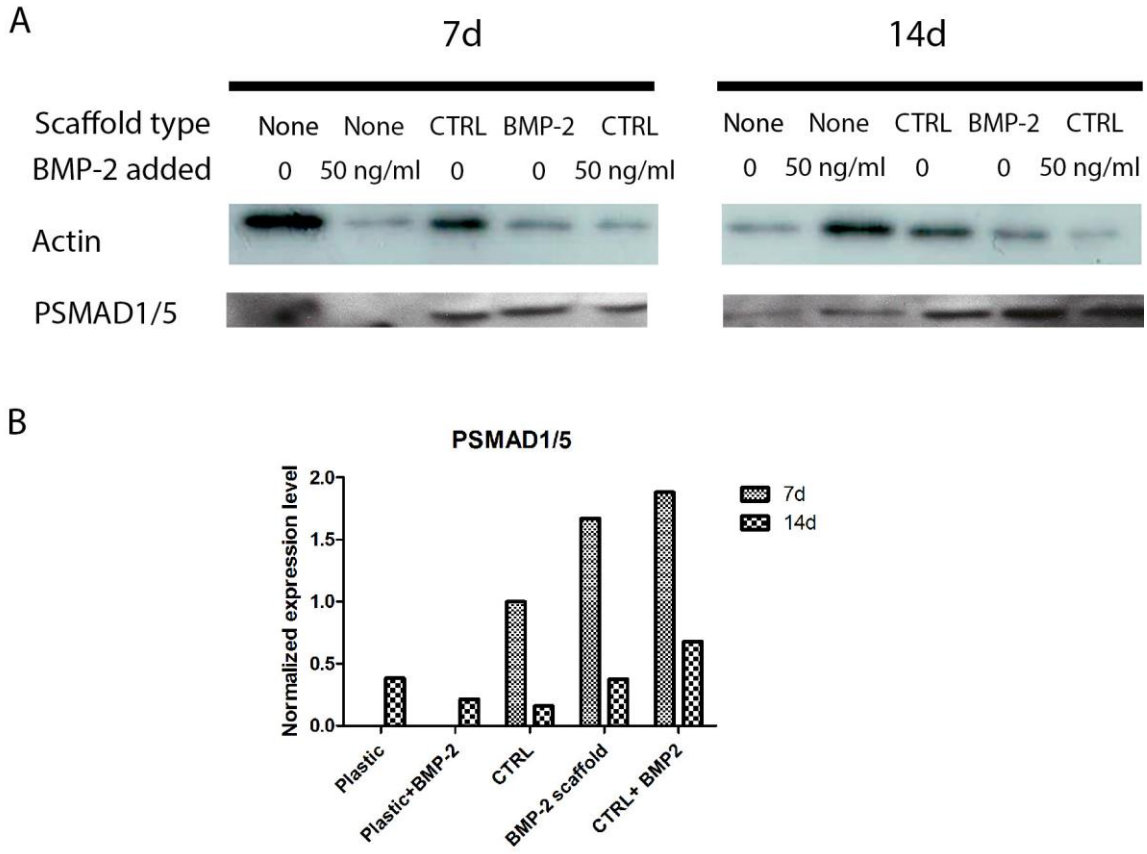

Figure 16. The results of WB immunodetection. A) Above, the relative β-actin levels between groups and below, the relative PSMAD levels at 7d and 14d. B) The expression levels of phosphorylated SMAD1/5 normalized to β -actin levels and related to CTRL group at d7.

From the β-actin expression results (Figure 16 A) it seems that at 7d the cells fare best in groups without any BMP-2. Furthermore the cells seem to grow best either the bare well or on the control scaffold. At 14d plastic+BMP-2 group has the most cells followed by CTRL scaffold group.

From Figure 16 it can be seen that SMAD phosphorylation has not occurred at 7d in plastic and plastic + BMP-2 groups, but at 14d the signaling pathway has been activated in all the groups. The PSMAD levels are however highest at 7d groups and there seems to be a trend, that shows phosphorylation to be directly related to BMP-2 concentration in cells grown on the substrate. Interestingly at 14d it looks as if BMP-2 has an adverse effect on the SMAD phosphorylation in cells grown on plastic, but an opposite effect if the cells are cultured on the substrate.

Unfortunately the blots stained with anti-noggin antibodies had so much background that they could not be scanned with our equipment. The films could only be interpreted when looked at against direct light. The noggin WB results were similar to the qPCR results (Figure 17 E), showing that the protein expression was highest in BMP-2 containing groups.

5.6 Expression of marker genes

The results of qPCR are summarized below in Figure 17. Osteogenic marker gene expressions are highest at 7d in the groups where BMP-2 has been added to the medium, while the opposite seems to be true for the adipogenic ones. This is most obvious when looking at *OSX* and *PPARg,* at 7d. *hAP2* levels stay relatively constant on the substrates. At 14d the *hAP2* expression follows a similar trend to that of *PPARg* on 7d. The highest *PPARg* expression can be seen on CTRL scaffolds without additional BMP-2.

When comparing the *PPARg* and *DLX5* results (Figure 17 C and B respectively) it seems that the material by itself is adipogenic, but the addition of BMP-2 turns the expression levels around from adipogenic to osteogenic. *hAP2* expression, in contrast to this, is showing the plastic to be more adipogenic than the material at 7d.

The *noggin* expression is highest in groups with added BMP-2 at 7d and also higher in the cells cultured on bare wells than on control or BMP-2 scaffolds. At 14d the expression is low in all groups.

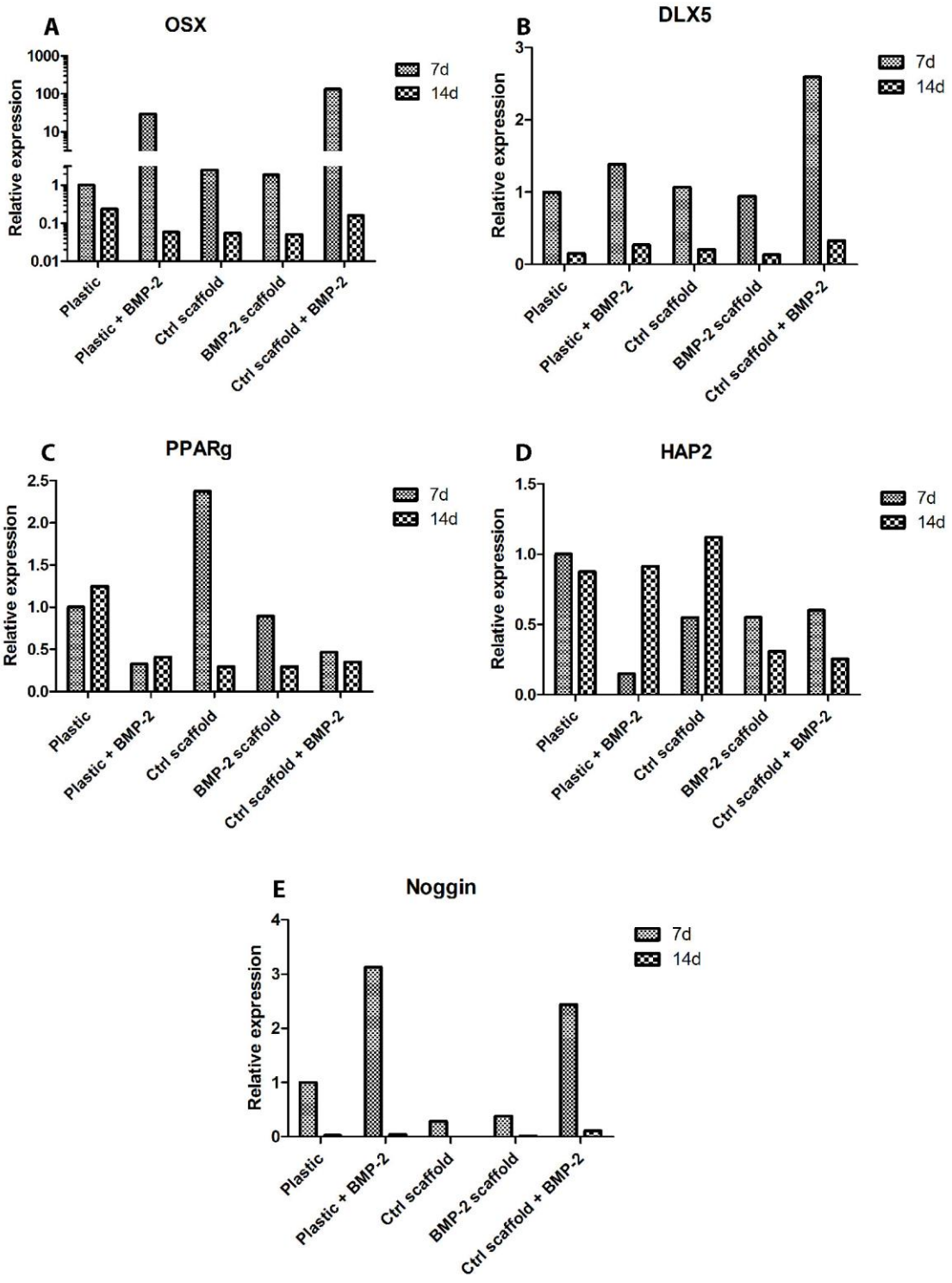

Figure 17. relative expression levels of marker genes for osteogenesis (A, B) adipogenesis (C, D) and BMP-2 antagonist Noggin of cells at 7d and 14d cultured in set up conditions II. The expression levels are normalized to RPLP0 and related to Plastic groups at 7d.

6. DISCUSSION

BMP-2 releasing scaffolds were used in this study in order to differentiate adipose stem cells into osteoblasts. This study is a part of a series of investigations to search for good materials, growth factors and their combinations to optimize osteogenic differentiation of hASCs. The results of this study will help to narrow down said combinations.

The effects of the material, BMP-2 and their combination were assessed using techniques that are a standard in the field of adipose stem cell research. Proliferation was assessed using CyQuant, which quantifies the total DNA and RNA in a sample. Osteogenic differentiation was assessed using alkaline phosphatase activity assay and quantitative alizarin red staining, as well as qPCR of key regulator genes *DLX5* and *OSX* and WB of PSMAD, a key component of a BMP-2 related signaling route important for osteogenesis. Adipose differentiation was evaluated using qPCR for *hAP2* and *PPARG*, important marker genes for differentiation. In addition to the aforementioned methods, the cells were observed using fluorescence microscopy to see their shape, growth patterns and intracellular fat, which are qualitative indicators for differentiation.

As can be seen from the results section no statistically significant relation between BMP-2 concentration and any of the parameters such as ALP-activity, Alizarin red staining or cell numbers was observed from the pooled data. The individual stem cell lines had different reactions to the conditions so no generalizable conclusions can be drawn from the data. While the general trends seen in the behavior of the cells is not statistically significant, the results themselves are significant in the sense that combined with literature they give us ideas in which direction to focus our efforts when designing future studies.

6.1 Evaluation of results

BMP-2 is a growth factor known well in the field of bone differentiation and has even been used in clinical treatments (Sandor GK, et al. 2013; Sandor, et al. 2014). There are contradicting views and results in literature concerning the effects of BMP-2 on hASC differentiation that must be kept in mind when evaluating the results. Some studies say that BMP-2 is beneficial for osteogenesis (Dragoo, et al. 2003), while others say it isn't (Peterson B, et al. 2005; Zuk, et al. 2011). Many studies have investigated the different outcomes of using BMP-2 for osteogenic

differentiation and tried to make sense of the results that sometimes indicate the effects to be more beneficial for adipogenesis (Chen, et al. 1998; Kang, et al. 2009). Vanhatupa and coworkers in their recent study came to the conclusion that the effects are patient or donor specific (Vanhatupa, et al. 2015). This makes sense in the light the effects that genetic variability or epigenetic changes of donated cells can have on the effects of BMP-2 on hASCs (Takada, et al. 2012): for example the expression of different BMPRs, that favor either osteogenesis or adipogenesis (James. 2013).

6.1.1 The Effects of BMP-2 on hASC proliferation and differentiation

High amounts of BMP-2 have been shown to decrease the proliferation or even cause apoptosis of different cell types, including hASCs (Tirkkonen, et al. 2013a) and osteocytes (Kim, et al. 2013). The decrease in proliferation is not unexpected, as cells tend to lose their proliferative capabilities after differentiation. In some studies BMP-2 has likewise been shown to increase the amount of intracellular fat(viite), but here oil red o -staining could not be used quantitatively to assess if BMP-2 affects the amount of intracellular fat in the current study, because the biomaterial itself binds the staining agent.

BMP-2 has been shown to have varying effects on hASC differentiation (Vanhatupa, et al. 2015). Similarly the potency of BMP-2 has been shown to be dependent on the organism it has been produced in. The BMP-2 that was added into the culture media in this study was recombinantly produced in *E. coli*. BMP-2 produced in E.coli has been shown to be less bioactive than that produced in eukaryotic organisms (Vanhatupa, et al. 2015). The BMP-2 used in the scaffolds was also produced in E. coli. In the literature BMP-2 have been added to culture media in greatly varying amounts for MSC differentiation. The amount of BMP-2 released by the scaffolds in current study is many folds lower than that typically used in bone differentiation: as mentioned in section 4.3, the BMP-2 release rate of the scaffolds was 0,45ng/10mg/day for up to 15d and their masses varied between 1-10 mg. With the medium changes taking place every third day, the concentrations remained very low. Lysdahl and coworkers tested extensively the BMP-2 stimulation time and concentrations on BMSCs and suggest that exposure to concentrations of as low as 10 ng/ml for 15min were more effective for osteogenesis than continuous exposure to BMP-2 in concentrations similar to those used in current study (Lysdahl, et al. 2014). At another extreme, in the clinical treatment reported by Sandor and co-workers the scaffold was incubated in a solution with 12mg BMP-2 (Sandor GK, et al. 2013). While the drug release profile was not reported in the study, it could be

assumed that the hydrophilic surface adsorbed more than the amount reported in the current study. Unfortunately in the current study no statistically significant differences between groups exposed to different amounts of BMP-2 were found when proliferation, ALP activity and mineralization were studied. This is partly due to the great variance between the individual cell lines. There were not enough individual samples to make statistical studies from the qPCR and WB results, but the former showed great differences in the expression of marker genes between different culturing conditions.

In the current study, the cells grow in greater numbers in OM than in BM, which can be seen both in the fluorescence images and the quantitative cell number analysis results (Figure 10 and Figure 9 respectively). Cell numbers are smaller in in BMP-2 containing groups, which is logical as BMP-2 is a signal molecule that should promote differentiation and not proliferation. This is also supported by β-actin WB results at 7d in set up conditions **II** (Figure 16 A). Similar results were gained by Kyllönen and co-workers concerning BMP-2 and especially BMP-7 (Kyllonen, et al. 2013). From Alizarin red and qALP results it can be seen that osteogenic differentiation occurs in OM better than in in BM, as expected. The choice of medium seems to have more effect on the differentiation outcome than the amount of BMP-2 present. This is consistent with studies performed by Tirkkonen and co-workers (Tirkkonen, et al. 2013b)**.** The qPCR results in the current study indicate a similar relationship, but from Figure **17** it seems that adipogenesis occurs better without BMP-2. However as discussed before, no statistical analysis could be performed from the qPCR results.

The WB results show that PSMAD were present in higher concentrations at 14d time point than at 7d and that the group that was grown on plain well without BMP-2 had the least PSMAD of any group at both time points (Figure **16**). PSMAD activation could not be quantified in groups that grew on plain wells with or without additional BMP-2 at 7d, due to nonspecific stains on the blot, but the levels were so low that it is likely there was very little activation. Likewise it could be seen that the BMP-2 scaffold and CTRL+ BMP-2 groups had the most SMAD phosphorylation, which is logical as they had the most BMP-2.

From Figure **11** one can see that the amount of intracellular fat decreases, but does not disappear, when comparing 3d images with no BMP-2 in BM to 14d images with BMP-2 in OM. It is possible that at 2 weeks the differentiation has not advanced to a stage where fat has disappeared as from the Alizarin red data we can see that at 21d the mineralization is much higher than at 14d. It would have been interesting to be able to continue the study until 21d,

take images and compare the amount of fat to the Alizarin Red results. Another possibility is that faster differentiating adipocytes have already disappeared and with them the greater fat vacuoles.

Taking into account all the results in literature and in this study, it seems that BMP-2 reduces proliferation of hASCs and increases osteogenic differentiation. This is true for both set up conditions **I** and **II**, although it is good to keep in mind the variation between the cell lines and that SMAD phosphorylation and gene expression were only studied on a single cell line (HFSC5/12).

6.1.2 The effects of the substrate on proliferation and differentiation

The choice of scaffold material and its manufacturing process have been shown to be important to the viability and differentiation of MSCs (D'Angelo F, et al. 2011). Both the physical and chemical properties as well the topography of the materials have an effect on the behavior of cells (Lee, et al. 2014a). Ceramics, especially the highly polar and stiff ones, such as bioactive glasses, have been shown to be beneficial for osteoblast differentiation (Haimi S, et al. 2009a; Sandor, et al. 2014; Ojansivu M, et al. 2015). Polymers, having lower Young's moduli compared to most ceramics and being hydrophobic, have been found in studies to be more adipogenic than osteogenic in general. Special patterning, however can tip the scales in the favor of osteogenesis (Choi, et al. 2006). All the polymers their copolymers and blends used in the current study have also been successfully used in in earlier studies or in bone fixation devices (Garvin & Feschuk. 2005; Woodruff & Hutmacher. 2010). The ease of shaping and producing different topographies on polymers coupled with their drug loading and releasing properties makes them very tempting materials for bone applications, especially in combination with more osteogenic ceramics.

The topographical properties of polymers can be made to resemble that found in bone ECM using for example electrospinning such as in this study: The formed mesh is loose enough to allow for ingrowth of lamellopodia and filopodia, as can be seen from the images. While electrospinning is used mostly with polymers, also composites of polymers and ceramics have been used. Polini and coworkers studied osteogenic differentiation of MSCs on PCL and PCL composites *in vitro* without growth factors and noticed that osteogenesis can be achieved also in BM, but polar composite substrates were better than plain PCL (Polini, et al. 2011). This also is consistent with *in vivo* studies with PCL blends (Padalhin AR, et al. 2014). This sort of

composite material mimics very closely the properties of live bone: the ceramics are like the mineralized part of bone ECM and the polymers are like the collagens that provide the bones with tensile strength and gives the structure greater fracture toughness (Rho, et al. 1998; Weiner, et al. 1999). This kind of composite material if made more three-dimensional could be the best choice for bone differentiation, but not necessarily with these particular constituents.

Classic studies have shown that forcing the shape of cells to change can make BMSCs differentiate into either adipocytes or osteoblasts: if they are forced into a round shape, the cell differentiates into an adipocyte and if the shape is more spread, the cells differentiate into osteoblasts (Thomas CH, et al. 2002). Similar results have also been reached with ASCs (Kilian, et al. 2010). In addition to micro patterning they also discussed the importance of the stiffness of substrates on osteogenesis. On a flat surface, porous or not, the shape is determined by the cells ability to spread on the material (Cassidy, et al. 2014). Due to this, the topography and mechanical properties of the scaffold become important: the surface must be stiff enough to withstand the forces the cells exert on it as they pull themselves into a spread shape, and porous enough to allow for the cell adhesion (Kilian, et al. 2010; Lee, et al. 2014a).

Based on the topography, the material used in this study should be beneficial for osteogenesis as the cells should be able to spread out. While electrospinning provides a topography that would seem beneficial for osteogenic differentiation, the material and its individual threads might not be stiff enough: especially as polycaprolactone is a constituent of the blend (Woodruff & Hutmacher. 2010; Kurniawan D, et al. 2011). Hints of insufficient mechanical support is given by the immunofluorescence images taken early in the study, where it seems that the cells have tried to attach to the material by forming focal adhesion complexes and left behind parts of themselves on the material while searching for good binding sites.

Bead formations in the fibers have a smaller surface area to volume ratio which slows down degradation (Vo TN, et al. 2012). The material is bulk eroding, which should in theory be better than surface eroding materials for cell adhesions: The cells should be able to attach better to a surface, which is not constantly detaching from the bulk. As the material has varying thickness the control of drug release is not easily controlled on the other hand the beads create more continuous surfaces to which the cells can adhere more easily. However, microspheres made of PLGA, with or without adipogenic drugs, have been used successfully *in vivo* to differentiate hASCs into adipocytes (Choi, et al. 2006; Rubin, et al. 2009; Kelmendi-Doko, et al. 2014). This once again underlines the importance of the topographical cues for the cells.

Unfortunately, there are no results to compare only the effects of CTRL scaffolds and plain wells on hASCs on the morphology of the cells in this study, as no images were taken in set up conditions **II** and no cells were cultured on plain wells in conditions **I**. As a result we can only look at the combined effects of the substrate and BMP-2 on morphology in the next section. Whether the changes seen are due to the biological activity of BMP-2 released from the scaffold, or due to the effects of drug loading to the mechanical properties of the scaffolds cannot be determined and will thus be discussed in the next section, section 6.1.3.

The cells cultured in set up conditions **II** expressed higher levels of both *PPARg* and *OSX* on CTRL scaffolds than on plain wells, but also lower levels of *HAP2* (Figure 17). Based on the qPCR results it is hard to discern which differentiation outcome is more likely, but factoring in the WB results, showing PSMAD on the CTRL scaffolds but not on plain wells, the material seems to favor osteogenesis (Figure 16). Whether this is a reflection of the serum poor conditions, the actual material properties or their combination, is unclear. The β-actin results show that cells proliferate better on plain wells than on the scaffolds at 7d, but not at 14d.

When comparing cells grown on plain wells and on CTRL scaffolds without added BMP-2, the latter seem to be more osteogenic than adipogenic, at least on 7d based on SMAD phosphorylation. While there are opposing signaling pathways activated, the available evidence tips the imaginary see-saw in Figure 2 in favor of osteogenesis. If morphological data, or quantifiable ORO was available from set up conditions **II**, we could be more confident on the matter.

6.1.3 The combined effects of BMP-2 and scaffold material on hASCs

The effects of combining the scaffold and BMP-2 were studied with qPCR and WB in cell culture conditions **II**, where cells were grown on plain plastic as well as on the CTRL scaffold with or without BMP-2 and on BMP-2 scaffold. Data on morphology as well as proliferation, ALP activity, and mineralization is available from set up conditions **I**, but cannot be used to evaluate the effects of the material in comparison to plain wells.

WB and qPCR results (Figure **16** and Figure **17**) indicate that the cells grow best on plastic, but also fare well on the CTRL scaffolds. The addition of BMP-2 makes the cells proliferate less and activates the osteogenic differentiation process by increasing SMAD phosphorylation.

PSMAD can also be seen on the CTRL scaffolds and at 7d it seems that the phosphorylation increases with rising BMP-2 concentration (Figure 16).

The morphological data from set up conditions **I** indicates that the BMP-2 scaffolds are more adipogenic than CTRL scaffolds. While there is opposing evidence from the qALP and Alizarin red quantification except for mineralization results at 21d in OM (Figure 15), the growth patterns of the cells resemble more those of adipocytes' than osteoblasts' (Figure **10**).

Bead structures are clearly visible both in the control scaffolds and the BMP-2 releasing ones. The width of threads is 400-600 nm, but the beads are up to 7 µm thick. Due to this it is likely that the difference in growth patterns of cells on the control scaffolds and the drug releasing ones seen in Figure **10** are not caused by topographical factors, but rather by the effects of drug loading weakening the mechanical properties of the material, or the small BMP-2 concentrations themselves. This would be consistent with a study conducted by Chew and co-workers, who showed that drug-loading can affect the mechanical properties of electrospun polymers (Chew, et al. 2006).

The isoelectric point of BMP-2 is around pH 8.21 (sequence from http://www.rcsb.org/pdb/explore.do?structureId=3BMP and parameters from http://web.expasy.org/cgi-bin/protparam/protparam(02.02.16)). This would make the protein have a net positive charge at around pH 7. The polar protein inside the substrate could destabilize some of the hydrophobic interactions that hold the polymers together, explaining the weakening. As a result it would make sense for the cells to seek support from one another, resulting in the growth pattern seen on the BMP-2 scaffolds. Another factor, which might affect the pattern seen in Figure **10**, is the BMP-2 concentration, which can be high in the microenvironment, near the scaffold surface where it is being released. The concentration in the BM an OM media, when added was however so much greater that it is hard to imagine that this could be the case in this study.

Recently it has been shown that *HAP2* down regulates *PPARg* expression (Garin-Shkolnik, et al. 2014). In this light it is logical that in the control scaffold group without BMP-2, where *PPARG* expression is highest at 7d, *HAP2* is expressed highest at 14d (Figure **17**). As was discussed before, the results at 14d might not be very reliable, due to low nutrient conditions that are abnormal for the cells, but the results are logical and indicate that the material, by itself is more adipogenic than osteogenic at 14d, but the addition of BMP-2 tips the scale towards

osteogenesis. Unfortunately due to the scarcity of the scaffolds not enough parallel samples could be made to make statistical analysis. However, as these results are only from one cell line and as we have already discussed the cell line specificity to reaction with BMP-2 no conclusions can be drawn for hASCs in general.

James and co-workers have shown in an earlier study that BMP-2 can also induce adipogenesis (James. 2013). In light of this the properties of the scaffold become exceedingly important if osteogenic differentiation is desired. Mechanotransduction is a very important activator of osteogenesis. Huang and co-workers showed that osteogenic differentiation of ASCs takes place more readily in 3D-cultures than in traditional 2D-cultures (Huang, et al. 2008) and Tirkkonen and co-workers showed that in 3D- cultures OM was a more potent inducer of osteogenesis than BMP-2, BMP-7 or VEGF and that having these growth factors in BM or OM had no significant effect on the differentiation (Tirkkonen, et al. 2013b). From the latter study it seems that at least in 3D the scaffold properties are more important than BMP-2. This might not however be directly comparable to this study, where the material was somewhere between 2D and 3D with respect to cells, as they grew in several layers.

As briefly discussed before, the amount of cells seeded has been shown to be one factor for the differentiation direction taken by MSC cells (McBeath R, et al. 2004). As MSCs are plastic adherent, it can be expected that the cells might at least initially grow better on bare wells than on the hydrophobic biomaterial. It is difficult to determine the optimal number of cells seeded on a new biomaterial and in the case of this study the amount was chosen without optimization as a result of the lack of scaffold material. This has to be taken into account when comparing the results between groups in set-up **II**. The β-actin levels go a long way to help with this in the WB results. Similarly, as the qPCR results are related to the large ribosomal gene the level of which is generally considered to be stable in cells regardless of their type the normalization is done appropriately.

The noggin WB could not be scanned due to high background. However, the noggin expression levels were logical, as the groups with the highest BMP-2 concentrations had the highest amount of anti-noggin antibody binding. If nothing else, this confirms that the growth factor has biological activity, as the suggested model of noggin expression is a negative feedback loop: the more BMP-2 is present, the more noggin is excreted to counter act it (Chen, et al. 2012). The idea of using noggin inhibitors for boosting the activity of bone morphogenetic proteins has been studied and patented (Wan DC, et al. 2007; Chen, et al. 2012), so it would be

interesting to see if their use would make the effects of BMP-2 statistically significant. Fan and coworkers combined BMP-2 agonists and noggin antagonists with genetic modification and BMP-2 in a recent study and gained great results in differentiating hASCs into osteoblasts(Fan, et al. 2016). Such a set up with could potentially lessen the variation between cells from different donors and thus be more generalizable.

When all the data is analyzed, the results indicate that the CTRL scaffolds are more osteogenic than plain wells and that the BMP-2 scaffolds are more adipogenic than the CTRL ones. Whether this is due to the weakening of the scaffolds mechanical properties due to drug loading, possible adipogenicity of low BMP-2 concentrations, or both is hard to discern at this time. Addition of BMP-2 to the medium increases osteogenic differentiation more on CTRL scaffolds than on plain wells. In conclusion it seems that CTRL scaffolds combined with BMP-2 in culturing medium, especially in OM is suitable for osteogenic differentiation of hASCs.

6.2 Evaluation of used methods

All the techniques used in this study have been used excessively in the field. Due to this, using these methods makes it possible to compare the results to literature.

6.2.1 Cell proliferation and viability

CyQuant is based on total ribonucleic acid (both DNA and RNA) amount and not absolute cell count such as staining of the nuclei. Due to this it has its inaccuracies, but steps were taken to minimize them. These steps included washing the biomaterials with PBS prior to lysing the cells as well as doing the sample collecting from the scaffolds systematically the same way with all the samples. This should be sufficient to get rid of the influence of dead cells.

While Cyquant proliferation assay is a good method to measure the relative amount of cells in a sample, it would have been informative to utilize a method to assess the viability of the cells. Cyquant cannot be used to distinguish between live and dead cells, so it is possible that some residual from dead cells remained in the samples after removing the medium and the PBS washes. qALP and Alizarin red -staining are related to cell numbers so the interpretation of those analysis results are also affected. Light microscopy could not be used to follow the cells during the majority of the tests, due to the material not being translucent. A live dead assay utilizing fluorescence could have been useful for checking the viability of the cells for more accurate results. Another option would have been to use FACS to count the live and dead cells

from a cell suspension, but detaching the cells from the material in a consistent way would have required additional testing beforehand. While such analysis would have been possible, it is worth noting that as the number of scaffolds was limited adding more analysis would have been impossible without omitting something else. Furthermore some of the cells were firmly embedded in the material, so there was no good way to detach them for FACS.

6.2.2 Cell-material interactions and morphology

Oil Red O stains not only the intracellular fat vacuoles but also to the material. This is useful for imaging and studying the scaffold-cell interaction with fluorescence microscopy. While the binding to fat vacuoles is not specific *per ce*, but rather due to hydrophobicity, there are virtually no other structures inside the cell that the stain can bind to.

It would have been more informative to obtain confocal microscopy images of the cells on the material to see how deep into the material the cells could invade. From the 2D images it can only be seen, that some appendages of the cells grow under the threads of the material. Scanning electron micrographs of the materials with and without the cells would also have been informative. It would have been more accurate to measure the thread and bead diameters with micrographs than from fluorescence microscopy images, as the resolutions are quite different.

Actin labeling is good for studying cell shapes, but in hindsight it might have been more interesting to focus on other aspects such mechanobiology, which could be better explored, if immunofluorescent labeling was used on talins, vinculins or other components of focal adhesion complexes. This would make it possible to compare their numbers and give qualitative information about the mechanically activated cell signaling pathways, such as the non-canonical wnt-pathways that have been shown to be important for osteogenic differentiation. This kind of analysis would however be more easy and accurate to conduct with the help of confocal microscopy. Due to the limited number of filters in the fluorescent microscope and the scarcity of the scaffolds, this could not have been done simultaneously with morphological studies that required phalloidin.

Actin labeling is extremely useful for studying cell shapes. Similarly ORO staining is a simple method to see the intracellular fat and the material at the same time, however, this simplicity comes at the cost of not always being able distinguish between intracellular fat and the biomaterial: the vacuoles were distinguishable from the material only by their shape, which is

qualitative. ORO staining just as Alizarin red staining can be quantized and has been used to study adipogenesis. However ORO quantitation could not be carried out in this study, as the stain binds to the scaffold material. The products of the biomaterial hydrolysis are likewise stained with the stain: their size can be in the same order of magnitude as the fat vacuoles, causing further confusion. Nevertheless the many washes during sample preparation and the shape of the vacuoles, made it possible to identify intracellular fat most of the time without problems. To obtain better images it could have been a good idea not to use a mounting agent with DAPI and just stain the nuclei separately. DAPI has the tendency to overstain, creating shadows on the images. Applying the same amount of staining agent on all the preparations is not easy, due to the low viscosity of the reagent and the scaffolds not being flat. As a result DAPI overstaining occurred in several samples. However while this made obtaining quality images harder, it did not affect the results of the study.

6.2.3 Early differentiation and mineralization

The differentiation towards osteogenic lineage was assessed using qALP- assay and Alizarin red staining, as previously discussed. The final quantification from the samples was done by using colorimetric methods with a plate reader. As such the limitations of colorimetry apply to the reliability of the results. Plate readers have upper and lower limits of detection for different wavelengths. Below the lower detection limit nothing can be measured. Similarly above the upper limit, the samples cannot be quantified. These limits greatly dependent on the plate reader.

Alizarin red staining, while the most used method for studying mineralization, can be biased, as it does not differentiate between calcium from the ECM and calcium released from dead cells. Therefore it is important to relate the results to cell numbers. Optimally the cell count should be taken from the same wells, as the samples for Alizarin Red, but as the conditions are the same, the error is not great even in this study. Furthermore steps were taken to minimize influence from the dead cells in the sample preparation protocol, as there were several washes before and after fixation. The upper detection limit was reached several times in the last time point measurements in this study, which might downplay the accuracy of the normalized results and explain the discrepancy seen in OM BMP-2 scaffold group at 21, where the results indicate opposite things.

ALP activity is important for mineralization as it provides the ECM with phosphate ions. It has been used to detect early differentiation and is a standard in the field. It is important to remember that enzyme activity can fall with multiple freeze thaw cycles, so in order to avoid differences between the samples due to ALP deactivation, the number of freeze thaw cycles was always the same. Other things to keep in mind are the detection limits of this method. In the assay, the enzyme incubation time has to be kept constant between samples, however it is possible that some samples have such high enzyme activity that the detection limits are exceeded. In this study the detection limit was not reached with ALP samples.

6.2.4 Serum starvation conditions

Serum starvation is used most often for cells intended for WB samples, when studying phosphorylation in response to stimulus. The condition is stressful for the cells and culturing in these conditions is not normally used for more than 7 days. In this study the effects of BMP-2 on SMAD phosphorylation are highlighted when the cells are deprived from other signal molecules they would normally receive from serum in the culturing media. Starvation also synchronizes cell cycles. The differences in protein and gene expression become clearer, but at the same time one might question if the results are relevant as the conditions differ greatly from normal ones. Furthermore, results vary from cell line to cell line, as for example some cancer cells thrive in the absence of serum, whereas detrimental effects can be seen in the survival of normal cells. Considering this study, one might argue that as adipose cells act as energy reservoirs of the body and adipose differentiation occurs in vivo when the body is in a state of anabolism, that in starvation, the cells are unlikely to differentiate into adipocytes. The qPCR results are interesting in this sense, as they nonetheless hint at higher adipogenesis than osteogenesis under these conditions.

The amount of cells seeded might be one factor that affects the outcome of this study. Regularly MSCs are seeded onto scaffolds in higher concentrations than on plain wells, as many of the cells do not properly attach to the scaffold at first. The seeding concentrations in previous studies performed in our group have ranged from 5000 to 10000 cells/1,9cm^2, while considerably lower seeding concentrations have been used for plain wells. One reason why more cells were seeded in condition **II** was because the lower serum concentration is harmful for cell survival. However, as higher cell concentration is considered beneficial to adipogenic differentiation, the conditions the results are not readily comparable to those received from condition **I.** While the effects of BMP- are somewhat clear from the qPCR results, due to the

difference in cell numbers between two experimental setups should be taken into account when considering the adipogenic properties of the material alone. However as MSCs proliferate better on plain plastic than on the scaffold material, this is a necessary compromise.

Moreover, looking at Figure **17**, which summarizes the results of qPCR results, it becomes obvious why serum starvation is not usually carried out for longer periods of time. The measurements at 14d should not be considered too rigorously, but it is interesting to note that at 14d osteogenic markers are down regulated, whereas the adipogenic ones up-regulated. This makes sense, when considering the metabolic activities of osteoblasts and adipocytes: Osteoblasts need to produce considerable amounts of collagen and other extracellular proteins, whereas adipocytes specialize in energy preservation.

6.2.5 Western Blotting

The biomaterial used seemed to hinder WB analysis. Previously used PLA-scaffolds gave no background on the films with the same SOP, whereas the scaffolds used in this study made the films difficult to read. It is a possibility that partly degraded negatively charged polymer particles in the samples loaded to the gel also travel to the membrane as the proteins are transferred. However, a more likely scenario is that the background was caused by a poor choice of blocking medium: Phospho-sensitive antibodies often bind to casein in the milk, resulting in nonspecific binding (www.abcam.com/technical(12.12.2015)). However, in earlier studies conducted in the Adult stem cell group, contrary to literature, milk blocking has yielded better results.

Another thing to consider with WB is the use of nitrocellulose membrane instead of PVDF membranes. While PVDF membranes bind more proteins than nitrocellulose this can cause a lot of noise in the signal. The protocol for transfer was however optimized for PVDF membranes, but had worked well before so there was no reason to try to change it for these particular samples. Especially as the PSMAD levels has proven to be hard to detect: The volume of sample loaded for PSMAD detection was as high as possible for the used gels –and six times higher than for β-actin detection. This justifies the use of the higher binding PVDF membranes over the more accurate nitrocellulose ones.

It might have however been more informative to use enzyme linked immunosorbent assay (ELISA) detection instead of, or in parallel with WB. While WB confirms the masses and identities of the proteins, ELISA is a more quantitative and a more sensitive method. The

protein and DNA-level results could have been related even better if the methods were both quantitative. One way to make WB more quantitative is to use software to compare the levels of luminescence instead of using x-ray films and old-fashioned developing machines. This semi-quantification can be done afterwards, as was done in the current study. The background was high in the blots, so semi-quantification might not be very accurate. It is however good to keep in mind that WB even at its best is only semi-quantitative. Noggin WB results could not be scanned as discussed earlier, but they were in agreement with the qPCR results, showing higher expression with higher BMP-2 concentrations.

6.2.6 qPCR

While looking at qPCR results, it is important to realize that mRNA-levels do not always correlate with protein expression levels. They do however indicate the activation of signaling routes as mRNA is not transcribed without the action of transcript factors that themselves need activation through signaling. As such the results are a good indication of differentiation, if the genes under surveillance are well chosen. In this study *DLX5* and *OSX* were chosen as markers for osteogenesis where as *HAP2* and *PPARg* were chosen for adipogenesis. These same markers have been used in earlier studies (Tirkkonen L, et al. 2011; Tirkkonen, et al. 2013a).

The timing of each genes' activation in the process of differentiation have been extensively studied. *OSX* is expressed prior to *DLX5* and is typically not expressed after the first week, due to methylation(Hagh, et al. 2015). Likewise, *PPARg* is expressed prior to *AP2*.

The use of SYBR green is not the most accurate method in qPCR. SYBR green binds to double stranded DNA and this binding can be detected in the form of fluorescence. As the amount of DNA increases, so does the fluorescence. SYBR green does not differentiate between primer dimers and double stranded DNA. In this kind of study, where the absolute DNA concentrations are not as important as their relative amounts between groups, it is however sufficiently accurate method.

7. CONCLUSIONS

Electrospun PLGA-PCL scaffolds with loaded BMP-2 do not give enough mechanical support to induce osteogenic differentiation of hASCs. Instead the material seems to support adipogenic differentiation. The same material without drug loading however seems like a good material for osteogenic differentiation, especially with BMP-2 added in the culture medium. This result supports the claim that the mechanical properties of the cell substrate are a very important factor in deciding the cell type hASCs differentiate into. The experiment also shows that BMP-2 has less effect on osteogenic differentiation than osteogenic medium, which contains a number of other soluble factors that are associated with osteogenesis. The combination of BMP-2 with OM however seems beneficial for osteogenesis. While no statistical significance was found in this study between the different conditions, assumably due to great variation caused by differences in cells derived from different patients, the overall trends were clear. However, for future studies a more hydrophilic and stiff material should be used for scaffolds and noggin antagonists and BMP-2 agonists could further help the osteogenic differentiation. Alternatively another growth factor, one that inhibits adipogenesis rather than promotes osteogenesis, could be tried in combination with the material.

REFERENCES

Ahn JO, Coh YR, Lee HW, Shin IS, Kang SK, Youn HY. Human adipose tissue-derived mesenchymal stem cells inhibit melanoma growth in vitro and in vivo. Anticancer Res 2015;35(1):159-68.

Ali AT, Hochfeld WE, Myburgh R, Pepper MS. Adipocyte and adipogenesis. Eur J Cell Biol 2013;92(6-7):229-36.

Alvarez CV, Garcia-Lavandeira M, Garcia-Rendueles ME, Diaz-Rodriguez E, Garcia-Rendueles AR, Perez-Romero S et al. Defining stem cell types: understanding the therapeutic potential of ESCs, ASCs, and iPS cells. J Mol Endocrinol 2012;49(2):R89-111.

Angelos MG, Kaufman DS. Pluripotent stem cell applications for regenerative medicine. Current Opinion in Organ Transplantation 2015;20(6):663-70.

Becker Aj, McCULLOCH EA, Till Je. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 1963;197:452-4.

Berdasco M, Esteller M. DNA methylation in stem cell renewal and multipotency. Stem Cell Research & Therapy 2011;2(5):42.

Berendsen AD, Olsen BR. Regulation of adipogenesis and osteogenesis in mesenchymal stem cells by vascular endothelial growth factor A. J Intern Med 2015;277(6):674-80.

Bhardwaj N, Kundu SC. Electrospinning: a fascinating fiber fabrication technique. Biotechnol Adv 2010;28(3):325-47.

Carragee EJ, Hurwitz EL, Weiner BK. A critical review of recombinant human bone morphogenetic protein-2 trials in spinal surgery: emerging safety concerns and lessons learned. Spine J 2011;11(6):471-91.

Cassidy JW, Roberts JN, Smith CA, Robertson M, White K, Biggs MJ et al. Osteogenic lineage restriction by osteoprogenitors cultured on nanometric grooved surfaces: the role of focal adhesion maturation. Acta Biomater 2014;10(2):651-60.

Cassidy JW, Roberts JN, Smith C, Robertson M, White K, Biggs MJ et al. Osteogenic lineage restriction by osteoprogenitors cultured on nanometric grooved surfaces: The role of focal adhesion maturation. Acta Biomaterialia 2014;10(2):651-60.

Chen C, Uludag H, Wang Z, Jiang H. Noggin suppression decreases BMP-2-induced osteogenesis of human bone marrow-derived mesenchymal stem cells In Vitro. J Cell Biochem 2012;113(12):3672-80.

Chen D, Ji X, Harris M, Feng J, Karsenty G, Celeste A et al. Differential roles for bone morphogenetic protein (BMP) receptor type IB and IA in differentiation and specification of mesenchymal precursor cells to osteoblast and adipocyte lineages. J Cell Biol 1998;142(1):295-305.

Chen Q, Shou P, Zheng C, Jiang M, Cao G, Yang Q et al. Fate decision of mesenchymal stem cells: adipocytes or osteoblastsquest]. Cell Death Differ 2016.

Chew SY, Hufnagel TC, Lim CT, Leong KW. Mechanical properties of single electrospun drug-encapsulated nanofibres. Nanotechnology 2006;17(15):3880-91.

Choi YS, Cha SM, Lee YY, Kwon SW, Park CJ, Kim M. Adipogenic differentiation of adipose tissue derived adult stem cells in nude mouse. Biochem Biophys Res Commun 2006;345(2):631-7.

Choudhery MS, Badowski M, Muise A, Pierce J, Harris DT. Donor age negatively impacts adipose tissue-derived mesenchymal stem cell expansion and differentiation. J transl med 2014;12:8.

D'Angelo F, Tiribuzi R, Armentano I, Kenny JM, Martino S, Orlacchio A. Mechanotransduction: tuning stem cells fate. J funct biomater 2011;2(2):67-87.

Darlington G, Ross S, MacDougald O. The role of C/EBP genes in adipocyte differentiation. J Biol Chem 1998;273(46):30057-60.

Dechat T, Pfleghaar K, Sengupta K, Shimi T, Shumaker DK, Solimando L et al. Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. Genes Dev 2008;22(7):832-53.

Djouad F, Bouffi C, Ghannam S, Noel D, Jorgensen C. Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases. Nat Rev Rheumatol 2009;5(7):392-9.

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315-7.

Dragoo J, Choi J, Lieberman J, Huang J, Zuk P, Zhang J et al. Bone induction by BMP-2 transduced stem cells derived from human fat. Journal of Orthopaedic Research 2003;21(4):622-9.

Engh CA, Bobyn JD, Glassman AH. Porous-coated hip replacement. The factors governing bone ingrowth, stress shielding, and clinical results. J Bone Joint Surg Br 1987;69(1):45-55.

Fan J, Im CS, Guo M, Cui Z, Fartash A, Kim S et al. Enhanced Osteogenesis of Adipose-Derived Stem Cells by Regulating Bone Morphogenetic Protein Signaling Antagonists and Agonists. Stem cells transl med 2016;5(4):539-51.

Garin-Shkolnik T, Rudich A, Hotamisligil GS, Rubinstein M. FABP4 Attenuates PPAR gamma and Adipogenesis and Is Inversely Correlated With PPARg in Adipose Tissues. Diabetes 2014;63(3):900-11.

Garvin K, Feschuk C. Polylactide-polyglycolide antibiotic implants. Clinical Orthopaedics & Related Research 2005(437):105-10.

Gimble JM, Katz AJ, Bunnell BA. Adipose-Derived Stem Cells for Regenerative Medicine. Circ Res 2007;100(9):1249-60.

Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. Cytotherapy 2003;5(5):362-9.

Gir PMD, Oni GMD, Brown SAPD, Mojallal AMD, Ph.D., Rohrich RJMD. Human Adipose Stem Cells: Current Clinical Applications. Plastic & Reconstructive Surgery 2012;129(6):1277-90.

Gutierrez-Fernandez M, Rodriguez-Frutos B, Otero-Ortega L, Ramos-Cejudo J, Fuentes B, Diez-Tejedor E. Adipose tissue-derived stem cells in stroke treatment: from bench to bedside. Discov medicin 2013;16(86):37-43.

Hagh MF, Noruzinia M, Mortazavi Y, Soleimani M, Kaviani S, Abroun S et al. Different Methylation Patterns of RUNX2, OSX, DLX5 and BSP in Osteoblastic Differentiation of Mesenchymal Stem Cells. Cell J 2015;17(1):71-82.

Haimi S, Moimas L, Pirhonen E, Lindroos B, Huhtala H, Raty S et al. Calcium phosphate surface treatment of bioactive glass causes a delay in early osteogenic differentiation of adipose stem cells. J Biomed Mater Res A 2009a;91(2):540-7.

Haimi S, Suuriniemi N, Haaparanta AM, Ella V, Lindroos B, Huhtala H et al. Growth and osteogenic differentiation of adipose stem cells on PLA/bioactive glass and PLA/beta-TCP scaffolds. Tissue Eng Part A 2009b;15(7):1473-80.

Halvorsen YD, Franklin D, Bond AL, Hitt DC, Auchter C, Boskey AL et al. Extracellular matrix mineralization and osteoblast gene expression by human adipose tissue-derived stromal cells. Tissue Eng 2001;7(6):729-41.

Hamam D, Ali D, Kassem M, Aldahmash A, Alajez NM. microRNAs as Regulators of Adipogenic Differentiation of Mesenchymal Stem Cells. Stem Cells and Development 2015;24(4):417-25.

Heldin CH, Miyazono K, ten Dijke P. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature 1997;390(6659):465-71.

Hoffman LM, Garcha K, Karamboulas K, Cowan MF, Drysdale LM, Horton WA et al. BMP action in skeletogenesis involves attenuation of retinoid signaling. J Cell Biol 2006;174(1):101-13.

Horwitz EM, Le Blanc K, Dominici M, Mueller I, Slaper-Cortenbach I, Marini FC et al. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. Cytotherapy 2005;7(5):393-5.

Hu Y, Fu Y, Tabata Y, Gao J. Mesenchymal stem cells: A promising targeted-delivery vehicle in cancer gene therapy. J Controlled Release 2010;147(2):154-62.

Huang CK, Huang W, Zuk P, Jarrahy R, Rudkin GH, Ishida K et al. Genetic markers of osteogenesis and angiogenesis are altered in processed lipoaspirate cells when cultured on three-dimensional scaffolds. Plast Reconstr Surg 2008;121(2):411-23.

James AW. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. Scientifica (Cairo) 2013;2013:684736.

Kang Q, Song W, Luo Q, Tang N, Luo J, Luo X et al. A Comprehensive Analysis of the Dual Roles of BMPs in Regulating Adipogenic and Osteogenic Differentiation of Mesenchymal Progenitor Cells. Stem Cells and Development 2009;18(4):545-U33.

Kelmendi-Doko A, Marra KG, Vidic N, Tan H, Rubin JP. Adipogenic factor-loaded microspheres increase retention of transplanted adipose tissue. Tissue Eng Part A 2014;20(17-18):2283-90.

Kilian KA, Bugarija B, Lahn BT, Mrksich M. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci U S A 2010;107(11):4872-7.

Kim HKW, Oxendine I, Kamiya N. High-concentration of BMP2 reduces cell proliferation and increases apoptosis via DKK1 and SOST in human primary periosteal cells. Bone 2013;54(1):141-50.

Kurniawan D, Nor FM, Lee HY, Lim JY. Elastic properties of polycaprolactone at small strains are significantly affected by strain rate and temperature. Proc Inst Mech Eng [H] 2011;225(10):1015-20.

Kyllonen L, Haimi S, Sakkinen J, Kuokkanen H, Mannerstrom B, Sandor GKB et al. Exogenously added BMP-6, BMP-7 and VEGF may not enhance the osteogenic differentiation of human adipose stem cells. Growth Factors 2013;31(5):141-53.

Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther 2013;4(5):117.

Langer R, Vacanti J. Tissue Engineering. Science 1993;260(5110):920-6.

Lee JS, Jin GH, Yeo MG, Jang CH, Lee H, Kim GH. Fabrication of electrospun biocomposites comprising polycaprolactone/fucoidan for tissue regeneration. Carbohydr Polym 2012;90(1):181-8.

Lee J, Abdeen AA, Kilian KA. Rewiring mesenchymal stem cell lineage specification by switching the biophysical microenvironment. Sci rep 2014a;4:5188.

Lee SY, Lee JH, Kim JY, Bae YC, Suh KT, Jung JS. BMP2 Increases Adipogenic Differentiation in the Presence of Dexamethasone, which is Inhibited by the Treatment of TNF-alpha in Human Adipose Tissue-Derived Stromal Cells. Cellular Physiology and Biochemistry 2014b;34(4):1339-50.

Li W, Yeo LS, Vidal C, McCorquodale T, Herrmann M, Fatkin D et al. Decreased bone formation and osteopenia in lamin a/c-deficient mice. PLoS ONE 2011;6(4):e19313.

Li C, Vepari C, Jin H, Kim HJ, Kaplan DL. Electrospun silk-BMP-2 scaffolds for bone tissue engineering. Biomaterials 2006;27(16):3115-24.

Lian JB, Stein GS, van Wijnen AJ, Stein JL, Hassan MQ, Gaur T et al. MicroRNA control of bone formation and homeostasis. Nature Reviews Endocrinology 2012;8(4):212-27.

Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. J Cell Biochem 2011;112(12):3491-501.

Liu Y, Liu Y, Zhang R, Wang X, Huang F, Yan Z et al. All-trans retinoic acid modulates bone morphogenic protein 9-induced osteogenesis and adipogenesis of preadipocytes through BMP/Smad and Wnt/beta-catenin signaling pathways. Int J Biochem Cell Biol 2014;47:47-56.

Liu W, Konermann A, Guo T, Jaeger A, Zhang L, Jin Y. Canonical Wnt signaling differently modulates osteogenic differentiation of mesenchymal stem cells derived from bone marrow and from periodontal ligament under inflammatory conditions. Biochim Biophys Acta-Gen Subj 2014;1840(3):1125-34.

Lovell-Badge R. Many ways to pluripotency. Nat Biotechnol 2007;25(10):1114-6.

Luo J, Lee OS, Liang L, Huang CK, Li L, Wen S et al. Infiltrating bone marrow mesenchymal stem cells increase prostate cancer stem cell population and metastatic ability via secreting cytokines to suppress androgen receptor signaling. Oncogene 2014;33(21):2768-78.

Lysdahl H, Baatrup A, Foldager CB, Bunger C. Preconditioning Human Mesenchymal Stem Cells with a Low Concentration of BMP2 Stimulates Proliferation and Osteogenic Differentiation In Vitro. BioRes open access 2014;3(6):278-85.

Madhavan Nampoothiri K, Nair NR, John RP. An overview of the recent developments in polylactide (PLA) research. Bioresour Technol 2010;101(22):8493-501.

Mathieu PS, Loboa EG. Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. Tissue Eng Part B Rev 2012;18(6):436-44.

McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell 2004;6(4):483-95.

Mikami Y, Lee M, Irie S, Honda MJ. Dexamethasone modulates osteogenesis and adipogenesis with regulation of osterix expression in rat calvaria-derived cells. J Cell Physiol 2011;226(3):739-48.

Mou H, Brazauskas K, Rajagopal J. Personalized Medicine for Cystic Fibrosis: Establishing Human Model Systems. Pediatr Pulmonol 2015;50(Supplement 40):S14-23.

Ojansivu M, Vanhatupa S, Bjorkvik L, Hakkanen H, Kellomaki M, Autio R et al. Bioactive glass ions as strong enhancers of osteogenic differentiation in human adipose stem cells. Acta Biomater 2015;21:190-203.

Osyczka AM, Diefenderfer DL, Bhargave G, Leboy PS. Different effects of BMP-2 on marrow stromal cells from human and rat bone. Cells Tissues Organs 2004;176(1-3):109-19.

Padalhin AR, Thuy Ba Linh N, Ki Min Y, Lee BT. Evaluation of the cytocompatibility hemocompatibility in vivo bone tissue regenerating capability of different PCL blends. Journal of Biomaterials Science, Polymer Edition 2014;25(5):487-503.

Parssinen J, Hammaren H, Rahikainen R, Sencadas V, Ribeiro C, Vanhatupa S et al. Enhancement of adhesion and promotion of osteogenic differentiation of human adipose stem cells by poled electroactive poly(vinylidene fluoride). J Biomed Mater Res A 2015;103(3):919-28.

Peterson B, Zhang J, Iglesias R, Kabo M, Hedrick M, Benhaim P et al. Healing of critically sized femoral defects, using genetically modified mesenchymal stem cells from human adipose tissue. Tissue Eng 2005;11(1-2):120-9.

Piskin E. Biodegradable polymers as biomaterials. Journal of Biomaterials Science, Polymer Edition 1995;6(9):775-95.

Polini A, Pisignano D, Parodi M, Quarto R, Scaglione S. Osteoinduction of human mesenchymal stem cells by bioactive composite scaffolds without supplemental osteogenic growth factors. PLoS ONE 2011;6(10):e26211.

Porter JR, Ruckh TT, Popat KC. Bone tissue engineering: A review in bone biomimetics and drug delivery strategies. Biotechnol Prog 2009;25(6):1539-60.

Rho J, Kuhn-Spearing L, Zioupos P. Mechanical properties and the hierarchical structure of bone. Med Eng Phys 1998;20(2):92-102.

Ridge M, Ridge SM, Ryan AE, Finn S, O'Leary J, Giles FJ et al. Prostate Cancer Mediated Differentiation of Bone-Marrow Derived Mesenchymal Stem Cells Facilitates Prostate Cancer Cell Invasive and Migratory Capacity: P15. BJU Int 2014;113(Supplement 5):28.

Rohner D, Hutmacher D, Cheng T, Oberholzer M, Hammer B. In vivo efficacy of bone-marrow-coated polycaprolactone scaffolds for the reconstruction of orbital defects in the pig. Journal of Biomedical Materials Research Part B-Applied Biomaterials 2003;66B(2):574-80.

Rubin JP, DeFail A, Rajendran N, Marra KG. Encapsulation of adipogenic factors to promote differentiation of adipose-derived stem cells. J Drug Target 2009;17(3):207-15.

Sandor GK, Tuovinen VJ, Wolff J, Patrikoski M, Jokinen J, Nieminen E et al. Adipose stem cell tissue-engineered construct used to treat large anterior mandibular defect: a case report and review of the clinical application of good manufacturing practice-level adipose stem cells for bone regeneration. J Oral Maxillofac Surg 2013;71(5):938-50.

Sandor GK, Numminen J, Wolff J, Thesleff T, Miettinen A, Tuovinen VJ et al. Adipose Stem Cells Used to Reconstruct 13 Cases With Cranio-Maxillofacial Hard-Tissue Defects. Stem Cells Transl Med 2014;3(4):530-40.

Satish L, Krill-Burger JM, Gallo PH, Etages SD, Liu F, Philips BJ et al. Expression analysis of human adipose-derived stem cells during in vitro differentiation to an adipocyte lineage. BMC Medical Genomics [Electronic Resource] 2015;8:41.

Schlaeger TM, Daheron L, Brickler TR, Entwisle S, Chan K, Cianci A et al. A comparison of non-integrating reprogramming methods. Nat Biotechnol 2015;33(1):58-63.

Seppanen R, Miettinen S. [Bone to the chin from adipose-derived stem cells]. Duodecim 2014;130(19):2009-16.

Shan T, Liu W, Kuang S. Fatty acid binding protein 4 expression marks a population of adipocyte progenitors in white and brown adipose tissues. Faseb Journal 2013;27(1):277-87.

Shu B, Zhang M, Xie R, Wang M, Jin H, Hou W et al. BMP2, but not BMP4, is crucial for chondrocyte proliferation and maturation during endochondral bone development. J Cell Sci 2011;124(Pt 20):3428-40.

Sill TJ, von Recum HA. Electrospinning: applications in drug delivery and tissue engineering. Biomaterials 2008;29(13):1989-2006.

Solheim E. Growth factors in bone. Int Orthop 1998;22(6):410-6.

Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. Genes Dev 2010;24(20):2239-63.

Strioga M, Viswanathan S, Darinskas A, Slaby O, Michalek J. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. Stem Cells Dev 2012;21(14):2724-52.

Takada I, Yogiashi Y, Kato S. Signaling Crosstalk between PPAR gamma and BMP2 in Mesenchymal Stem Cells. Ppar Research 2012:607141.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al. Induction of Pluripotent Stem Cells From Adult Human Fibroblasts by Defined Factors. Obstet Gynecol Surv 2008;63(3):153.

Takedachi M, Oohara H, Smith BJ, Iyama M, Kobashi M, Maeda K et al. CD73-generated adenosine promotes osteoblast differentiation. J Cell Physiol 2012;227(6):2622-31.

Thesleff T, Lehtimaki K, Niskakangas T, Mannerstrom B, Miettinen S, Suuronen R et al. Cranioplasty With Adipose-Derived Stem Cells and Biomaterial: A Novel Method for Cranial Reconstruction. Neurosurgery 2011;68(6):1535-40.

Thomas CH, Collier JH, Sfeir CS, Healy KE. Engineering gene expression and protein synthesis by modulation of nuclear shape. Proc Natl Acad Sci U S A 2002;99(4):1972-7.

Tirkkonen L, Halonen H, Hyttinen J, Kuokkanen H, Sievanen H, Koivisto AM et al. The effects of vibration loading on adipose stem cell number, viability and differentiation towards bone-forming cells. J R Soc Interface 2011;8(65):1736-47.

Tirkkonen L, Haimi S, Huttunen S, Wolff J, Pirhonen E, Sandor GK et al. Osteogenic medium is superior to growth factors in differentiation of human adipose stem cells towards bone-forming cells in 3D culture. Eur Cell Mater 2013a;25:144-58.

Tirkkonen L, Haimi S, Huttunen S, Wolff J, Pirhonen E, Sandor GK et al. Osteogenic medium is superior to growth factors in differentiation of human adipose stem cells towards bone-forming cells in 3D culture. Eur Cell Mater 2013b;25:144-58.

Toyoda M, Matsubara Y, Lin K, Sugimachi K, Furue M. Characterization and comparison of adipose tissue-derived cells from human subcutaneous and omental adipose tissues. Cell Biochem Funct 2009;27(7):440-7.

Tseng Y, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature 2008;454(7207):1000-4.

Tsuji K, Bandyopadhyay A, Harfe BD, Cox K, Kakar S, Gerstenfeld L et al. BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. Nat Genet 2006;38(12):1424-9.

Tsuji W, Rubin JP, Marra KG. Adipose-derived stem cells: Implications in tissue regeneration. World Journal of Stem Cells 2014;6(3):312-21.

van Amerongen R. Alternative Wnt Pathways and Receptors. Cold Spring Harbor Perspectives in Biology 2012;4(10):a007914.

Vanhatupa S, Ojansivu M, Autio R, Juntunen M, Miettinen S. Bone Morphogenetic Protein-2 Induces Donor-Dependent Osteogenic and Adipogenic Differentiation in Human Adipose Stem Cells. Stem cells transl med 2015;4(12):1391-402.

Vidal C, Bermeo S, Fatkin D, Duque G. Role of the nuclear envelope in the pathogenesis of age-related bone loss and osteoporosis. Bonekey Rep 2012;1:62.

Viswanathan P, Ondeck MG, Chirasatitsin S, Ngamkham K, Reilly GC, Engler AJ et al. 3D surface topology guides stem cell adhesion and differentiation. Biomaterials 2015;52:140-7.

Vo TN, Kasper FK, Mikos AG. Strategies for controlled delivery of growth factors and cells for bone regeneration. Adv Drug Deliv Rev 2012;64(12):1292-309.

Wan DC, Pomerantz JH, Brunet LJ, Kim JB, Chou YF, Wu BM et al. Noggin suppression enhances in vitro osteogenesis and accelerates in vivo bone formation. J Biol Chem 2007;282(36):26450-9.

Wang YK, Chen CS. Cell adhesion and mechanical stimulation in the regulation of mesenchymal stem cell differentiation. J Cell Mol Med 2013;17(7):823-32.

Waselau M, Patrikoski M, Juntunen M, Kujala K, Kaariainen M, Kuokkanen H et al. Effects of bioactive glass S53P4 or beta-tricalcium phosphate and bone morphogenetic protein-2

and bone morphogenetic protein-7 on osteogenic differentiation of human adipose stem cells. J Tissue Eng 2012;3(1):2041731412467789.

Weiner S, Traub W, Wagner H. Lamellar bone: Structure-function relations. J Struct Biol 1999;126(3):241-55.

Wiley LA, Burnight ER, Songstad AE, Drack AV, Mullins RF, Stone EM et al. Patient-specific induced pluripotent stem cells (iPSCs) for the study and treatment of retinal degenerative diseases. Progress in Retinal & Eye Research 2015;44(Supplement C):15-35.

Woodruff MA, Hutmacher DW. The return of a forgotten polymer-Polycaprolactone in the 21st century. Progress in Polymer Science 2010;35(10):1217-56.

Wu H, Mahato RI. Mesenchymal stem cell-based therapy for type 1 diabetes. Discov medicin 2014;17(93):139-43.

Wurmser AE, Gage FH. Stem cells: cell fusion causes confusion. Nature 2002;416(6880):485-7.

Zara JN, Siu RK, Zhang X, Shen J, Ngo R, Lee M et al. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation in vivo. Tissue Eng Part A 2011;17(9-10):1389-99.

Zhang R, Wang D, Xia Z, Chen C, Cheng P, Xie H et al. The role of microRNAs in adipocyte differentiation. Front med 2013;7(2):223-30.

Zielins ER, Luan A, Brett EA, Longaker MT, Wan DC. Therapeutic applications of human adipose-derived stromal cells for soft tissue reconstruction. Discov medicin 2015;19(105):245-53.

Zuk PA, Zhu M, Mizuno H, Huang J, Futrell JW, Katz AJ et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001a;7(2):211-28.

Zuk P, Zhu M, Ashjian P, De Ugarte D, Huang J, Mizuno H et al. Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 2002;13(12):4279-95.

Zuk P, Zhu M, Mizuno H, Huang J, Futrell J, Katz A et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. Tissue Eng 2001b;7(2):211-28.

Zuk P, Chou Y, Mussano F, Benhaim P, Wu BM. Adipose-derived Stem cells and BMP2: Part 2. BMP2 may not influence the osteogenic fate of human adipose-derived stem cells. Connect Tissue Res 2011;52(2):119-32.