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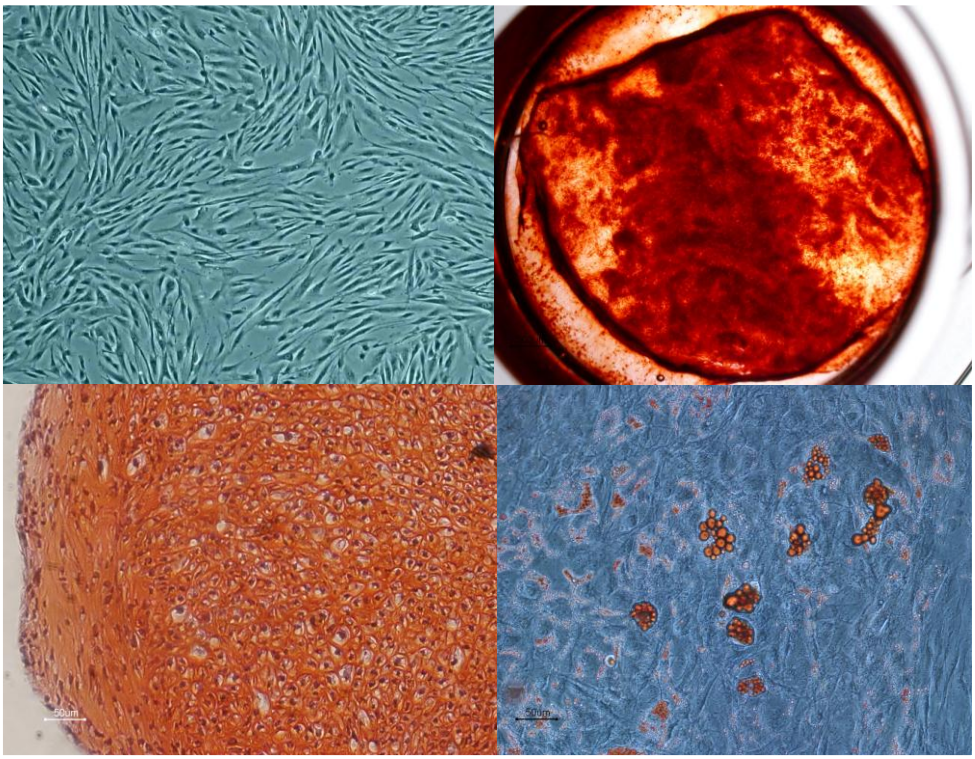

Differentiation of human pluripotent stem cell-derived mesenchymal progenitors into osteogenic, chondrogenic and adipogenic lineages

Master of Science Thesis in the Master Degree Program Biotechnology

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Göteborg, Sweden, 2012

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Cover:

[Upper left picture – Mesenchymal Progenitor line hES-MP002.5; upper right picture – Alizarin Red stained osteogenically induced hES-MP002.5 (see page 29); lower left picture – Safranin O stained histological section of chondrogenically induced dedifferentiated chondrocytes (see page 31); lower right picture – Oil Red O stained adipogenically induced dedifferentiated chondrocytes (see page 34)]

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The project was carried out at the company Cellectis Stem Cells under the supervision of Catharina Ellerström (Cellectis Stem Cells) and Jenny Johannisson (Cellectis Stem Cells).

The examiner was Julie Gold (Chalmers University of Technology, Department of Applied Physics, Division of Biological Physics).

Abstract

Differentiated cell types are of great interest in applications such as tissue engineering and drug screening. As harvesting of fully differentiated, adult cells neither is a safe nor scalable alternative, *in vitro* differentiated cells have become an attractive option. hES-MP002.5 (mesenchymal progenitors) is a cell type established by and at Cellectis Stem Cells from human embryonic stem cells (hESCs), that carries adult mesenchymal stem cell like characteristics. An examination of hES-MP002.5's mesenchymal differentiation potential was performed in this project, in particular into the osteogenic, chondrogenic and adipogenic lineage. A second objective was to establish a mesenchymal progenitor cell line from a human induced pluripotent stem cell line (ChiPSC4) and compare its characteristics to those of hES-MP002.5. Results were obtained primarily through the use of differentiation in cell culture, histological stains, immunocytochemistry and quantitative PCR.

An osteogenic differentiation study concluded that hES-MP002.5 show vast osteogenic potential when a previously established protocol was used. However, hES-MP002.5 showed near to no responsiveness to variations of commonly used chondrogenic or adipogenic treatments in terms of visible chondrogenic extracellular matrix deposition or intracellular lipid accumulation, respectively. Yet, hypoxic culture conditions ought to be considered if chondrogenic differentiation is attempted in future studies. Gene expression studies of adipogenically induced cells implied that a gene essential for adipogenic differentiation was down- regulated for both MP-lines; a fact that could help to explain the low differential response.

With an established protocol for hESCs, a cell population resembling hES-MP002.5 was developed from ChiPSC4 (ChiPSC4-MP), but the cell population seemed less homogenous and had lower proliferative potential. hES-MP002.5 and ChiPSC4-MP seemed to respond similarly to mesenchymal differentiation inductions – however ChiPSC4-MP consistently showed a weaker response. This might be due to heterogeneity in the cellular population obtained.

With the broad laboratory investigations in mind, it is not recommended that hES-MP002.5 is marketed as a mesenchymal progenitor with the ability of differentiating into chondrogenic and adipogenic lineages. A recommendation is that the protocol for mesenchymal progenitor establishment is reviewed and further developed, since the MP-cells established from both embryonic and induced pluripotent stem cells share the same strengths and weaknesses.

Keywords: Stem cell, mesenchymal progenitor, pluripotency, differentiation, osteogenic, chondrogenic, adipogenic, extra cellular matrix

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List of abbreviations

ACAN	Aggrecan
ALP	Alkaline phosphatase
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenic protein 4
C/EBPa	Ccaat-enhancer-binding proteins
ChiPSC4	Cellartis human induced pluripotent stem cell line 4
ChiPSC4-MP	Cellartis human induced pluripotent stem cell line 4 derived mesenchymal progenitor
COL2A1	Collagen type II
ECM	Extra cellular matrix
GLUT4	Glucose transporter type 4
hESC	Human embryonic stem cell
hES-MP	Human embryonic stem cell derived mesenchymal progenitor
IBMX	Isobutylmethylxanthin
ICC	Immunocytochemistry
MEF	Mouse embryonic fibroblast
MP	Mesenchymal progenitor
PPARg	Peroxisome proliferator-activated receptor gamma
qPCR	Quantitative polymerase chain reaction
SOX9	Sex-determining region Y box 9
TGF-β	Transforming growth factor beta

TABLE OF CONTENTS

1 INTRODUCTION

In this section, a background to the project is presented, as well as statements of the aims and boundaries of the project. A set of specific questions that are answered in the conclusion (section 7) is also stated.

1.1 Background

Cellectis Stem Cells (previously Cellartis AB) is a biotechnology company specializing in human pluripotent stem cells and their derived products. The company has developed a straightforward protocol to produce mesenchymal progenitor lines from human embryonic stem cells (hES-MP) with satisfying reproducibility[1]. The hES-MPs bare high resemblance to adult human mesenchymal stem cells (hMSCs)[2] and are multipotent stem cells which should have the potential of differentiating into several mesodermal lineages *in vitro*[1]. Differentiated cells are of great interest in various applications in the medical industry. For example, osteogenic/chondrogenic lineages (bone and cartilage, respectively) have potential to be used in tissue engineering applications[2], and adipogenic cells (fat cells) grown *in vitro* are of importance for organizations that perform research on metabolic diseases and pharmaceutical companies developing drugs. To ensure purity and functionality of cells in any applications, efficient protocols of differentiation is essential. There are numerous scientific articles that contain protocols of differentiation into the discussed lineages; however, different cell types may require different management in order to reach successful differentiation.

Cellectis Stem Cells was in need of customized procedures for the differentiation of the commercial cell line hES-MP002.5 into osteogenic, chondrogenic and adipogenic lineages, why they requested an evaluation on the efficacy of the current procedures and an attempt to improve the protocols used. As the use of embryonic stem cells is ethically questionable in many countries, mesodermal experiments on a lineage of human induced pluripotent stem cells was also of interest to reach a broader spectrum of customers, as well as to develop methodology that could be used to derive patient specific stem cells.

1.2 Aim

The primary objective of this project was to investigate how well the cell line hES-MP002.5 can be differentiated into the osteogenic, chondrogenic and adipogenic lineages. Regarding osteogenic differentiation, emphasis was to map the stages of the differential process using previously established protocols, while an increase of efficiency from current protocols was attempted in order to obtain chondrogenic and adipogenic cells.

A second objective was to attempt differentiation of a human induced pluripotent stem cell line named ChiPSC4 (Cellartis human induced pluripotent stem cell line 4) into mesenchymal progenitor cells, based on previously established protocols for differentiating hESCs into MP cells. If successful, these MP-cells would also be used in order to try to establish the three discussed mesenchymal lineages and a comparative study of the hiPS-MP and hES-MP cells would be performed. Figure 1.1 illustrates the two objectives.

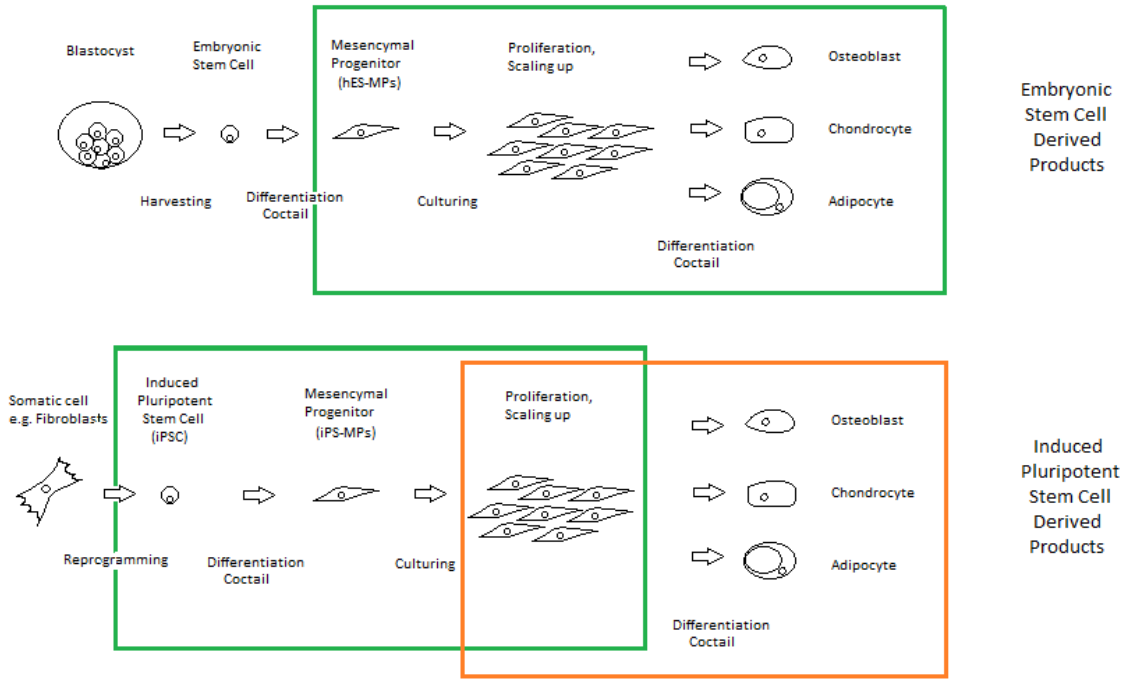


Figure 1. 1 ***The squared upper panel shows the first objective of the thesis – to evaluate, and if possible enhance, mesodermal potential of the commercially available cell type hES-MP002.5. The lower squared panels shows the second objective of this thesis – to establish a mesenchymal progenitor line from the human induced pluripotent stem cell line ChiPSC4, and, if successful, carry out mesodermal experiments of the resulting cell type (orange square).***

1.3 Specific questions to be answered

- How well can Cellectis Stem Cells embryonic stem cell derived mesenchymal progenitor line hES-MP002.5, with the previously used protocols, be differentiated into:
  - Osteoblasts
  - Chondrocytes
  - Adipocytes

- When and how does osteoblast differentiation from hES-MP002.5 start and proceed?

- Is it possible to enhance Cellectis Stem Cells protocols for differentiation of mesenchymal progenitors into chondrogenic and adipogenic lineages by changing additives/concentrations/procedures of differentiation?

- Is it possible to differentiate Cellectis Stem Cells induced human pluripotent stem cell line ChiPSC4 into a mesenchymal progenitor line? If so:
  - How well does the cell population resemble hES-MP002.5? If not, why so?
  - Does the cell population have the capability of differentiating into osteoblasts, chondrocytes and adipocytes *in vitro* with the protocols used to induce hES-MP002.5?

- In summary, what conclusions can be drawn from the work with hES-MP002.5 and ChiPSC4?

1.4 **Limitations**

- Optimization of the differentiation procedures will not be made, as there are too numerous factors that can be altered to increase differentiation efficiency. Attempts to improve these protocols with other agents/ideas/experimental setups will however be made.

- To be able to perform experiments with reasonable workload, restrictions have to be made in the chosen analysis method for the cell types concerned in the project. Therefore, characteristics that are essential for a certain cell lineage might be absent while the results obtained indicate that a functional differentiated cell has been produced.

- Due to the broad scope of the project, experiments will be performed to generate indicative results, and statistical analysis will not be a part of this project. Thus, plausible trends or responses presented in the results and discussion should be confirmed in other experiments.

2 LITERATURE REVIEW

2.1 Embryonic and adult stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of fertilized eggs[3]. These cells are pluripotent, which means that they have not yet differentiated into more specialized cell types, and therefore can give rise to all three germ layers that human cells are derived from; the endoderm, ectoderm and mesoderm[3]. Embryonic stem cells have virtually unlimited proliferative capacities, as compared to somatic cells that usually stops dividing after maturation, but also compared to many adult, stem cells that loses proliferation potential after extended culture *in vitro*.[5] Therefore, embryonic stem cells constitute a potentially inexhaustible source of cells for e.g. tissue engineering applications[5]. However, in many countries ethical issues have resulted in laws regulating the use of embryonic stem cells[6].

Another source of stem cells is adult stem cells, residing in many different tissues throughout the body where they can increase the number of somatic cells[3]. These cells are multipotent, which means that they have the possibility to differentiate into multiple mature cell types, but are restricted in their differentiation potential when compared to pluripotent stem cells. Mesenchymal stem cells (MSCs) are an example of an adult stem cell that belongs the mesodermal germ layer[4]. MSCs can for example be found in bone marrow and fatty tissue, and have the ability to differentiate into bone, cartilage and fat cells[4]. For an overview of stem cell differentiation and potency terms, see Figure 2.1 below.

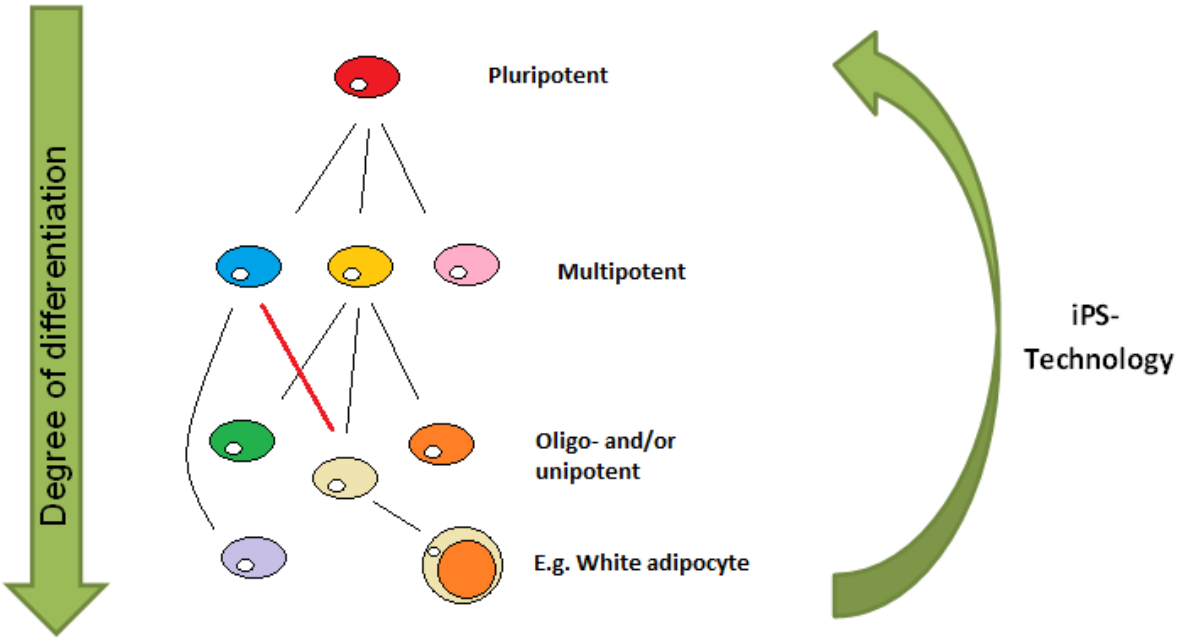


Figure 2. 1 ***General scheme of stem cell differentiation. Pluripotent stem cells have the capability of differentiating into all lineages of the human body, and directly give rise to multipotent stem cells. These can give rise to several cell lineages, but are more restricted in their differentiation potential when compared to pluripotent stem cells, as indicated by the red line above. Multipotent stem cells give rise to oligo- or unipotent cells, which finally mature into somatic cells, such as a white adipocyte, indicated above. The conversion of somatic to pluripotent cells is possible since 2006 through iPS-technology (see section 2.2).***

Differentiation of stem cells *in vivo* is a highly complex process, and is hard if not impossible to study and map in detail. The divisions of stem cells are intrinsically regulated by the environment found in the "stem cell niche", but also impacted by paracrine as well as endocrine signals that affect differentiation responses[64]. When a stem cell

divide, it mainly can go through either symmetric or asymmetric division, where the first indicates that two new stem cells are formed, while the latter gives rise to one stem cell and one more specialized cell type[3].

When keeping embryonic stem cells in culture, one wants to promote symmetric division to amplify the number of pluripotent cells. Until recently, 2D-culture of embryonic stem cells required co-culturing on a layer of quiescent feeder-cells (e.g. mouse embryonic fibroblasts) in order to retain their capacity for self-renewal.[7]. The last few years, however, feeder and xenogenic free choices of culturing have become available that allows for pluripotent stem cell expansion[8], and Cellectis Stem Cells have among others created such an option: the DEF-CS™ Platform.

In an *in vitro* culturing system, stem cells can be induced to differentiate into a specific lineage if treated with the appropriate stimuli. These stimuli usually consist of soluble factors, such as growth factors and small molecules that help to induce expression of genes that are specific for the wanted lineage[7]. However, other aspects, such as the use of cell adhesive substrates, regulation of cell seeding densities and biomechanical signaling may also be of importance to mount an appropriate differentiation response[7].

2.2 Induced pluripotent stem cells and ChiPSC4

Induced pluripotent stem cells are reprogrammed somatic (adult) cells from which all three germ layers can be established (see figure 2.1). The reprogramming procedure typically involves the introduction of a small set off genes, e.g. by viral transduction, coding for transcription factors which have been shown to be crucial for the maintenance of pluripotency in embryonic stem cells[9]. The resulting iPS cells show close resemblance to hESCs in many aspects ranging from morphology (e.g. high nucleus to cytoplasm ratio, generates flat compact colonies in culture with defined edges) and gene expression (e.g SSEA3/4, Tra-1-60/80, Oct4, Sox2, Nanog) to differentiation potential *in vitro*, and their ability to form teratomas *in vivo*[10].

The procedure was first shown by Yamanaka et al. in 2006, when they managed to pin down four transcription factors – Oct3/4, Sox2, c-Myc and Klf4 – that were able to transform mouse embryonic and adult fibroblasts into a pluripotent state when introduced with viral vectors[9]. In 2007, they showed that human somatic cells could be converted with the same set of factors, while other groups revealed that a different sets of reprogramming factors could be used, such as the Thomson group using the factors Oct4, Sox2, Nanog and Lin28[10]. Today, the general knowledge is that Oct4 is an essential factor, Sox2 and Klf4 can be omitted only if endogenous expression of the genes are high in the somatic cells, alternatively if replaced by a suitable chemical additive (such as small molecules and epigenetic modulators), while Nanog, c-Myc and Lin28 are dispensable, but they do act to enhance conversion rate[10]. Gurdon and Yamanaka received the Nobel Prize in medicine in 2012 for their discoveries in somatic cell plasticity and iPS-technology[71].

By this date, several human cell types have been successfully reprogrammed into pluripotency[10]. Many different approaches for the introduction of reprogramming factors have also been explored, such as viral based genetic integration, genetic integration with subsequent excision, episomal transfection with vectors that do not integrate into the host genome, repeated exposure to reprogramming proteins or synthetic mRNA[10]. The developmental of a clean iPS-technology (induction without non-integrating reprogramming factors) with improved efficiencies are highly sought after, where reprogramming with synthetic mRNA has been shown to do well in both aspects[11].

iPS-technology has produced great hope for an unlimited source of patient specific stem cells for tissue regenerative applications and the possibility to study disease specific cells and developmental processes without ethical concerns[12]. However, there are still several obstacles that have to be overcome before the technique can settle as a clinically relevant alternative. Among others, induced pluripotent cells have been shown to retain parts of the epigenetic memory from their originating cell type, which might give rise to batch-to-batch variation in iPS-quality and affect the ability of the cells to differentiate into functional cell types[13].

Cellectis Stem Cells have established an induced pluripotent stem cell line named ChiPSC4 (Cellartis human induced Pluripotent Stem Cell line 4) from human fibroblasts with viral transduction technique. The cell line has been passed extensive quality check-ups and is for example used to produce functional hepatocytes sold as a commercial product (hiPS-HEP™)[70]. Catharina Ellerström, Senior Principal Scientist and Department Head at Cellectis Stem Cells, confirms that ChiPSC4 displays several stem-ness markers expected from pluripotent cells, that it has a diploid normal karyotype, and has also been shown to give rise to all three germ layers. Furthermore, qPCR analysis has been able to show that the exogenously expressed factors have been silenced in both undifferentiated and differentiated ChiPSC4. However, since no published materials are available, no thorough comparison of ChiPSC4 and hESC-lines can be made in this study.

2.3 Embryonic stem cell derived mesenchymal progenitors

Mesechymal progenitors (hES-MP™002.5, also known solely as hES-MPs) is a product sold by Cellectis Stem Cells. It is marketed and sold as a homogenous and robust source of mesenchymal stem cell like cells established from embryonic stem cells for *in vitro* research applications (both cell types are shown in Figure 2.2). See Section 2.4.4 for more details regarding the mesenchymal progenitor establishment procedure. Studies performed by de Peppo et al. confirm that the hES-MP cells in many aspects show resemblance to adult mesenchymal stem cells[2]. Among others, the mesenchymal progenitors exhibit hMSC-characteristic fibroblast like morphology and microarray data analysis demonstrates that the hES-MPs significantly decreased expression of most genes overexpressed in hESCs, while they showed a specific increase in expression of many genes commonly expressed by hMSCs (e.g. genes coding for mesodermal ECM-components) at levels comparable to hMSC expression[2].

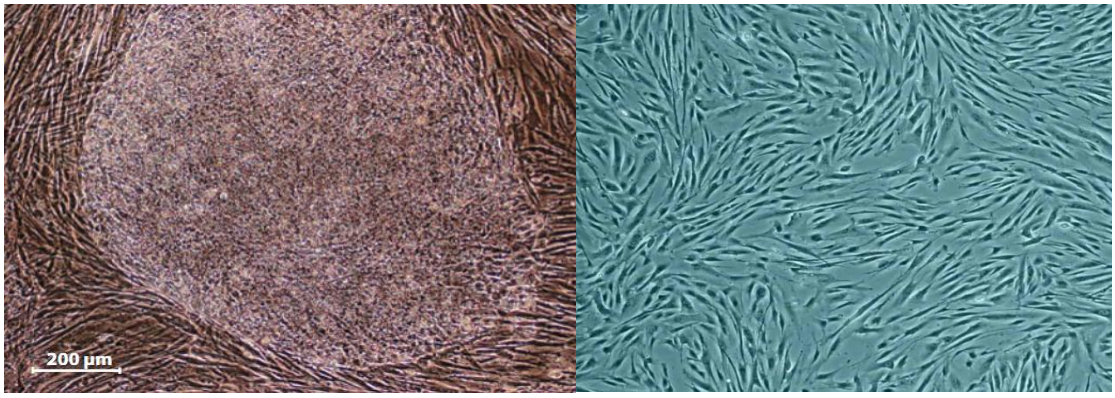


Figure 2. 2 ***Left: a human embryonic stem cell colony on human foreskin fibroblast feeders, 2 days after passage. Right: a human embryonic stem cell derived mesenchymal progenitor culture with fibroblast like morphology.***

Some advantages that hES-MP002.5 hold compared to hMSCs are that it demonstrates significantly higher proliferative capacity *in vitro* (see figure 2.3), which has been shown to be correlated to a longer telomere length, and they also constitute a reliable source of cells not depending on donor availability[2]. hES-MPs also show advantages for use in tissue engineering applications, such as having a lower expression of HLA class II proteins (triggers immune responses *in vivo*) when compared to hMSCs[2] and constitutes a safer option than direct hESC use, as one avoids risk of teratoma formation when implanting cells *in vivo*[2].

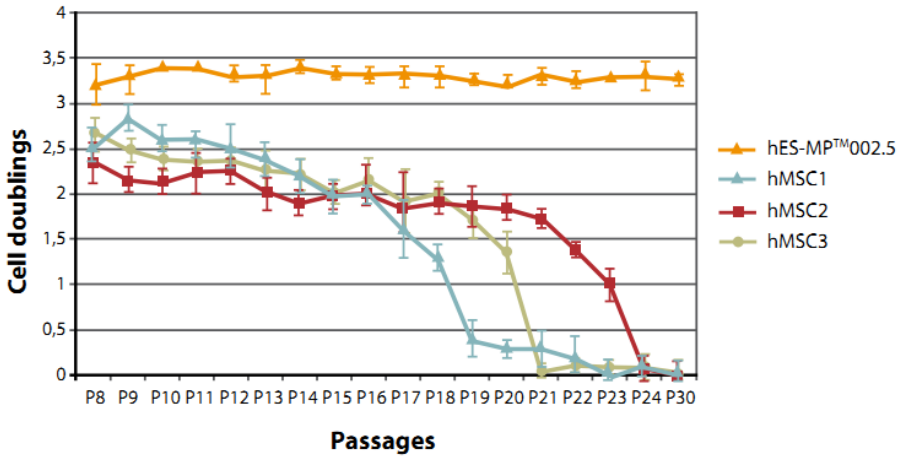


Figure 2. 3 ***hES-MP002.5 show a significantly higher number of cell doublings per time period compared to hMSCs***[2]

Mesenchymal stem cells have numerous times been shown to be able to produce osteogenic, chondrogenic and adipogenic cells *in vitro*[4]. Likewise, the mesenchymal progenitors produced at Cellectis Stem Cells have been shown to possess this ability[1]. Furthermore, hES-MP cells implanted *in vivo* (in SCID mice), has been shown to spontaneously differentiate into a range of different mesenchymal tissues, such as cartilage, but not other developmental lineages[1]. This indicates a commitment of the hES-MP line to mesodermal lineage. However, when differentiating hES-MPs and MSCs into chondrocytes and adipocytes *in vitro* using the same factors, efficiency of differentiation seems to be lower for hES-MPs as compared to MSCs[1].

2.4 Mesenchymal cell lineages, tissues and procedures of differentiation

The choices of methods are based on the following sections. The most prominent characteristics of osteogenic, chondrogenic and adipogenic cells and tissues will be discussed. Furthermore, common methods of differentiation of stem or progenitor cells into bone, cartilage and fat cells will be presented, as well as methods to derive mesenchymal like stem cells from pluripotent stem cells.

2.4.1 Osteogenic cells and differentiation

Bone is a supportive and protective structure primarily composed of mineral and proteins, finely organized to give a light yet strong tissue. Cells from the osteogenic lineage include osteoblasts and osteocytes. Osteoblasts are bone forming cells and osteocytes are maturated osteoblasts, entrapped within the extracellular matrix (ECM)[23]. Another important bone cell is the osteoclast, which is of hematopoetic origin, and acts as a bone resorber[23]. The objective of this project was to establish bone depositing osteoblasts.

Osteoblasts have cuboidal morphology *in vivo*[23], however, in cell culture they morphologically resemble fibroblasts[24]. The osteoblast can essentially be viewed as a specialized fibroblast since the two cell types have a very similar gene expression profile; however, the osteoblast has a competence for matrix mineralization[24]. Therefore one of the most evident characteristics of a successful osteogenic differentiation is to detect mineralized ECM (i.e. bone): a composite material consisting mainly of the proteinbundles of collagen type I and the mineral hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) at a dryweight ratio of 30-40% to 60-70%, respectively[3]. Osteoblasts uniquely express the protein osteocalcin[24] and also express alkaline phosphatase (ALP)[23], an enzyme which dephosphorylates a range of substrates, thus releasing inorganic phosphate that can be incorporated into the ECM.

Osteogenic differentiation in the adult individual occurs naturally from osteogenic progenitors present in the periosteum; a membrane that lines the outer surface of bones mature bone[31]. When osteogenic differentiation is initiated, regulatory transcription factors such as Runx2, β-Catenin and Cbfa1 are expressed, which all help to

commit progenitor cells to osteoblasts[25]. Runx2 is considered to be a master switch in osteogenesis, and binds to osteoblast-specific cis-acting element (OSE2) in promotor regions of genes such as collagen type I, osteocalcin, osteopontin and alkaline phosphatase, thus stimulating expression[32].

In vitro, osteogenic differentiation can be induced by supplementing stem cells or progenitors with growth factors, synthetic hormones or biochemical substances that affect protein synthesis, and is a well-studied process. In the majority of published protocols of differentiation, the substances L-ascorbic acid, dexamethasone and β-glycerophosphate is used as supplements and have known implications for the transformation of precursor cells into bone depositing osteoblasts[34]. L-ascorbic acid is a cofactor for and an inducer of collagen production[14], Dexamethasone is a synthetic member of the glucocorticoid class of steroid drugs and have numerous times been shown to stimulate osteogenesis and terminal differentiation of osteogenic cells[15]. β-glycerophosphate acts as a substrate for and an inducer of matrix mineralization[16]. Dexamethasone is essential for differentiation and has been speculated to exert its effect through the upregulation of a β-Catenin-resembling molecule, which in turn switches on the expression of Runx2 and similar transcripotion factors[33]. In some applications, bone morphogenic proteins and Vitamin D3 are also utilized as inducers of osteogenic differentiation[35, 36]. In figure 2.4, an overview of how to stimulate mesenchymal differentiation *in vitro* is outlined, where additives used to induce osteogenic differentiation is included to the left.

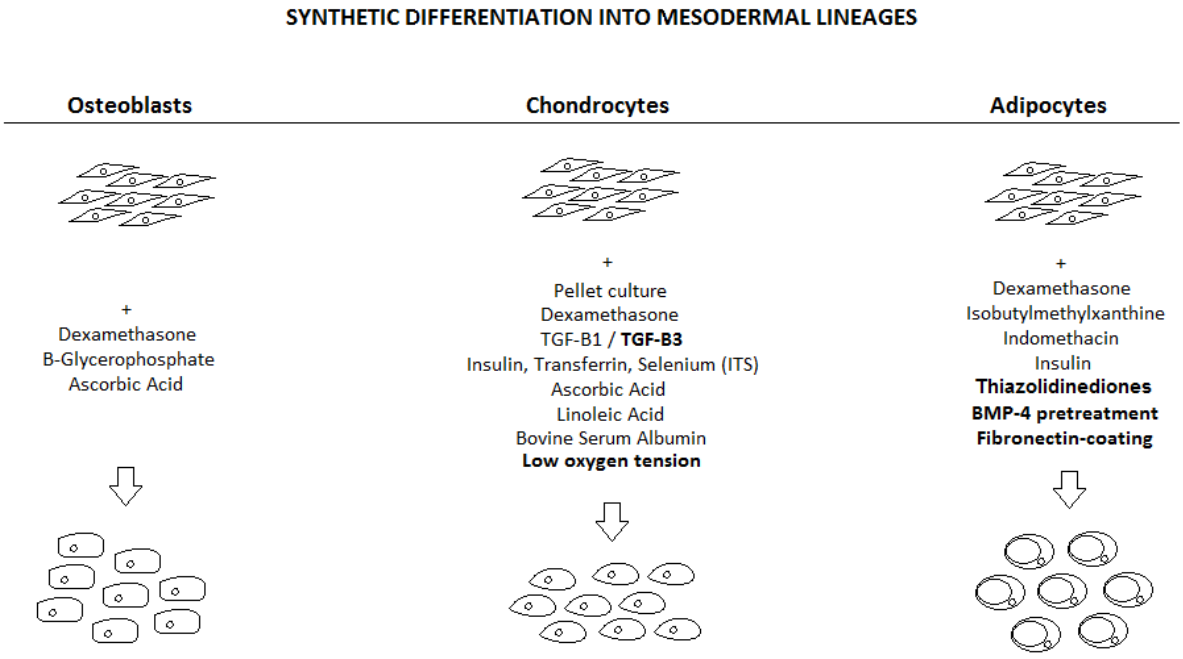


Figure 2. 4 ***An overview of how to stimulate differentiation of mesenchymal stem or progenitor cells into the mesodermal cell types osteoblasts, chondrocytes and adipocytes in vitro. The bold notations of differentiation factors indicates that they are not yet evaluated by Cellectis Stem Cells.***

2.4.2 Chondrogenic cells and differentiation

Cartilage is a connective tissue that consists mainly of proteins and glycosaminoglycan, which yields a support resistant to compression and friction. The only type of cell present in cartilage is the chondrocyte, primarily recognized by its matrix producing function and a highly developed actin-based cytoskeleton[3]. It takes care of all functions regarding the cartilaginous extracellular matrix: deposition, maintenance and degradation[26]. As opposed to bone, cartilaginous tissue has very low self-repair capability in an adult vertebrate, and chondrocytes have limited proliferative potential[3]. This is largely due to the fact that cartilage completely lacks vascularization and

that resident chondrocytes are trapped in the rigid matrix in empty spaces termed lacunae, without possibility for migration[3].

There are three distinct kinds of chondrogenic tissue: hyaline, elastic and fibrous cartilage, which all have different ECM character[3]. Hyaline cartilage, which for example can be found in joints, is frequently damaged due to mechanical stress or diseases, thus of great interest to repair with tissue engineered constructs[3]. It's a tissue mainly consisting of the proteins aggrecan and type II collagen[3]. Aggrecan is a proteoglycan composed of a core protein to which large, sulphated glycosaminoglycans (GAGs) are bound, while collagen II is a fibrillary element that strengthens the mesh structure of the cartilage[22]. The GAGs are bound to hyaluronic acid, which both are negatively charged and thus bind water, which makes the cartilage resistant to friction and compression[26].

To characterize chondrogenic differentiation, one usually examines the formed extracellular matrix directly or quantifies the expression of cartilage ECM-specific genes (such as Aggrecan, Versican, Collagen type II, IX, and XI [21]). Another target is to investigate how regulatory gene expression is affected, such as Sox9 ((sex determining region Y)-box 9) – the master regulator of chondrogenesis. Sox9 has been shown to be essential for commitment into chondrogenic cell lineage, and regulates expression of chondrocyte-specific proteins such as Aggrecan and collagen type II, IX and XI[37]. Furthermore, individuals suffering from the joint disease osteoarthritis have been noted to have decreased expressional levels of Sox9 when compared to healthy controlled[37].

In vitro, two-dimensional culturing of mature chondrocytes changes both morphological and expressional markers of the cells, an event called dedifferentiation[55]. Thus, special techniques to culture and differentiate chondrocytes *in vitro* have been established. Pellet culture is a technique originating from the 1960's[38]. The technique has been refined since its foundation, and today it is one of the most common ways for culture of chondrocytes and differentiation of stem cells into chondrocytes. The material of the culture vessel is a critical factor of the process since it should not support cell adhesion (commonly polypropylene)[39]. The resulting cell aggregates are free-floating, meaning that cell-cell interactions are dominant, mimicking the conditions occurring under pre-cartilage condensation under embryonic development[40].

To differentiate mesenchymal stem cells into chondrocytes, soluble cues are also required. Many protocols show that transforming growth factor beta (TGF-β) and dexamethasone are the most influential in mounting a differential response. Johnston et al. showed that leaving out dexamethasone from an original protocol containing both TGF-β and dexamethasone when differentiating mesenchymal progenitors from bone marrow into chondrocytes reduced efficiency of differentiation, while leaving out TGF-β failed to result in positive differentiation[40]. Several studies also suggest that different forms of TGF-β mount responses that differ in strength: TGF-β2 and TGF-β3 seems to generate a quicker and stronger chondrogenic response than TGF-β1 do[17]. Furthermore, additives such as ascorbic acid, ITS$^+$ (insulin, transferrin, selenium), bovine serum albumin (BSA) and linoleic acid are used[1]. Ascorbic acid is, as mentioned previously in the osteogenic chapter, a cofactor for collagen production. ITS+ is a complex additive commonly added as a defined serum replacement, where insulin is a hormone important for cellular functions due to its involvement in fatty acid and glycogen synthesis, transferrin a protein that complex with iron and increase its bioavailability, and selenium is a cofactor for glutathione peroxidase; an enzyme important in the cellular protection against oxidative damage[41]. BSA acts as a stabilizer for other proteins, thus ensuring their functionality, while linoleic acid has been shown to have a great stimulatory effect on chondrogenesis in combination with TGF-β[41].

An increasing amount of studies also point out oxygen tension to be of critical importance for chondrogenic differentiation. In the physiological environment of both articular cartilage and bone marrow, the oxygen tension is reported to be 1-7% O_2; a condition that can be mimicked under *in vitro* culturing and has been shown to be beneficial both for differentiation and function of chondrocytes[21]. One explanation for this is that hypoxia

upregulates expression of Sox9[42]. For an overview of how to stimulate chondrogenic differentiation *in vitro*, see Figure 2.4.

2.4.3 Adipogenic cells and differentiation

Adipogenic tissue is a connective tissue designed to cushion and isolate other areas in the body, and constitute the largest energy storage facility in the body. Fat cells include two different cell types: white and brown adipocytes. The white adipocyte has spherical morphology, comprising one large triaglycerol droplet that presses the nucleus and other organelles towards the cell membrane[29]. The brown fat cells are smaller, contain multiple lipid droplets as well as numerous mitochondria, and are specialized for heat production[27]. Fat tissue has also been recognized as an important endocrine organ, regulating various whole-organism aspects such as energy balance, blood pressure and vascular haemostasis[27]. The objective of this project was to find and analyze differentiation into white adipocytes.

Adipose tissue is a highly vascularized organ containing several different cell types, such as fibroblasts, endothelial cells and macrophages, as well as mesenchymal stem cells and preadipocytes[27]. The fully differentiated adipocytes do not proliferate, but can however expand in size to about 4 times the average volume (hypertrophy)[29]. If this limit is reached, tissue residing stem cells or progenitors can expand in number, thus generating more fat cells (hyperplasia)[29]. However, proliferation of fat cells is less common in adulthood than during development and childhood, when cell number is highly regulated by the nutritional content of the diet[29]. Fat cells created during childhood are kept throughout adulthood[29].

There have been extensive work performed that map the adipogenic differentiation process during *in vitro* culturing[43]. There are two particularly important gene families involved in the adipogenic differentiation: C/EBP (CAAT/enhancer-binding proteins) and PPAR (peroxisome proliferation-activated receptors)[28]. The isoforms C/EBP beta and delta is expressed shortly after induction is begun and decreased towards the end of the process[44]. The two factors can form dimers that help to induce expression of transcription factor PPAR gamma (from here on denoted PPARg) and C/EBP alpha (from here on denoted C/EBPa) [45] – two genes of critical importance for terminal differentiation[44]. PPARg and C/EBPa both positively regulate their own and each other's expression, and in cooperation (such as dimeric binding to promotor regions of genes) helps to express many adipocytes specific genes that finalize the differentiation, such as aP2 (adipocyte protein 2, a carrier for fatty acids) and GLUT4 (glucose transporter protein 4, a membrane located transporter that imports glucose in response to insulin)[44]. Figure 2.5 illustrates the relationships between differentiation and transcriptional regulation. Fully differentiated adipocytes are straightforwardly characterized by the accumulation if lipids inside the cells, and can be further determined through the quantification of gene expression specific to that of adipocytes, such as leptin, adiponectin, ap2, lipoprotein lipase, GLUT4, FABP4, PPARg and C/EBPa[30].

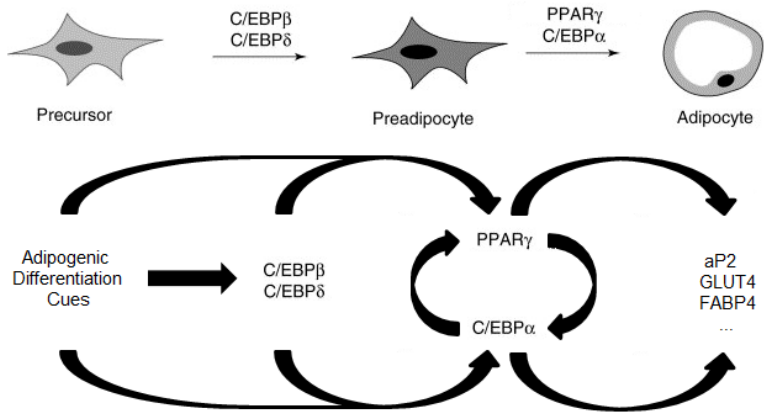


Figure 2. 5 ***Schematic overview of how genes important for adipogenic differentiation are regulated during transformation of a precursor cell to a mature adipocyte.***

A number of different factors have been shown to be influential in the differentiation of adipogenic cells, such as dexamethasone, isobutylmethylxanthin, insulin and indomethacin[1]. As for osteogenic and chondrogenic differentiation procedures, dexamethasone helps to improve the efficiency of the procedure: in the specific case presumably through the induction of transcription factor C/EBP delta[46]. Isobutylmethylxanthin is a chemical compound known to work as an inhibitor for the phospodiesterases that breaks down cyclic AMP, resulting in activation of the transcriptions factor C/EBP beta occur[46]. Thus, the combined usage of IBMX and dexamethasone should induce expression of PPARg and C/EBPa. Insulin is an endocrine factor which naturally affects energy balance of the adipocytes, which *in vitro* also have been shown to help mount a differentiation response when administered at high concentrations (when compared to physiological conditions)[28]. Insulin is partially believed to enhance adipogenic differentiation through activation of PI3-kinase and impact on Akt activity[47], and is believed to exert its effect through the IGF-1 receptor[28], but the complete mechanism of action remains unclear. Indomethacin is a non-steroidal anti-inflammatory drug that inhibit prostaglandin production, and it is commonly is used as an additive during adipogenic differentiation, which significantly upregulates PPARg expression in C3H10T12 cells (murine mesenchymal stem cell like cells) [48].

A certain class of drugs called thiazolidinediones used to treat diabetes mellitus type II have also been shown to increase adipogenic differentiation[49]. They act as PPARg-agonist, which directly bind to the nuclear receptor PPARg and help to express adipogenic specific genes[49]. Two of these substances are Roziglitazone and Pioglitazone.

The growth factor BMP-4 has been proposed to be able to commit previously mentioned C3H10T12 cells (previously mentioned) into adipogenic prescursors[18]. These cells naturally do not show any responsiveness to the adipogenic treatment MDI (IBMX, dexamethasone and insulin)[18]. However, after a pre-treatment of proliferative cells with BMP-4 (until 2 days postconfluent), these cells have been found to be receptive to adipogenic MDI-treatment, with a degree of differentiation of more than 90% of the present cells[18].

The material used for culture can also be of importance for the degree of differentiation. Studies were found indicating that fibronectin coated culture plastic can improve efficiency of adipogenic differentiation in human preadipocytes[67], while other suggest that fibronectin inhibits adipogenesis in murine adipocytes cell lines[57,58]. For an overview of how to stimulate adipogenic differentiation *in vitro*, see Figure 2.4.

2.4.4 Mesenchymal progenitor differentiation

Differentiation into a mesenchymal stem cell like cell requires a partial differentiation, in terms of cell lineage commitment. The obtained cell type should show the typical markers and behavior of a mesenchymal stem cells – cells should still be proliferative, but leave pluripotent stem cell characteristics behind. At the same time, they must remain the capability to be converted into several finally differentiated mesenchymal cell types, but not into other developmental lineages.

Characterization of mesenchymal stem cells is not foolproof, as efforts to find any unique markers have failed so far[68]. Immunological markers expressed by mesenchymal stem cells, such as vimentin, are also commonly expressed by their matured offspring[1]. Therefore, an immune characterization of a presumed established mesenchymal progenitor line (that determines loss of pluripotency, gain of mesenchymal features and failure to show specific ectodermal and endodermal motifs) or equivalent method should be performed in combination with an evaluation of morphology and specific capacities for differentiation into osteogenic, chondrogenic and adipogenic cells[1].

There are several teams that have attempted to establish mesenchymal stem cells artificially, both from embryonic stem cells and induced pluripotent stem cells. As mentioned in section 2.3, Cellectis Stem Cells have developed a reproducible protocol for the establishment of mesenchymal progenitors from embryonic stem cells, where over 10 different embryonic stem cell lines have been used to derive MP-cells that are able to develop several different connective tissues *in vivo* without causing teratomas[1]. The method of establishment is based on selective pressure, where fast growing cells with ability to attach and proliferate in a feeder-free environment are favored[1]. Thus, both pluripotent stem cells and slow growing cells are eliminated. The culture conditions used at differentiation support the growth of mesenchymal stem cells and progenitors, such as gelatin-coating and addition of the growth factor bFGF (basic fibroblast growth factor), which is expected to eliminate other stem cell types. The method is outlined in section 3.2.2.

Efforts to establish MP-cells from induced pluripotent stem cells have also become increasingly prevalent in recent years. Teramura et al. did obtain a mesenchymal progenitor like cell type with good chondrogenic performance from mouse iPS-cells through a process selecting and culturing cells (in MSC-supporting media) showing plastic adherence within an hour subsequently after a enzymatic digestion of embryoid body cultures exposed to a weeklong retinoic acid treatment[51]. Lian et al. obtained functional mesenchymal stem cells from human iPS cells that could be shown to attenuate limb ischemia in mice by using a medium containing the growth factors bFGF), PDGF (platelet derived growth factor) and EGF (epidermal growth factor) for enrichment of MSC outgrowth[20]. FACS-sorting for $CD24^-$ and $CD105^+$ (surface markers shown to be overexpressed at hESCs and hESC-MSCs, respectively) and limiting dilution was performed to obtain pure populations that were shown to be able to give rise to adipogenic, osteogenic and chondrogenic cells to a high degree[20].

2.5 Cell culture

Cell culture is commonly referred to the growing of mammalian cells *in vitro*. Human cells are in general cultured in a humidified incubator at the physiological temperature of 37°C, at levels of 21% oxygen and 5% carbon dioxide. The high levels of carbon dioxide mimic the *in vivo* situation, where carbon dioxide is dissolved in the blood as bicarbonate (HCO_3), which acts as a buffer to prevent great pH changes due to gas, nutrient and metabolite fluctuations. One might adjust gas levels, such as oxygen, for optimal growth of a certain cell type.[3]

Cells can be grown in many different formats (culture flasks, different sized wells, etc.), but in general tissue culture treated plastic is utilized. The plastic is produced in a way that lets most cells adhere to the surface, which is critical for most cell survival and growth. The material that cells adheres to can provide stimulate cues to the

cells which affect cell behaviour. Thus, different coating solutions may be used to cover the culture plastic, such as extracellular matrix components, which in turn can produce a desired behaviour in the cultured cell type.[3]

Cells get their nourishment from cell culture media: a solution containing all essential nutrients that support cell growth, such as amino acids, inorganic salts, vitamins, as well as a source of energy. Usually commercially available culture media also contains a pH-indicator, such as phenol red, that will change colour when the pH is changed, which indicate that a change of media is in order. To the cell culture media, antibiotics (PEST; penicillin and streptomycin) and fetal bovine serum (FBS) are regularly added to keep microorganisms from contaminating the cell culture and as complex supplement of proteins and growth factors, respectively. Specific additions of growth factors are also commonly added depending on cell requirements for growth, such as basic fibroblast growth factor, that for example are essential to keep pluripotent in a non-differentiated state *in vitro*.[3]

As cells grow confluent in two dimensions, meaning that there is no space left for newly dived cells to attach to, they tend to alter their characteristics, such as losing their proliferative abilities. To prevent this, one regularly subcultures, or passages, the cells to a new culture container by enzymatically digesting the protein attachment cells have to the culture material and each other. Once a single cell suspension is obtained, an estimate of the total number of cells can be obtained by using a haemocytometer.[3]

3 METHOD

In this chapter, all of the experimental work that has been performed in this project will be presented. This includes specific experimental setups as well as conceptual descriptions of methodology, materials, tools and techniques used.

3.1 Cell types and culture requirements

Below, a brief description of the cell types used in this project is presented, as well as their culture requirements.

3.1.1 Human embryonic mesenchymal progenitors (hES-MP002.5)

hES-MP002.5 were cultured on gelatine-coated tissue culture plastic in DMEM supplemented with 1% PEST, 10% FBS and bFGF at 4 ng/ml (referred to as MP-media, see 2.5 for details of ingredients). The cells were passaged with TrypLE™ every 3-4 day when cells had grown confluent and showed a swirly pattern, at a seeding density of 10.000 cells/cm^2.

3.1.2 ChiPSC4

ChiPSC4 is a human induced pluripotent stem cell line that originates from fibroblasts established by Cellectis Stem Cells and show characteristics typical for embryonic stem cells (no published material available, see section 2.2 for more details). It was either grown on a quiescent feeder layer of MEF (mouse embryonic fibroblasts unable to divide) or on coated tissue culture plastic. Both culture techniques utilized Cellectis Stem Cells own culture medium DEF-CS™ that support pluripotent growth.

3.1.3 ChiPSC4-MP

ChiPSC4-MP is a cell type created in this project, with ChiPSC4 as starting material. The cells were cultured on gelatine coated tissue culture plastic in DMEM supplemented with 1% PEST, 10% FBS and bFGF at 4 ng/ml. The cells were passaged with TrypLE™ (Life Technologies) when the cells had grown confluent, at a seeding density of 10.000 cells/cm^2.

3.1.4 Thumb

Thumb is a cell line that serves as positive control in the mesodermal experiments of the project, received from Sahlgrenska University Hospital, Institute of Biomedicine/Neuroscience and Physiology, and was established from neonatal articular chondrocytes isolated from the distal end of metal carpal phalangeal (MCP) bone[59]. When grown in 2D-culture, they dedifferentiate and start to proliferate. They should acquire the ability to differentiate into osteogenic, chondrogenic and adipogenic lineages. Thumb was cultured in DMEM/F12 supplemented with 1% Glutamax, 1% PEST, 10% FBS and 140 uM Ascorbic Acid. The cells were passaged with Trypsin/EDTA (more invasive than TrypLE™) when 80% confluence was reached, at a seeding density of 5000 cells/cm^2.

3.2 Enzymatic passage

Enyzymatic passages in this project have been performed according to the following protocol:

1. Aspirating culture medium and washing with PBS- (phosphate buffered saline) once.
2. Adding TrypLE™, alternatively Trypsin +EDTA for Thumb-cells, and incubating at 37°C for approximately 5 minutes or until singelcell-suspension is obtained.
3. Transferring cell suspension to a 15-ml tube, washing culture container with PBS+ and adding it to rest of cell suspension, pipetting up and down to achieve homogenous solution.
4. Transferring cell suspension to Bürkerchamber, counting cell and calculating cell concentration.
5. Transferring an appropriate volume (i.e. number of cells) for passage to a new tube, centrifuging the sample at 300g/5min.

6. Aspirating supernatant gently, dissolving cell pellet into fresh culture medium, and transferring of cell suspension into new culture container.

3.3 Differential inducement

In this project, differentiation of pluripotent stem cells toward mesenchymal lineage have been investigated, as well as a more specialized differentiation towards the mesodermal lineages of bone, cartilage and fat. Details regarding the procedures follow in the sections below.

3.3.1 Preparation of additives

Several different additives have been used as supplements to the culture medium during differential inducement. Below in table 3.1 the soluble components for differentiation used in this project are listed, the solvents used, concentrations obtained and what application the solution was used in.

Table 3. 1 *Used additives for differential inducement*

Compound	Solvent	Concentration	Use
Dexamethasone	DMSO	0,1 mM	Osteogenic, Chondrogenic, Adipogenic
β-Glycerophosphate	DMEM-LG	1 M	Osteogenic
Ascorbic Acid	dH_2O	45 mM	Osteogenic, Chondrogenic
Linoleic Acid	EtOH	5 mg/ml	Chondrogenic
Human Serum Albumin	DMEM with Glutamax	1 mg/ml	Chondrogenic
TGF-β1/ TGF-β3	Citric Acid 10 mM (in dH_2O); 0,1% BSA (in dH_2O)	1 ug/ml	Chondrogenic
Isobutylmethylxanthin (IBMX) + Indomethacin	DMSO	0,5M 60 mM	Adipogenic
Insulin, human	0.01 M HCl (in dH_2O)	5 mg/ml	Adipogenic
Rosiglitazone	DMSO/DMEM-LG	0,1 mM	Adipogenic

3.3.2 Establishment of mesenchymal progenitors from ChiPSC4

Cellectis have established a commercially available mesenchymal progenitor cell line (hES-MP002.5) from embryonic stem cells, and has standard operating procedures for the establishment. In this project, the possibility to utilize the existing protocol to establish mesenchymal-like cells from the induced pluripotent stem cell line ChiPSC4 is investigated. Figure 3.1 shows an overview of the procedure.

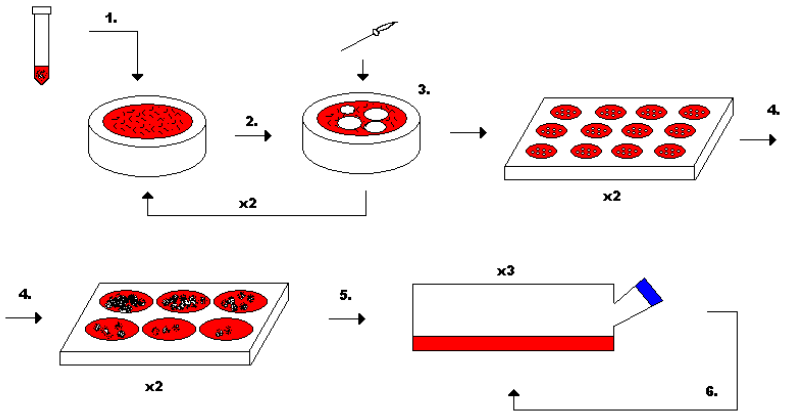


Figure 3. 1 *Procedure of mesenchymal progenitor establishment: 1. Freely growing ChiPSC4 enzymatically passaged to MEF, 2. Stem cell colony formation, 3. Mechanical passage to 12-hole format and start of mesenchymal induction, 4. Enzymatical passage to 6-hole format with different cell densities, 5. Enzymatical passage to flask-format, 6. Enzymatical passages for continued growth and maturation*

3.3.2.1 Colony formation on MEF

Intitially, ChiPSC4 in passage 16 were enzymatically passaged from a feeder free container and seeded on MEF growing in IVF-dishes at a density of 1000 cells/cm^2 (figure 2.1, point 1). Cells were mechanically passaged two times onto MEF when colonies grown big (figure 2.1, point 2), yielding the starting product for differentiation: ChiPSC4 in passage 16 in feeder-free culture and passage 3 on MEF. The transferral of cells from feeder-free to a feeder environment is due to the fact that the establishment-protocol was created earlier than the development of DEF-CS™.

The mechanical passages were performed by using Stem Cell Cutting Tool and Suction Pipette (Vitrolife). By making perpendicular cuts in the cell matt, a grid was created and squares of coherent, undifferentiated cell were scraped loose (approx. 200µm x 200µm). The squares were then transferred to a fresh, MEF-covered IVF-dish.

3.3.2.2 Mechanical Passage/MP inducement

When the pluripotent cells produced big enough colonies for the third time, the cells were mechanically passaged from several IVF-dishes for differential inducement (figure 2.1, point 3). About 15-20 cell-squares (approx. 200µm x 200µm) were placed in each well of a gelatine-coated 12-well plate, to which MP-medium had been added. Media were not changed for three days to let the cell-pieces firmly attach to the bottom of the wells, thereafter; media was changed every second to third day.

3.3.2.3 Enzymatic passage and maturation

After one week, the entire population in 12-well format was enzymatically passaged to two 6-well plates, and seeded with a split ratio of 1:1 - 1:4 (figure 2.1, point 4) in order to assess the impact of seeding density on cell growth (not according to protocol: usual procedure is to collect all cells and passage when confluent). Three days later, one of the two 6-well plates was further enzymatically passaged into two T75-flasks: the three wells seeded at lowest density and at highest density were pooled together (figure 2.1, point 5). After another three additional days, the three "sparse wells" on the second plate were pooled together and enzymatically passaged to a T75-flask, while the "dense wells" were discarded. The best proliferating flasks were then passaged at every time point when confluence had been reached (figure 2.1, point 6). The "pooling-procedures" were a result of time deficiencies during laboratory work, and was not according to established protocol.

The cells established in these experiments are analysed in an immunocytochemical assay to examine mesodermal lineage potential (see section 3.4.4.1), as well partly used as starting material for secondary rounds of mesodermal induction experiments (see section 3.3.3 – 3.3.5).

3.3.3 Osteogenic culture

The differentiation of hES-MP002.5 cells into the osteogenic lineage has been successfully and efficiently performed by de Peppo et al.[2], why this protocol is used to promote osteogenesis throughout the entire project.

3.3.3.1 Media composition

The osteogenic media consisted of:

- DMEM-LG supplemented with:
    - 1% PEST
    - 10% FBS
    - 1 µM Dexamethasone
    - 45 µM Ascorbic Acid
    - 20 mM β-Glycerophosphate

1 ml medium was used per well, and medium was changed twice a week. Culturing was performed under normoxic incubation conditions.

3.3.3.2 Experimental setup

To induce osteogenic differentiation, all cells to be tested are seeded and grown on tissue culture treated plastic 24-well plates, seeded at 10.000 cells/cm^2. Two runs of inductions were made:

1. For 8 weeks, hES-MP002.5 in passage 9 (p9) was induced with osteogenic medium, comparing osteogenic potential on gelatin- or non-coated plates, since specifications were not given by the author of the protocol. Sample analysis was performed two times for the first three weeks, then once every week for the resulting 5 weeks.

2. For 6 weeks, hES-MP002.5 (p9), ChiPSC4-MP (p7) and Thumb cells (p8) were induced with osteogenic medium on gelatin-coated plates, comparing mesodermal potential. hES-MP cells were also cultured and used as a negative control, administered MP-media throughout the whole culture period. Sample analysis was performed twice each of the first three weeks, and thereafter once every week.

Analysis methods were histological staining with Alizarin Red and an activity assay of the enzyme alkaline phosphatase. See section 3.4.2.1 and 3.4.3 for further information.

3.3.4 Chondrogenic culture

Chondrogenic differentiation is commonly induced by the addition of a growth factor supplemented media to cells in pellet culture, discussed below. The protocol initially used is obtained from an article where several hES-MP lines established at Cellectis Stem Cells were characterized, including hES-MP002.5[1].

3.3.4.1 Pellet culture

Cells were obtained through enzymatic passaging, and were evenly distributed in differentiation medium. For each pellet, cells were transferred to a 15 ml polypropylene tubes commonly used for centrifugation. The cells were spun at 500 g for 5 minutes, yielding a condensed cell mass in the bottom of the tube that aggregated into a free floating, spherical pellet within 24h.

3.3.4.2 Media composition

The chondrogenic media consisted of:

- DMEM-HG with GlutaMAX supplemented with:
    - 1% PEST
    - 10% FBS
    - 1% ITS^{+} (Insulin, Transferrin, Selenium)
    - 0.1 µM Dexamethasone
    - 80 µM Ascorbic Acid
    - Linoleic Acid: 5 µg/ml
    - BSA (Bovine Serum Albumin): 1 mg/ml
    - TGFB-1: 20 ng/ml

    - Modification: 20 ng/ml TGF-β3 instead of TGF-β1 (see section 2.4.2 for motivation)
    - Modification: Hypoxic incubation culture (see section 2.4.2 for motivation)

0,5 ml medium was used per well, and medium was changed twice a week. Culturing was performed both under normoxic and hypoxic incubation conditions (5% O_2, 5% CO_2) .

3.3.4.3 Experimental setup

To induce chondrogenic differentiation, all cells to be tested were pelleted as described above, and grown in 15-ml propylene tubes with lids allowing for gas exchange. The pellets created in this project consisted of approximately 400.000 cells each (except for point 1.b below). Two runs of inductions were made:

1. hES-MP002.5 in passage 13 was induced with chondrogenic medium containing TGF-β1 under four different circumstances:
    a. Regular medium and treatment
    b. Half sized pellet (200.000 instead of 400.000 cells)
    c. Double TGF-β1 concentration during pellet formation
    d. Hypoxic conditions (5% oxygen instead of atmospheric 21%)

    Samples were gathered for sectioning and histological staining after 26 days of culture.

2. hES-MP002.5 (p11), ChiPSC4-MP (p9) and Thumb-cells (p9) were induced with chondrogenic medium containing TGF-β1, and hES-MP002.5 (p11) was also treated with media containing TGF-β3. All samples were made in duplicates, where one of the groups was cultured under hypoxic conditions. Samples were gathered for sectioning and histological staining after 4 and 24 days, respectively. Samples were gathered every week for analysis with qPCR.

Analysis methods were histological staining with Alcian Blue van Gieson and Safranin O for all samples, and for the second run of chondrogenic inductions, qPCR analysis were performed using 3 assays. See section 3.4.2.2 and 3.4.5.1 for further information.

3.3.5 Adipogenic culture

Adipogenic differentiation is commonly induced by regular two-dimensional culture with supplemented medium. The protocol initially used is obtained from an article where several hES-MP lines established at Cellectis Stem Cells were characterized, including hES-MP002.5[1].

3.3.5.1 Media composition

The adipogenic media consisted of:

- DMEM-LG supplemented with:
  - 1% PEST
  - 10% FBS
  - 1 µM Dexamethasone
  - 0.5 mM Isobutylmethylxanthin (IBMX)
  - 60 µM Indomethacin
  - Insulin, human: 5 µg/ml

  - Modification: 1 week pre-treatment of cells to be induced with BMP-4: 50 ng/ml (see section 2.4.3 for motivation)
  - Modification: induction medium supplementation with Roziglitazone: 1 or 10 µM (see section 2.4.3 for motivation)

1 ml medium was used per well, and medium was changed twice a week. Culturing was performed under normoxic incubation conditions.

3.3.5.2 Experimental setup

To induce adipogenic differentiation, all cells to be tested are seeded and grown on tissue culture treated plastic 24-well plates, seeded at 10.000 cells/cm^2. Two runs of inductions were made:

1. For 3 weeks, hES-MP002.5 (p12) was induced with either regular adipogenic medium, or with the addition of Roziglitazone at 10 µM. The MP-cells used for induction had either been seeded 2 or 7 days prior to induction. The cells seeded 7 days prior to induction were either cultured in MP-media supplemented with BMP4 at 50 ng/ml or in regular MP- media. All cells were seeded on gelatine-coated dishes. Samples were taken for histological staining once every week.

2. For 3 weeks, hES-MP002.5 (p11), ChiPSC4-MP (p8) and Thumb cells (p8) were induced with regular adipogenic medium supplemented with Roziglitazone at 1 µM. hES-MP cells were also cultured and used as a negative control, administered MP-medium throughout the whole culture period. The cells used for induction had either been seeded on gelatine- or fibronectin-coated dishes. Samples were taken for histological staining and qPCR every week.

Method of analysis were histological staining with Oil Red O for all samples, and for the second run of adipogenic inductions, qPCR analysis were performed using 3 assays. See section 3.4.2.3 and 3.4.5.2 for further information. Aditionally, Alizarin Red staining was performed during the first round of experiments (section 3.4.2.1).

3.4 Analysis

3.4.1 Microscopic inspection and documentation

Throughout the whole project, light microscopes have been the most frequently used tool in determining whether or not cells have differentiated into the right lineage. The camera Nikon Eclipse Ti-U has been used to document sequential progress of the cultured cells as well as the results from histological staining. The microscope Nikon SMZ1000 has also been of use for whole-well imaging as well as when cutting stem cell colonies. During

microscopic inspection, changes in morphology and growth rate have been recorded. Pictures was taken with digital Camera Nikon MXM1200C and processed with the software ATC-1C.

3.4.2 Histological staining

To confirm microscopical evaluations, histological staining is a straightforward technique to detect certain cellular features or products. A staining solution is prepared which is added to a sample of fixated cells for a period of time, after which excess colour is washed away and the sample can be viewed microscopically.

3.4.2.1 Alizarin Red

Alizarin Red is an organic compound that binds specifically to ionic calcium, which precipitates the compound. It is commonly used to confirm the presence of osteoblasts by detect mineralized deposits.

The Alizarin Red working solution was prepared fresh at each point of analysis by dissolving 0.2g of Alizarin Red powder in 10 ml of dH_2O, followed by sterile filtration to remove non-dissolved particles. Samples to be examined in this project were washed with PBS+, fixated with 2,5% glutaraldehyde for 15 min, washed twice with dH_2O (to prevent precipitations of calcium containing PBS+) before adding of Alizarin Red solution. Samples were incubated in 37°C for 15-20 min, stain solution was aspirated and samples washed twice with dH_2O before PBS+ addition and microscopic inspection.

3.4.2.2 Alcian Blue van Gieson/Safranin O/Sectioning

Alcian Blue van Gieson and Safranin O are both common stains to detect cartilage. Alcian Blue is a large cationic molecule with several positive charges which bind to the negative sites on polysaccharides found on glycosaminoglycan and consequently stain them blue. Van Gieson is a low molecular counter stain which penetrates the tissue to be investigated and stain connective tissue such as collagen red. Safranin O is a smaller cationic molecule with a single charge which stains negative sections of the proteoglycans, as well as nucleic acids, orange to red.

Cartilage pellets could only be evaluated macroscopically (e.g. taking notations of pellet hardness and size) during period of culture, since cellular differentiation take place inside the pellet. At harvest (day 4 and end of experiment), pellets were washed with PBS+ once, incubated in 4% formaldehyde solution overnight, followed by incubation in PBS+ for 4-6h, before final replacement and storage in 70% ethanol. Following this, sectioning of the pellet was outsourced to Histocenter, Gothenburg. Their procedure involves dehydration of the pellets in a series of alcohols of graded alcohols (increasing strength), paraffin embedding and sectioning into 5 µm slices before mounting them on a microscopic glass slide and rehydrating them. The sections were then stained with Alcian Blue van Gieson and Safranin O.

3.4.2.3 Oil Red O

Oil Red O is a fat-soluble dye which stains neutral lipids and triglycerides orange to red, a fact that can be used to identify adipogenic cells which accumulate intracellular lipid droplets.

An Oil Red O stock solution is prepared by dissolving 0.25 g Oil Red O powder in 50 ml 2-propanol. At each point of analysis, a proper amount of working solution is prepared fresh by mixing the stock solution with 2/3 dH_2O, and letting the solution rest for 10 minutes before filtrating it to remove precipitated particles. The staining is performed by washing cells with PBS+, fixating cells with Histofix for 15 min, washing twice with PBS+, incubating samples with Oil Red O in room temperature for about 1 h before removing excess dye with PBS+ for microscopic inspection.

3.4.3 Alkaline phosphatase activity assay

The levels of alkaline phosphatase (ALP) were chosen to be evaluated as a marker of ontogenesis, since it is an abundantly expressed enzyme in osteoblasts and allows for a quantitative measure of osteoblast activity. ALP is also expressed at high levels in pluripotent stem cells, but since state of pluripotency additionally is evaluated by immunocytochemistry, the ALP-activity is assumed to stem only from osteogenic activity.

The assay used in this project is a commercially available Alkaline Phosphatase Fluorometric Assay Kit (Abcam, ab83371), in which the substrate methylumbelliferyl phosphate disodium salt (MUP) can be processed by ALP to the compound 4-Methylumbelliferone (4-MU) which emit light with wavelength 440 nm when exposed to a wavelength of 360 nm (Ex/Em 360/440 nm). Fluorescence was measured using FLUOstar Omega (BMG Labtech)at ex/em 355/460 nm.

The processing of each sample included 1) the addition of 250 µl Assay Buffer to a PBS+ washed well, 2) mixing and scraping of the cells/ECM into solution, 3) transferral to centrifuge tube, 4) centrifugation of the cell suspension at 13.000g/3min, and 5) transferral of 110 µl of each sample into two separate wells: sample and background (i.e 220 µl in total), 6) adding of 20 µl of "stop solution" to each background-well to prevent any phosphatase activity, 7) adding 20 µl of 0.5 mM MUP-substrate to all samples and backgrounds and incubating the ongoing reactions in the dark at RT for 30 min, 8) adding 20 µl "stop solution" to all sample wells and results were obtained by measuring the fluorescence intensity. In all experiments, 3 or 4 samples per experimental treatment or time point were used to increase accuracy of the readings, and an average of activity was calculated.

One or more standard curves were created at each point of analysis: six known concentrations ranging from 0-0.5 nmol (recommended by producer), 0-5 nmol or 0-10 nmol MUP were set up in the same manner as the other samples, to which 10 µl of purified ALP-enzyme (from producer) was added. The ongoing reactions were incubated in the dark at RT for 30 min, followed by addition of 20 µl "stop solution" and results were obtained by measuring the fluorescence intensity, from which a standard curve could be calculated.

When the results had been obtained, the activity of the sample was obtained through the following calculations:

A = (((Sample mean) – (Background mean))/(Slope of standard curve)) =

nmol 4-MU generated by the sample taken

Note should be taken to the fact that only 110/250 µl of the solution containing all ALP from one examined well is used for sample reading, which should be corrected for. As the sample volume (110 µl) and reaction time (30 min) is constant, the final equation for calculating ALP-activity is:

ALP activity per examined well =

= (A / (Volume of sample added in assay) / (Reaction Time)) * (250/110) =

= (A / 0.11 ml / 30 nin) * (250 / 110) = 0.68817 A [mU/ml]

U stands for the unit definition, indicating the amount of enzyme causing the hydrolysis of 1 µmol of MUP per minute at pH 10.0 and 25°C.

3.4.4 Immunocytochemistry (ICC)

Immunocytochemistry is the process of detecting cellular markers in cultured cells through the use of antibody based techniques.

3.4.4.1 Mesenchymal assay

The cells obtained from the mesenchymal progenitor establishment experiments were characterized through a small immunocytochemical evaluation, set up with antibodies already present at Cellectis Stem Cells. Non-induced ChiPSC4, hES-MP002.5 and Thumb-cells were used as to obtain positive and negative controls. Table 3.2 contains detailed information on the antibodies used in the project.

Table 3. 2 ***Antibodies used in mesenchymal assay and specifications***

Type	Epitope	Kind	Specificity	Dilution	Localization
Primary	**Oct4**	IgG2b	Pluripotency	1:200	Intracellular
Primary	**hES-Cellect**	IgG1	Pluripotency	1:1000	Surface
Primary	**TRA-1-81**	IgM	Pluripotency	1:200	Surface
Primary	**ASMA**	IgG2a	Mesodermal (smooth muscle cells)	1:500	Intracellular
Primary	**Vimentin**	IgG1	Mesodermal	1:300	Intracellular
Primary	**FoxA2 (HNF3 β)**	IgG2a	Endodermal	1:500	Intracellular
Primary	**β-III-Tubulin**	IgG	Ectodermal (neuron)	1:100	Intracellular
Secondary	**Goat-anti-Mouse**	Anti-IgG	Primary antibody raised in mouse	1:1000	Depending on primary binding

The antibodies used to rule out pluripotency were targeted to Oct4 and TRA-1-81, and an in-house antibody product developed to detect pluripotent cells called hES-Cellect was also be used for this purpose[69]. The antibody hES-Cellect was raised against intact pluripotent cells and thus is a surface maker; however its specific target is not identified.

Antibodies to verify mesodermal motifs were targeted to Vimentin, while antibodies raised against ASMA (alpha smooth muscle actin) was used to rule out that the cells have furthered differentiated into the mesodermal cell type smooth muscle cell. Furthermore, antibodies recognizing endodermal phenotype (FoxA2 (HNF3beta)), as well as ectodermal phenotype (neuron specific β-III-Tubulin), were used to rule out differentiation into the wrong germ layer.

The procedure was initiated by blocking the fixated cells (48-well format) to be examined with a skim-milk-powder solution for 1h, washing with PBS+/+, followed by the addition of 100 µl of primary antibody solution (see used dilution in table) to each sample and incubation overnight in 4°C. The primary antibodies were dissolved in 1% BSA (bovine serum albumin), either in a solution containing 0.2% Triton X (a membrane permeabilizer) for internal epitope localization, or without Triton X for surface-antigens.

The second day of analysis, the samples were washed three times with PBS+ before the secondary antibody were added. The secondary antibody used was Goat-anti-mouse IgG, which was used for all samples (including for antibody directed against TRA-1-81: IgM). It was diluted in the same solutions used for the primary antibodies.

To the secondary AB-solution, the fluorescent chromosome-specific stain DAPI was also added (1:2000) enabling cell nuclei localization. The cells were incubated for 1.5 hours in room temperature before the samples were washed three times with PBS+, observed and photographed with a fluorescence microscope.

3.4.4.2 TGF-β-RII assay

As chondrogenic differentiation of hES-MP002.5 is problematic, one would like to study the possible ways for the cells to respond to the most influential differentiation signal administered during induction, namely TGF-β. An antibody raised against full-length, human, TGF-β-RII in mouse was purchased and evaluated (Santa Cruz Biotechnology) for this purpose. The dilution of the antibody used was 1:100 in 0.2% Triton X and 1% BSA, otherwise carried out as described in section 3.5.4.1, for all cell types discussed.

3.4.5 Quantitative polymerase chain reaction (qPCR)

Quantitative, or real-time, PCR is a method that enables quantitative measurements of the level of expression of any gene of interest, through the conversion of mRNA into cDNA by reverse transcription, followed by a real-time amplification of the cDNA in a PCR where fluorescently labelled primers specific for the gene of interest is added. The PCR-reaction proceeds for 40 cycles, and the fluorescent signal (conveying double stranded DNA-content) is measured continuously. Relative levels of gene expression can be acquired by correlating the signal to that of an internal reference – a gene that is rendered to have relatively stable expression irrespective of surrounding conditions. In this project, CREBBP (cyclic AMP-responsive element binding protein) was used as a reference gene.

The quantification is represented by a plot showing the fluorescence intensity against the numbers of PCR-cycles. The fluorescent signal from the sample will after a number of cycles generate a steep slope upwards if the investigated mRNA is present, which reach a cycle threshold set for the detection of DNA-based fluorescent called Ct (set slightly higher than background signal). The sooner this threshold is met, the higher the expression of the gene. Each obtained Ct-value for a sample is correlated to the Ct for the housekeeping gene in the same sample to normalize for differences in RNA-levels and quality between the examined samples, which allows for comparisons between samples.

In this project, values obtained were further normalized to a calibrator, which consists of a pool of RNA collected from different cell types and tissues that is used within Cellectis Stem Cells (such as pluripotent cells, cardiomyocytes and hepatocytes), in order to have a reference that show expression of most of the genes of interest. In the project, it is included since the use of a calibrator is standard operating procedure.

3.4.5.1 Cartilage specific qPCR

Three cartilage-specific genes were selected to be examined through RT-PCR, namely the regulatory gene SRY-Box 9 (SOX9), and the extracellular-matric proteins Aggrecan (ACAN) and Collagen Type II (COL2A1). All assays were purchased from Life Technologies (specification: Hs00264051_m1* for COL2A1, Hs00153936_m1* for ACAN and Hs01001343_g1* for SOX9).

Samples for RT-PCR were harvested each week (day 7, 14 and 21), of chondrogenic induction through instant freezing of pellets in liquid nitrogen, and storage at -80°C (24 samples in total). All samples were homogenized at Sahlgrenska University Hospital in a "TissueLyser" (Qiagen).

The cell lysates were centrifuged and used for RNA-purification in a MagMAX Express (Applied Biosystems) according to the manufacturer's protocol. The concentrations of the resulting RNA-samples were calculated from the absorbance at 260nm in a GeneQuant Pro (Amersham Biosciences, UK). Samples were also taken from un-induced cells to provide background levels, as described in section 3.4.5.2. cDNA-synthesis were performed in a Mastercycler gradient (Eppendorf), and RT-PCR in a 7500 RealTime PCR System (Applied Biosystems) by Tina

Nilsson, Cellectis Stem Cells employee. Only one sample were taken per treatment Two technical replicates were made per sample.

3.4.5.2 Adipose specific qPCR

Three adipose-specific genes were selected to be examined through RT-PCR, namely the regulatory genes CCAAT-enhancer-binding protein alpha (C/EBPa) and Peroxisome Proliferator-Activated Receptor gamma (PPARg) , as well as the functional membrane protein GLUT4 (also known as Solute Carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4)). All assays were purchased from Life Technologies (specification: Hs00269972_s1* for C/EBPa, Hs01115513_m1* for PPARg and Hs00168966_m1* for SLC2A4).

Samples for RT-PCR were harvested each week (day 7, 14 and 21) of adipogenic induction by adding of 250 µl RNA protect Cell Reagent (Qiagen) to each well. Cells were detached by scraping, and the cell suspension in was stored at -20°C until point of analysis. Samples were also taken from un-induced cells to provide background levels (referred to as zero-samples in the results section). Total RNA was prepared in a MagMAX Express (Applied Biosystems) according to the manufacturer's protocol. The concentrations of the RNA-samples were determined as described in previous section. cDNA synthesis and RT-PCR were performed by Tina Nilsson as described in previous section. Two technical replicates were made per sample.

4 RESULTS

4.1 Establishment of mesenchymal progenitors from ChiPSC4

Three batches of ChiPSC4-MP were induced during the time of the project. Batch number 2 was handled by the author and will be presented with detailed information regarding morphology and mesodermal induction. The different stages of mesenchymal induction from the second batch are outlined in Figure 4.1. The pictures taken from passage 3 and forward are taken from the cell population that grew the fastest after varying cell density.

4.1.1 Morphological evaluation

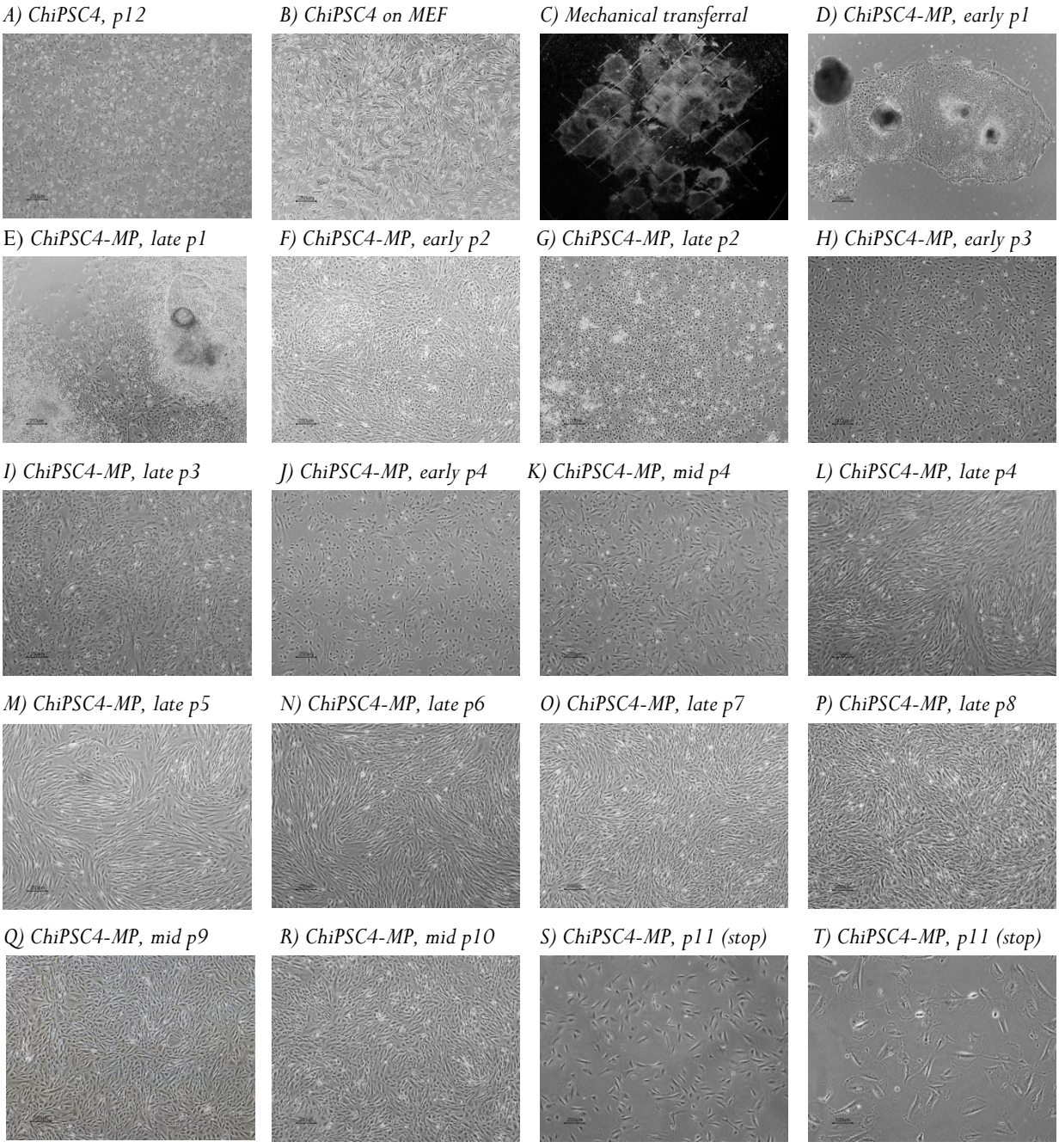


Figure 4. 1 ***Proceedings of mesenchymal induction of batch 2. From passage 3 and onwards, the pictures illustrates the quickest growing batch (high density culture).***

The MP-induction of ChiPSC4 started with the placement of mechanically cut pluripotent cell units (C) in MP-culturing medium. As the media supporting pluripotent growth was replaced, the cells growing out from the cell units showed a range of differentiated morphologies (D). Certain areas looked much like mesenchymal progenitors; stringy, elongated cells; while other was more condensed; rounded nucleuses with low degree of stretch. Some cells also looked finally differentiated, such as the larger cells with rounded nucleuses and wide cell attachments in all directions. This kind of cell type could also be seen at passage 11, when the population stopped proliferating (T). However, all cells were enzymatically passaged further after the induction was started. Cells were generally relatively small in passage 3-4, similar to hES-MP002.5 cells, seemed to become bigger in passage 5. At this point, the cellular population looked roughly homogenous, and exposed a swirly pattern that hES-MP002.5 also show at confluence. This characteristic was eventually diminished (O-P), and a more inhomogeneous cell population appeared – small, rounded cells parallel to larger and stretched cells, not appearing to follow a clear growth pattern. In later passages, the growth rate of the cells diminished, and cells completely stopped dividing after passage 11.

During the early period of culture, it appeared as a high cell density was beneficial for the growth rate of the population. From observations made at batch 1, where the population stopped growing at passage 6, a decision was made to vary seeding density and point of passage after the first enzymatic passage for batch number two in order to see if it could influence growth. During the second to the third passage, cells in the same format were either passaged at day 4 or 7 after previous passage. 2 days after the passage of the last group of cells (i.e. day 9), cells in both culture flasks were confluent, while cells in the flask passaged at day 4 were bigger. The two flasks were passaged simultaneously, and the flask passaged at day 7 was found to contain about 3 times as many cells, even if the starting cell number should have been roughly equal. The quick-growing batch was the only one taken for further experiments.

4.1.2 Growth rate

Three batches of ChiPSC4-MP were induced, where the first batch kept proliferating until passage 6, the second batch until passage 11 (the same batch used for mesenchymal induction studies), and the third batch to passage 12 before it was cryopreserved. Figure 4.2 show how many cell doublings the population made during each passage for batch number 3 of ChiPSC4-MP. The number of cell doublings seems to be consistent around 1.8 throughout the culture period.

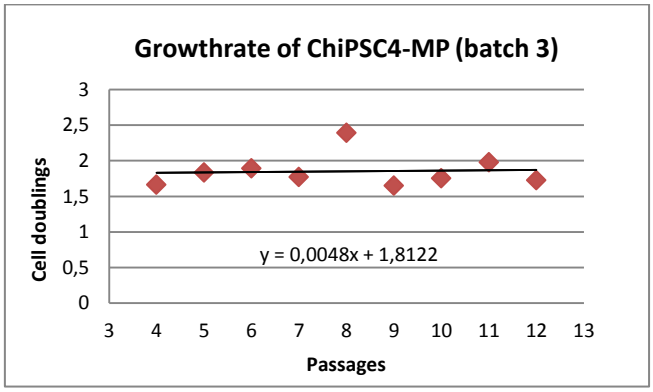


Figure 4. 2 *Cell doublings per period of time (mean passage number 3,44 days) for the third batch of MP-induced ChiPSC4-MP.*

ChiPSC4-MP appeared to grow slower than hES-MP002.5, however, no laboratory comparison was made. Batch number two of ChiPSC4-MP cells seemed to grow slower towards the end of the culturing period since passaging of cells became less frequent, while batch three appeared to have constant growth rate until they were frozen in passage 12, as indicated in the figure.

4.1.3 ICC – Mesenchymal evaluation

The results of immunocytochemical evaluation were inconclusive. Analysis was performed on all three batches of ChiPSC4-MP either in passage 8 or 9, and generated the same results. ChiPSC4-MP showed the same staining patterns as hES-MP002.5 with the exception that the epitopes recognized by hES-Select were not detected at all in ChiPSC4-MP, however vaguely in both hES-MP and Thumb. See summary of results for all cell types and investigated markers in Table 4.1.

Table 4. 1 *Outcome of mesodermal characterisation by immunocytochemicstry. The notations in the table are as follows: (-) negative, (+) slightly positive, (++) medium positive, (+++) completely positive.*

	ChiPSC4-MP (p8 & p9)	**hES-MP002.5 (p9)**	**ChiPSC4 (p18)**	**Thumb (p9)**
Oct4	-	-	+++	-
hES-Cellect	-	+	+++	+
TRA-1-81	-	-	++	-
Vimentin	+++	+++	+++	+++
ASMA	+	+	-	+
β-III-Tubulin	+++	+++	+++	+++
FoxA2	-	-	-	-

All cell types, including pluripotent stem cell line ChiPSC4-MP, showed strong positive signal for Vimentin and neurospecific β-III-Tubulin, results not correlating well to predicted results – Vimentin should not be positive for the pluripotent line ChiPSC4, while none of the investigated cell types should be positive for neurospecific β-III-Tubulin.

Both MP-lines and Thumb were also slightly positive for ASMA, as well as negative for FoxA2, which was according to prediction. Accordingly, ChiPSC4 were negative for ASMA and FoxA2, but strongly positive for all pluripotency markers (Oct4, hES-Cellect, TRA-1-81). All other cell types were negative for Oct4 and TRA-1-81, but as mentioned, a somewhat positive signal was generated for hES-Cellect in hES-MP002.5 and Thumb – cell types that should show negative results for all pluripotent motifs. The positive results from the immunocytochemical staining for ChiPSC4-MP are shown in Figure 4.3.

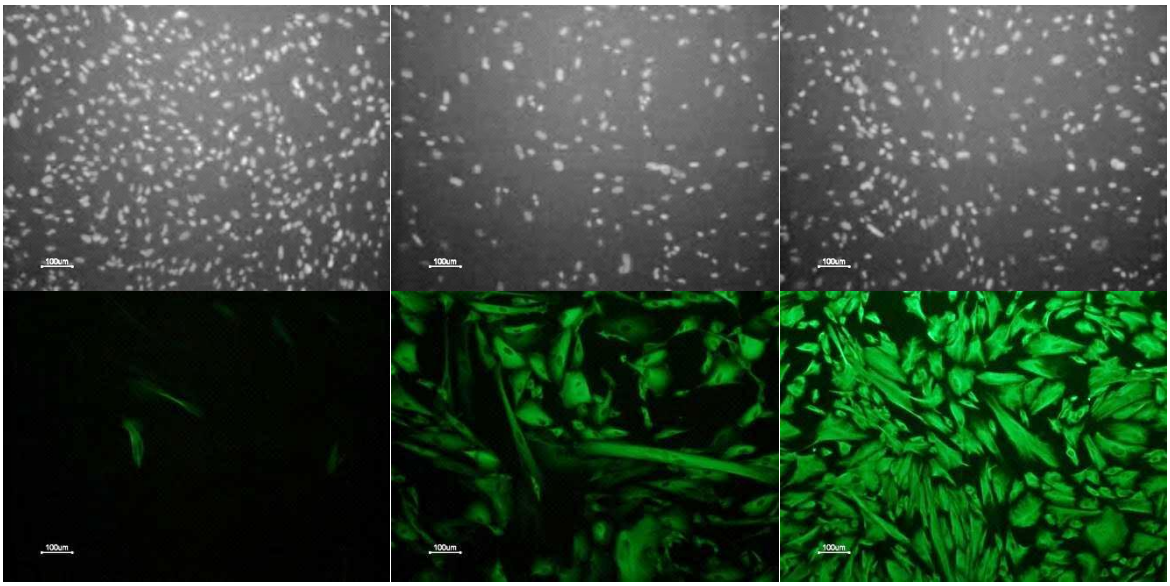


Figure 4. 3 *Positive immunocytochemical staining of ChiPSC4-MP. Upper row shows DAPI-staining (show nucleus), lower row shows corresponding positive antibody binding. From left to right: ASMA, β-III-Tubulin and Vimentin.*

4.2 Osteogenic differentiation

The results gathered from the osteogenic analysis are represented as graphs containing ALP-activity plotted against induction time from both experimental rounds, and sequential photos of Alizarin Red stained wells from the second experimental round. From the first of the two individual experiments made, it was concluded that inducing cells on gelatine gave a more homogenous growth, while non-coated plastic made the cells curl up and grow more "in lumps" (photos not shown). Thus, gelatine-coating was selected for further use in experiment number 2 since it visualize the cells in the wells better and make ALP-analysis more reliable.

4.2.1 ALP-activity

In figure 4.4, alkaline phosphatase-activity from both experimental rounds is depicted. The activity is *not* normalized to cell-number, due to problems of obtaining single-cell suspensions after deposition of ECM and mineralization, and is therefore not representable for the fluctuations of ALP expression in an individual cell and appropriate for comparisons between cell types.

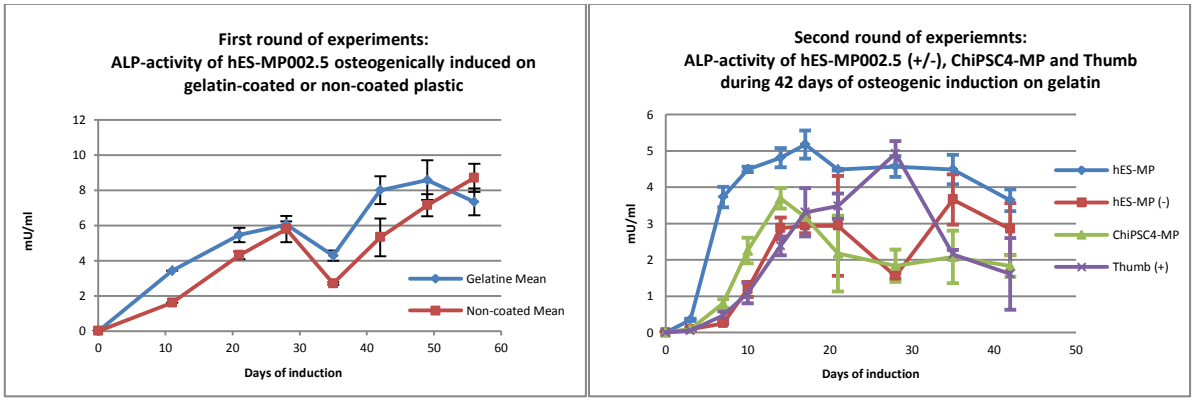


Figure 4. 4 *Graphs visualizing how ALP-activity varies with time in the two osteogenically induced batches of MP-cells and controls. hES-MP (-) represent a non-induced control for hES-MP002.5.*

The first experimental run compared hES-MP002.5 grown on gelatine or non-coated plastic, where ALP-levels increased for both conditions throughout the whole culture period of 8 weeks. Methodology was not completely

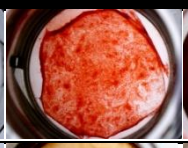
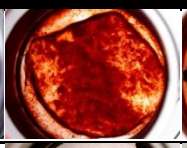
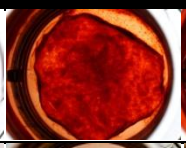
optimized for the procedure in round one, why the same standard curve was used for most of the weekly sample takings. From the obtained material, no greater difference could be seen in ALP-activity when comparing gelatine- and non-coated culture plastic during a long term induction.

In the second round of experiments, hES-MP002.5 appeared to respond to the treatment within 3 days, with an activity level about 10 times higher than the other examined cell types, and after 2 weeks, the measured activity seemed to reach a plateau, not surpassed by any other cell type except for Thumb. ChiPSC4-MP also seemed to respond to osteogenic treatment, though slower than hES-MP002.5 did. However, the cell type also showed less proliferative behaviour. At week 2.5-3, ChiPSC4-MP detached from the culture plastic and curled up, thus generating lower ALP-readings. Untreated hES-MP002.5 cells also showed increased levels of ALP-activity, however the cells proliferated so rapidly that they started to detach from the culture plastic after week 2, but were able to repopulate the wells a second time during the period of experimentation. Thumb showed increased ALP-activity until week 4, after which the cell sheet were too dense to keep in contact with the well.

4.2.2 Alizarin Red staining and morphology

All cells were seeded at 10.000 cells/cm2, and within three days, hES-MP cells and ChiPSC4-MP had grown confluent, while Thumb remained non-confluent until 1,5-2 weeks. Within one week, individual cellular morphology was difficult to detect in all MP-cells types, since all cells were pressing against each other longitudinally. ChiPSC4-MPs were slightly bigger than hES-MP cells, which also could be seen regular culture, but morphologically they looked similar, with not much difference to their fibroblastic shape seen in regular culture. The negative control of hES-MP showed longer and thinner cells than in treated wells, in many cases growing in perpendicular layers, due to the lack of space created by high proliferation. In table 4.2, a summary of Alizarin Red staining over experimental round number two is found.

Table 4. 2 ***Alizarin Red staining of secondary rounds of osteogenic experiments.***

	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
hES-MP002.5						
hES-MP002.5 (control)						
ChiPSC4-MP						
Thumb						

Alizarin Red staining was negative for all cell types during the first 2 weeks. However, an increase in light orange stain could be seen that seemed to be localized to the individual cells. At 2.5 to 3 weeks of induction, deposits of calcium could be visually observed in treated hES-MP wells even without staining, either through direct eye inspection or in the light microscope, and showed as a milky white film or greyish granularity preventing direct inspection of the cells, respectively. When stained, the colour was more intense than previously seen, and was

clearly localized outside the cells. The stain was increased over time in the hES-MP002.5, as can be seen in table 4.3. The non-induced hES-MP002.5 never showed any specific positive Alizarin Red staining.

ChiPSC4-MP started to detach from the culture plastic at week 3 and no specific stain had been detected at that point. At week 4, only one well remained with a non-curled cell sheet, which were used for staining and generated a slightly positive result. At the last and sixth week of differential inducement, ChiPSC4-MP cells had repopulated some of the wells fully. Alizarin Red staining then yielded positive results for one of two examined ChiPSC4-MP wells, but it is suspected that it was due to a contamination with hES-MP cells. The positive control Thumb did not show any noteworthy calcium deposition until week 5, when cells in most of the remaining wells had detached from the culture plastic.

4.3 Chondrogenic differentiation

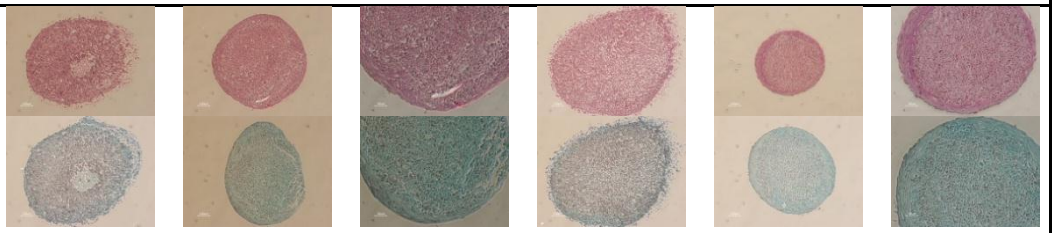
From the two chondrogenic experiments, only results from the second round will be described in detail in this section. Results from the first round of experiments analyzed through sectioning and histological staining concluded that none of the used treatments generated any positive chondrogenic differentiation. The sections investigated showed no obvious distinction between the differently treated cells, except that there was a hint of additional reddish staining on the Safranin O sections cultured under hypoxic conditions when compared to the rest, why hypoxia was selected for further investigation in round two.

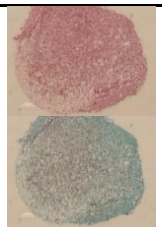
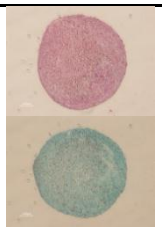
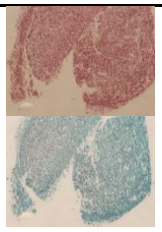
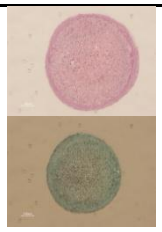
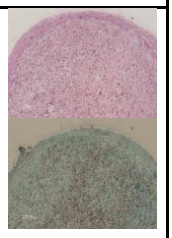
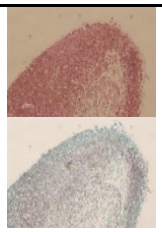
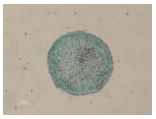
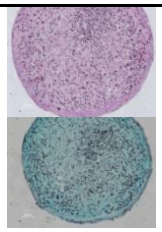
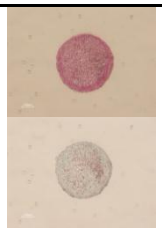
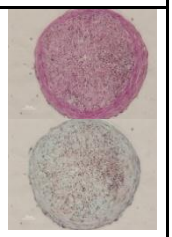
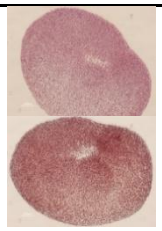
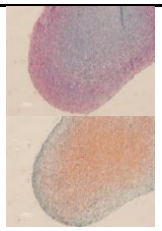
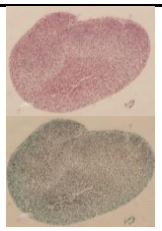
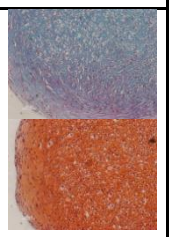
4.3.1 Alcian Blue van Gieson/Safranin O staining and morphology

In table 4.3, results obtained from sectioning and staining of the second round of chondrogenic inductions are presented. The results are univocal – no purposeful chondrogenic differentiation could be seen in either hES-MP treated with TGF-β1 or TGF-β3, and neither in ChiPSC4-MP, on the basis of positive Alcian Blue van Gieson or Safranin O. In contrast, Thumb showed a high degree of chondrogenic differentiation, mainly seen on the vast amounts of deposited chondrogenic-specific ECM which was heavily stained both by Safranin O and Alcian Blue van Gieson. Thumb cells cultured in hypoxic incubator also showed a substantial increase of deposited glycosaminoglycans when compared to regular conditions.

All MP-pellets harvested at day 4 were "fluffy", had ruffled edges and plenty of space in between cells, indicating that no supportive extra cellular matrix had been deposited. Pellets seemed slightly less coherent under hypoxic conditions. Conversely, Thumb-cells showed closely packed cells and coherent pellets at day 4 under both incubation conditions, without any detection chondrogenic specific ECM.

Table 4. 3 ***Results of histological staining of 5 um sections obtained from pellets harvested at day 4 and 24 of secondary rounds of chondrogenic differential inducement. All pictures on upper part of rows show pictures of Alcian Blue van Gieson stained sections, while the lower part of the rows contains pictures of Safranin O stained sections.***

	Regular conditions			Hypoxic conditions		
	Day 4 (10x)	**Day 24 (10x, 20x)**		**Day 4 (10x)**	**Day 24 (10x, 20x)**	
hES-MP002.5 TGF-β1						

	Regular conditions			Hypoxic conditions		
	Day 4 (10x)	Day 24 (10x, 20x)		Day 4 (10x)	Day 24 (10x, 20x)	
hES-MP002.5 TGF-β3						
ChiPSC4-MP						
Thumb (positive control)						

At final sectioning, all pellets look condensed with smooth edges. In most of the MP-pellets, cells closest to the pellet surface have stretched out much like in two-dimensional culture and created a protective capsule around the rounded cells in the middle of the pellets. In hES-MP pellets treated with TGF-β3 and ChiPSC4-MP pellets, areas of what looked like an abundance of pyknotic nuclei could be seen (condensed chromatin, darkly stained), whereas other areas of these pellets looked less condensed. The less condensed areas of MP-pellets did however not show any GAG-deposition either.

Thumb cells produced ECM with resemblance to hyaline cartilage, with chondrocytes residing in small empty spaces, and seemed to deposit matrix from the inside working outwards when regular and hypoxic conditions were compared. In some regions of regular cultured hES-MP002.5 supplemented with TGF-β3, a similar ECM formation could also be seen, however without obvious positive stain. Figure 4.5 visualizes ECM-patterns produced by hES-MP with TGF-β3 addition under regular conditions and Thumb under hypoxic conditions. hES-MP pellets cultured with TGF-β1 were not able to show structured ECM-deposition.

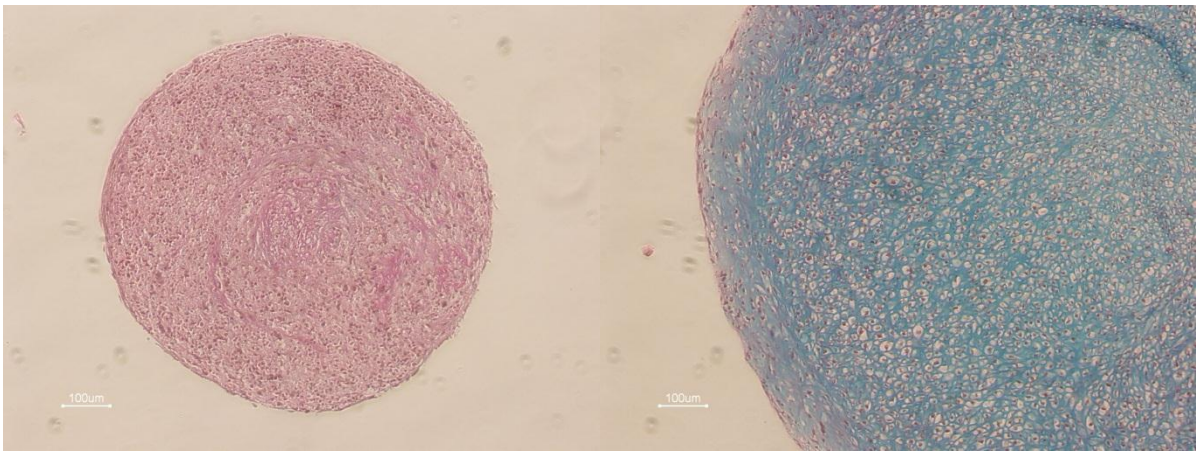


Figure 4. 5 ***Morphologic comparison of hES-MP002.5 regular pellet culture with TGF-β3 addition (left) and positively differentiated Thumb under hypoxic culture conditions (right), both stained with Alcian Blue van Gieson.***

Regarding pellet size, all treated hES-MPs were slightly smaller at day 24 compared to their size at day 4. No substantial amount of ECM was deposited, which otherwise would affect pellet size. However, a small deposition of ECM seemed to create a mesh that keeps the pellets coherent, but the character of this ECM remains unclear. Positive control Thumb show a great increase in size, especially under hypoxic conditions, which from the microscopic inspection can be attributed to the mass of excreted proteins.

4.3.2 Chondrogenic qPCR data

When chondrogenic RNA was purified, only six pellet samples were detected to have measurable concentrations: ChiPSC4-MP at week 1, both regular and hypoxic conditions; Thumb at week 1, 2 and 3 for regular conditions and Thumb at week 3 for hypoxic conditions. As samples in spite of this fact can contain mRNA at levels sufficient for cDNA-synthesis and the following qPCR-reaction, a set of extra samples were collected for qPCR: all hES-MP samples at week 3 as well as all ChiPSC4-MP and Thumb-samples. Some results indicating trends in expressional levels could be found, however as most of these data are not reliable, only data for originating cell types, Thumb cells and ChiPSC4-MP at week 1 are shown graphically. The vales shown are relative levels of the expressed genes: the quantified gene expression normalized against the reference gene CREBBP and further against a calibrator. Note should be taken to that the results present only are based on two technical replicates made on one sample per test group, why the results only are indicative on a relationship. At least three samples should be taken per test group in order to be able to obtain statistically supported results.

Figure 4.6 show that the expression levels of ACAN in non-induced Thumb cells are relatively high, while levels for COL2A1 and SOX9 seem to be low. It is indicated that all genes investigated more or less are positively regulated for cells induced under regular culture conditions during the culturing period. Among hypoxic conditions, results from week 3 are the most reliable. These consistently indicate that hypoxic culture induce a greater expression of the genes examined when compared to the third week of pellet cultures grown under regular conditions, especially when COL2A1 is considered.

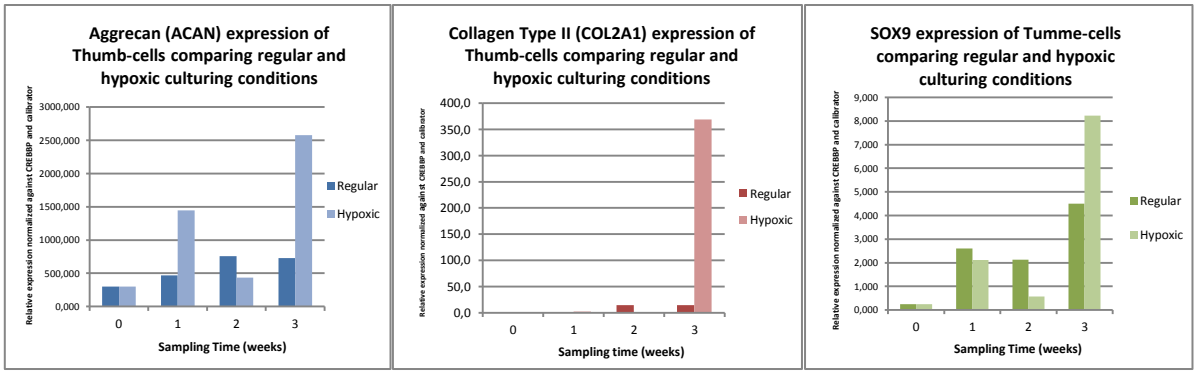


Figure 4. 6 ***qPCR results of genes ACAN, COL2A1 and SOX9 for chondrogenically induced positive control Thumb.***

qPCR results for the gene SOX9 expressed in ChiPSC4-MP samples (that were able to generate measurable RNA-levels) are presented in figure 4.7. It is indicated that the regulatory gene expression is lower during induction in pellet culture than in two dimensional cultured, non-induced cells. For the genes ACAN and Aggrecan, no difference could be seen when comparing non-induced ChiPSC4-MP to induced ones, where background levels were low for both genes.

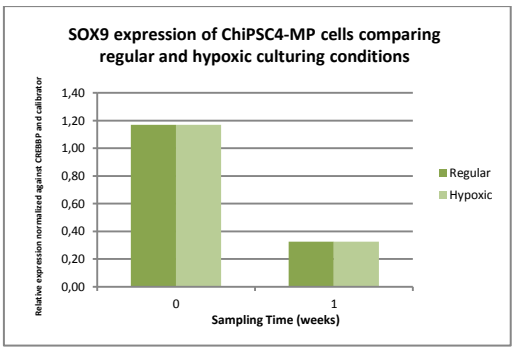


Figure 4. 7 ***qPCR results of gene SOX9 for chondrogenically induced ChiPSC4-MP***

4.3.3 ICC – TGF-β-RII presence

No positive result could be detected for the presence of the membrane bound receptor II for TGF-β on any of the cells in the assay: hES-MP, ChiPSC4-MP, ChiPSC4 and Thumb.

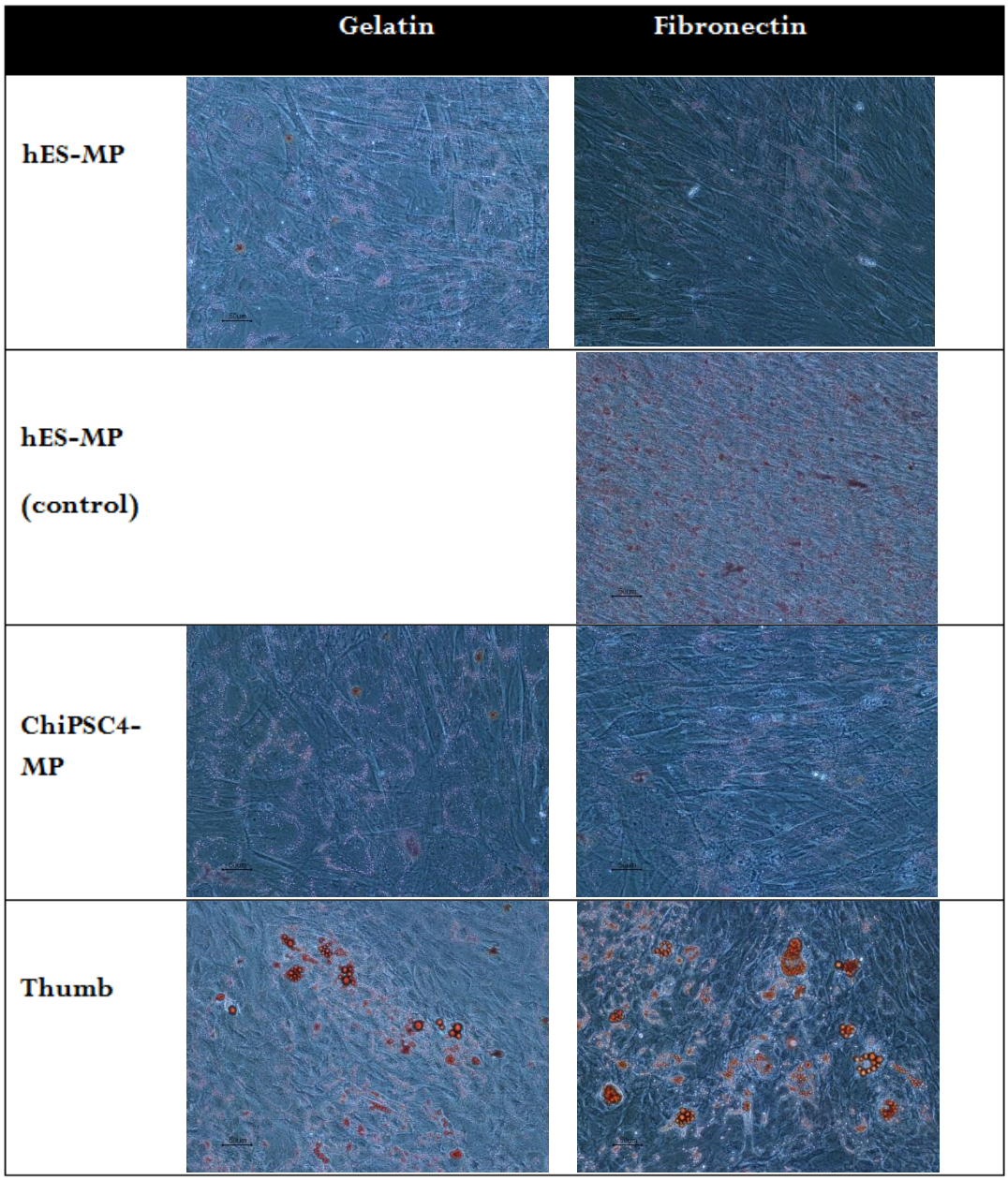
4.4 Adipogenic differentiation

From the two adipogenic experiments, only Oil Red O and adipogenic qPCR data from the second round of experimentation will be presented in detail. Results from the first round of adipogenic induction of hES-MP002.5 were only examined with Oil Red O, and all setups failed to show any positive differentiation.

4.4.1 Oil Red O staining and morphology

Oil Red O staining of second round of experiments at week 3 (final day of analysis) is depictured in table 4.4. The only induced cell type that showed an accumulation in intracellular lipids were the positive control Thumb. This could be detected microscopically from about day 10, appearing as light, spherical, small droplets; clearly within the borders of single cells. From thereon, the lipid content was increased over time until the experiment was terminated.

Table 4. 4 *Results of Oil Red O staining obtained from second round of adipogenic differential inducement. hES-MP (control) refers to non-induced cells. Magnification is 20 times in all photos.*



In Thumb cultures, only a slight percentage of the present cells were detectable with dense lipid accumulation, present in regionalized clusters. There seemed to be a higher fraction of Thumb cells accumulating lipids when cultured on fibronectin coated dishes.

Oil Red O staining of the MP-cell types did not reveal any results indicative of positive differentiation. At highest magnification (20x), all cell types showed small droplets stained red, which was uniformly distributed and could not be described as accumulated lipids. hES-MP002.5 cells on gelatin coated culture dishes detached from the culture plastic between week 2 and 3, why the picture are omitted.

4.4.2 Adipogenic qPCR data

All investigated samples from the second round of experiments yielded detectable concentrations of RNA. However, as no positive lipid-accumulation could be seen in any of the MP-lines (or control), only MP-cells grown on gelatin were taken further for qPCR analysis to get any indication whether the cells responded to the adipogenic treatment. Exception were made for the cell line Thumb, which demonstrated differentiation potential regardless of the substrate used, why samples grown on both gelatin and fibronectin were taken for qPCR analysis in order to see if a difference in expression could be detected. Samples of all the non-induced cell types were also examined.

The results from C/EBPa, PPARg and GLUT4 assays are presented graphically in figure 4.8 - 4.11. The values shown are relative levels of the expressed genes: the quantified gene expression normalized against the reference gene CREBBP and further against a calibrator. Note should be taken to that the results present only are based on two technical replicates made on one sample per test group, why the results only are indicative on a relationship. At least three samples should be taken per test group in order to be able to obtain statistically supported results.

All induced cell types seem to increase their C/EBPa-expression over the three weeks of induction, while the hES-MP control remains at a uniform level. As can be seen in the leftmost graph in figure 4.8, Thumb shows considerable higher response than the MP-cells do. The close-up of the lower part of the y-axis is depicted in the right graph, and shows that hES-MP seem to respond in the same fashion to the adipogenic inductive treatment, however much weaker. The response from ChiPSC4-MP is so low that it should not be considered to be releveant.

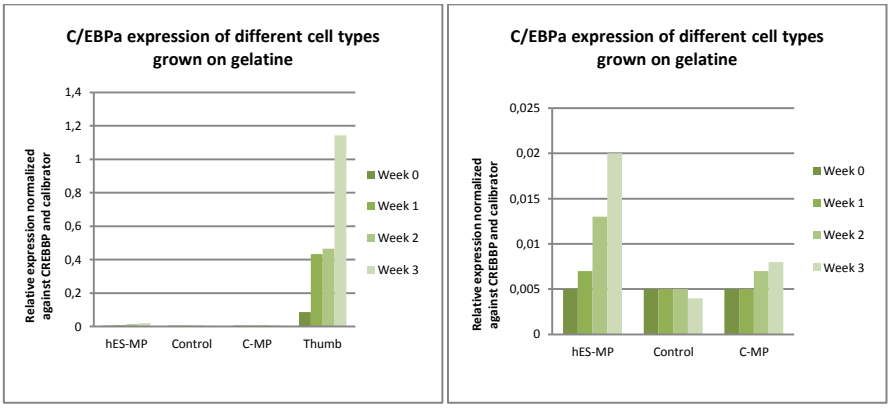


Figure 4. 8 ***qPCR results of gene C/EBPa showing expressional levels in adipogeniccaly induced hES-MP002.5, ChiPSC4-MP and Thumb. Control indicates non-induced hES-MP002.5 cells.***

As can be seen in figure 4.9, expression of PPARg in non-induced cell types are similar for all investigated cell types. For both MP-cell types investigated, PPARg-levels appear to be decreased throughout the period of induction, which is true also for the hES-MP control. Conversely, such a decrease cannot be seen for Thumb, but it is not possible to disuss if PPAR-expression is upregulated or not.

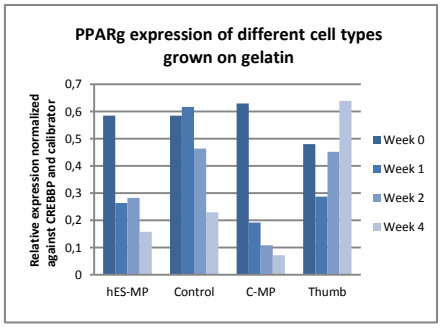


Figure 4. 9 *qPCR results of gene PPARg showing expressional levels in adipogenically induced hES-MP002.5, ChiPSC4-MP and Thumb. Control indicates non-induced hES-MP002.5 cells.*

As can be seen in the graphs in figure 4.10, the expressional changes of GLUT4 during the period of induction are marginal for MP-cells over the period of induction, with a very small incresase when compared to that of the untreated cell type. Conversely, Thumb initially showed a weekly increase in GLUT4-levels.

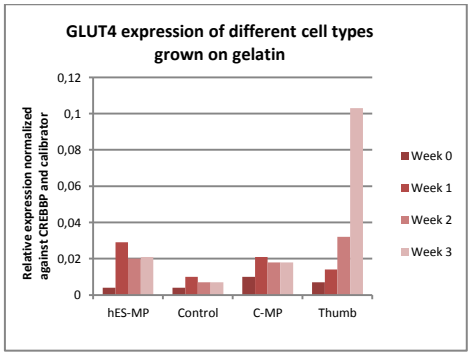


Figure 4. 10 *qPCR results of gene GLUT4 showing expressional levels in adipogenically induced hES-MP002.5, ChiPSC4-MP and Thumb. Control indicates non-induced hES-MP002.5 cells.*

Figure 4.11 show expressional differences in adipogenically induced Thumb for all investigated genes. A slightly higher expression can be seen for fibronectin-samples at week 3 when compared to gelatin for all genes, but the differences cannot be regarded as significant since the experiment has only been performed once.

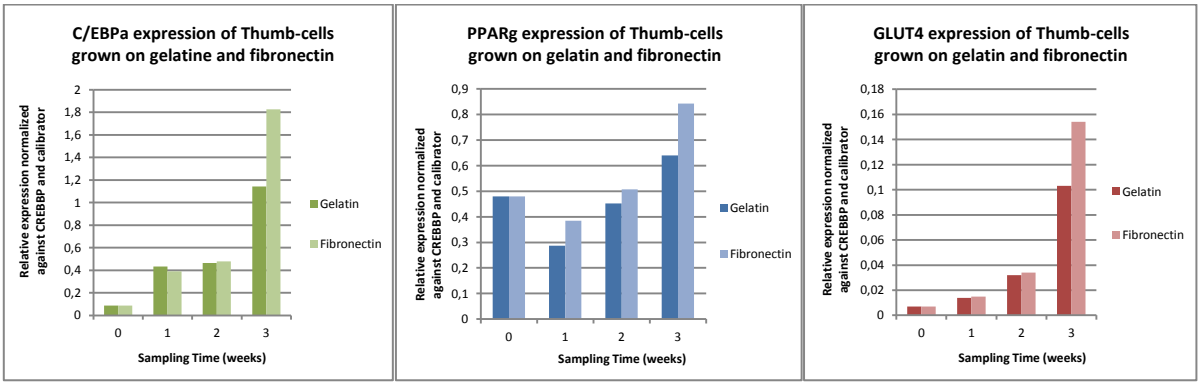


Figure 4. 11 *qPCR results of genes C/EBPa, PPARg and GLUT4 in adipogenically induced Thumb.*

5 DISCUSSION

5.1 Establishment of mesenchymal progenitors from ChiPSC4

The mesenchymal progenitors were established from the induced pluripotent stem cell line ChiPSC4 through a protocol previously used by Cellectis Stem Cells to establish MP-cells from embryonic stem cells. The similarities between hESCs and ChiPSC4 are hard to discuss in detail since no published material is available, but ChiPSC4 have gone through an extensive quality control ensuring that it has a similar profile to hESCs and pluripotent characteristics (see section 2.2). The cell types established in this project (ChiPSC4-MPs) did indeed share similar characteristics with hES-MP002.5, such as cell morphology, growth patterns, immunocytochemical markers and mesodermal potential (osteogenic, chondrogenic and adipogenic differentiation of ChiPSC4-MP is) discussed in detail in section 5.2-5.4).

However, differences could also be seen compared to hES-MP002.5: the ChiPSC4-MP cells from batch two appeared to be bigger in size and seemed to grow at a slower pace, and this population stopped proliferating at passage 11, whereas hES-MP002.5 in other studies have been recorded to keep proliferating beyond passage 30[2]. Batch number three (not handled by the author) did however show a constant growth rate and were proliferative at point of cryopreservation (passage 12). A comparison can be made between the growth rate recorded of batch three of ChiPSC4-MP and published results of the growth rate of hES-MP002.5 (see figure 2.2). ChiPSC4-MP exhibited a mean population doubling of 1.8 per passage between p3 and p12, while the number noted for hES-MP002.5 has been about 3.3 between p8 and p30. However, it is not recorded how often the hES-MP culture was passaged, but it is recommended that it is performed every 3-4 days.

A plausible reason for the decreasing proliferative behavior of second batch of ChiPSC4-MP compared to that of the third of could be variability in laboratory handling. The second batch was for example used for all mesodermal induction experiments, why the population had to be scaled up and was passaged at irregular time points – a fact that might have exposed the population to additional stresses. The third batch was on the other hand regularly passaged every third or fourth day.

A general explanation for differences in behavior between hES-MP and ChiPSC4-MP cells could lie in the fact that ChiPSC4 from the beginning originates from fibroblasts, and therefore retain some of the epigenetic modifications present in the originating cell. Epigenetic modifications in iPS-cells have been shown to be correlated to a "skewed" differentiation potential – meaning that it is easier to differentiate iPS-cells into the cell lineage from which it came[72]. It could be speculated that such parental cell epigenetic memories in a similar manner could make it hard to differentiate a fibroblast-derived induced pluripotent stem cell into other cell types, such as a mesenchymal stem cell. However, ChiPSC4 have been shown to give rise to functional hepatocytes[70], why other reasons could be responsible for the lesser presence of MSC-characteristics in ChiPSC4-MP. In addition, the way of culturing the parental strains (hES002.5 and ChiPSC4) may have an impact on offspring characteristics: hES-MP002.5 was established in 2007, and at that time, no feeder-free option of growing pluripotent stem cells was available, while ChiPSC4 mainly have been grown in the feeder-free system DEF-CS™.

In the established protocol, the cells are thought to become a homogenous population as passaging continues. In reality, however, this was hard to obtain. As no selection is made, cells with different characteristics can become part of the cellular population as long as they show the same degree of proliferative behavior or undergo spontaneous differentiation during culture. It could be seen during the period of MP-induction as larger and smaller cells growing side by side, the bigger "encapsulating" the smaller. It becomes more apparent when comparing the ChiPSC4-MP population obtained with hES-MP002.5, which have extremely high degree of homogeneity already at passage 7 (the passage the population were cryogenically preserved), while ChiPSC4-MP

at the same passage show a less structured cellular order. This inhomogeneity was perhaps influential during the mesenchymal inductions, and affecting growth capacity of the population as a whole.

To avoid the problems with a heterogeneous cell population, limiting dilution could have been performed – taking a single to few cells and letting them repopulate the culture vessel, from which a mesenchymal like population could be chosen for further experiments[20]. However, the procedure takes time, as well as leading the cells into a high passage number – a fact that could be unbeneficial during the mesodermal induction. Other options is to sort cells with equipment such as FACS and only make use of those that show surface markers connected to cells of mesenchymal origin (such as and that lacks motifs connected to other lineages[51]. Furthermore, the fact that mesenchymal stem cells show particular good plastic adherence could be made use of during the initial steps of differentiation, since cells that do not succeed to bind to the culture plastic within the first or second hour easily can be washed off after an enzymatic passage[51].

The comparative immunocytochemical study indicates that the separate batches of ChiPSC4-MP resembled hES-MP002.5 according to the investigated markers, but chiefly, it were able to provide results pointing out that the examined ChiPSC4-MP populations had lost pluripotency. Only a limited number of markers to deduce germ layer potential were able to generate reliable results (only FoxA2 and ASMA), since both Vimentin and B-III-Tubulin unexpectedly showed positive staining for all cell types (see discussion below). In order to generate more accurate results and to be confident in the purity of the population, several verified antibodies directed against each of the three different germ layers should be used. The reason for not including more of these markers in this project was due to time and economical limitations, and only antibodies present at Cellectis were used.

The inhouse antibody hES-Cellect is developed to detect pluripotent stem cells[69]. In this study hES-Cellect could be seen to vaguely bind to redifferentiated chondrocytes as well as hES-MP002.5. The occurrence of these binding-events do probably not indicate that Thumb and hES-MP002.5 are contaminated with pluripotent cells, since the antibodies detecting specific pluripotency markers (Oct4 and TRA-1-81) were negative for all investigated cells except for undifferentiated ChiPSC4. As mentioned in the methods-section, the precise target of the hES-Cellect is unknown, why it is plausible that it recognizes an epitope that differentiated cell types (lacking pluripotency) also express to a small extent. See hES-Cellect staning of ChiPSC4 and hES-MP002.5 in figure 5.1.

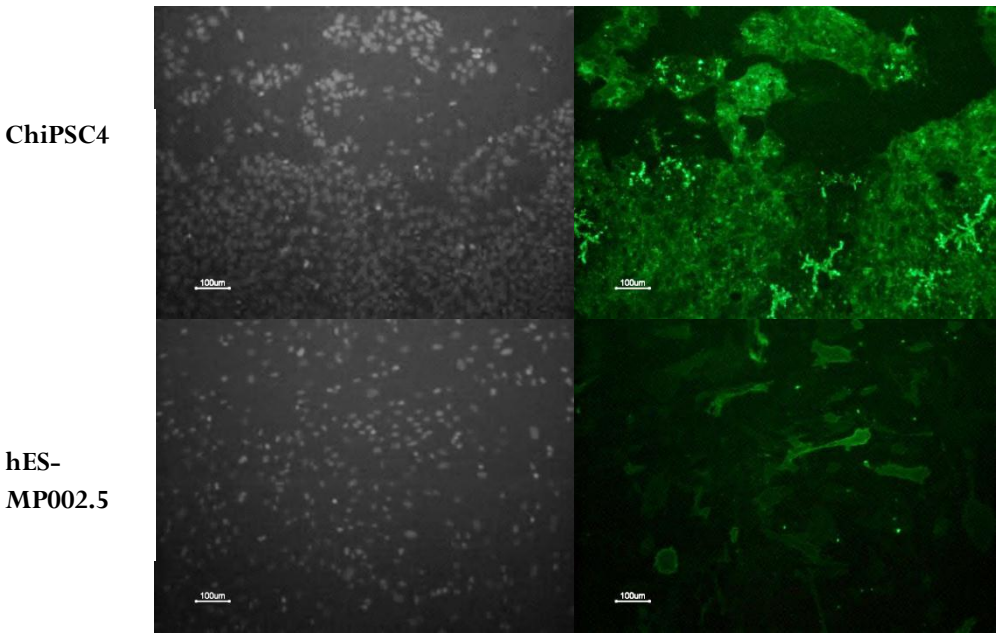


Figure 5. 1 ***hES-Cellect staining of ChiPSC4 and hES-MP002.5. The upper row shows DAPI and hES-Cellect staining of ChiPSC4, while the lower row shows the same results for hES-MP002.5.***

Vimentin was used as a marker to determine differentiation toward the mesenchymal lineage, the same used for the characterization of hES-MP002.5 and other MP-lines[1]. Vimentin is not a specific marker for mesenchymal progenitors or stem cells, but rather for all cells of mesenchymal origin as a part of intermediary filaments. The marker is therefore used in combination with a larger array of antibodies to get a complete picture to draw conclusions from. However, Vimentin also generated a positive result for ChiPSC4, the induced pluripotent stem cell line, which was unexpected. Reasons could be that the specificity of the antibody in itself is questionable, as well as that the ChiPSC4 cells were at a starting point of differentiation when they were fixed. The first option is the most reasonable, since pluripotent motifs such as Oct4 and TRA-1-81 quickly should be down regulated when differentiation is initiated. The antibody should be tried on several other pluripotent stem cells to get more reliable results. The outcome of β-III-Tubulin staining also raised questions. Results were throughout the whole assay positive, even though the antibody was marketed as a neurospecific indicator. The targeted motif in the cells was probably a kind of tubulin present in all cell types, since even undifferentiated cells were positive.

Alpha-smooth muscle actin was present only to a low degree, which partly rules out wrongful differentiation for majority of cells. Furthermore, cells in passage 11 were fixated after growth arrest had occurred and stained with ASMA-antibody. However, the hypothesis that these cells and other cells with broad morphology and centralized nucleus found within the proliferating population were ASMA-cells was not supported, since only a very small amount could be detected positive.

As a whole, both the morphological and immunocytochemical evaluation suggests that the cell line ChiPSC4-MP is of same character as hES-MP002.5, which indicates a successful differentiation according to the used protocol. Whether this represent a functional, multipotent mesenchymal progenitor cell type is another question, discussed closer in the following sections.

5.2 Osteogenic differentiation

The analysis methods used to evaluate osteogenic differentiation were histological staining with Alizarin Red and quantification through ALP-enzyme activity. Alizarin Red staining is demonstrated to be a straightforward and reliable method to detect mineral deposition. The quantification of ALP-activity is a commonly used technique to determine that osteoblast differentiation take place, but it also enzyme highly expressed in pluripotent cells. Since the previously discussed immunocytochemical assay failed to show any specific pluripotent markers in any of the cell types used in the mesodermal differentiation experiments, the ALP-activity measured in the osteogenic experiments can be assigned to osteoblast function.

However, it can be concluded that the ALP-enzyme activity assay used in this project does not constitute a suitable option to generate valid, quantitative results of osteogenic differentiation, partly due to the difficulty of normalizing to cellular number. As an example, in the first round of experiments, cells from both gelatin- and non-coated wells were counted at week 2 to compare cellular number. By this time it was hard to obtain a single-cell suspension due to retention of cells in the expressed ECM. When mineralization was initiated, it became even more difficult. Thus, as all cell types investigated have different proliferation rates, relative comparisons are impossible to make from the obtained results. Other problems related to the assay were that the assay showed great variability when establishing the standard curves, which in part could be due to instability of the substrate used. Furthermore, eluation of the ALP-enzyme from cells might also be an issue as soon as ECM-deposition and mineralization begins which physically entrap the cells. Thus, it is suggested that other methods of evaluation is used when examining osteogenesis, such as qPCR. This would enable normalization to the total amount of RNA detected in the sample, thus removing the issue of creating a single cell suspension.

The first experimental run only compared hES-MP002.5 grown on gelatine or non-coated plastic, since it was not specified in the protocol what to use, which both seemed to differentiate into ALP-expressing and mineralizing

osteoblasts, and increased mineral deposition in a time dependent manner. However, cells grown on gelatin were evenly distributed and showed a homogenous growth, while non-coated plastic made the cells curl up and grow "in lumps", and thus were harder to examine. In general, no greater difference could be seen in ALP-activity when comparing gelatine- and non-coated culture plastic during a long term induction, while Alizarin Red staining seemed to generate a heavier staining for gelatine cultured cells, which obviously correlated to the fact that cells were growing and depositing mineralized matrix within the cell cluster-lumps. The more homogenous growth seen on gelatin probably was due to the fact that hES-MP002.5 cells are used to grow on gelatin, and express proteins that enhance attachment to the protein in question.

In the second round of experiments, Alizarin Red staining was negative for all cell types during the first 2 weeks. However, an increase in light orange stain could be seen that seemed to be localized to the individual cells. As Alizarin Red can bind to ionic calcium, the intracellular deposits of calcium are probably responsible for this stain. The unspecific stain should logically increase with number of cells, why negative control hES-MP probably shows the fastest increase in colour during the first 2-3 weeks.

In the second round of experiments, hES-MP002.5 seem to respond to the treatment within 3 days, and after 1-2 week, maximum levels of activity appears to be reached. Thus, ALP thus seems to be a "quick indicator" of osteogenesis. As the readings made at day 3 and 7 show a 10-fold increase in activity, while the untreated control remained very low (which should have an equal or higher growth rate, since it also is administered bFGF, a growth factor stimulating proliferation), at least a part of the increase should be attributed to expressional changes of ALP. At 2.5 to 3 weeks of induction, deposits of calcium could be visually observed in treated hES-MP002.5 wells even without staining. When stained with Alizarin Red, the colour was more intense when compared to the unspecific stain, and is localized outside the cells, and was increased over time in the hES-MP. Mineral-deposition seemed to decrease cellular detachment, and consequently hES-MP002.5 remained in their wells through the entire period of induction. However, mineralization might also have made ALP-enzyme extraction more difficult.

ChiPSC4-MP seemed to respond to osteogenic treatment, though slower than hES-MP002.5 do. The lower ALP-readings are not reliable in such a comparison, since it cannot be excluded that the difference is correlated to cell number (reinforced by the fact that the examined ChiPSC4-MP batch probably had a slower growth rate than hES-MP002.5). At week four, however, a low level of deposition of calcium was seen, while hES-MP002.5 showed heavy deposition. The low degree of deposition could not prevent curling of the ChiPSC4-MP cell sheet after the cells had grown beyond confluent (~ week 3), which led to detachment and in turn made it hard to tell whether further mineralization could be performed by the cell type. This fact shows that the cells proliferative potential is retained even if differential induction is on-going. This might complicate the differentiation procedure in another aspect than that physical retention is hard to achieve, but rather relating to that cellular focus of the population lie in dividing rather than differentiating. For ChiPSC4-MP, the problem of continued growth might be addressed by using optimized seeding densities allowing full retention in the wells until mineral deposition is induced, which physically can retain the cells in the wells. Alternatively, reduced levels of FBS or completely omitting the serum might be an option, since the complex serum may contain an abundance of growth factors that stimulate expression of transcription factors[3], which may act to keep the cells in a proliferative stage. The presence of undefined components in FBS may also affect cells ability to respond to soluble differential cues: if FBS-components bind to cellular receptors, it may act to shield signalling intended to generate a differentiation response in the cells.

Untreated hES-MP002.5 cells also showed increased levels of ALP-activity, however the cells proliferated so well that they detached from their wells and repopulated them a second time. It is plausible that the increase in ALP-activity is correlated to cellular number, but it could also indicate that hES-MP002.5 has an ease of osteogenic responsiveness; meaning that the cell line possibly start to express bone-specific proteins solely through the

process of growing confluent. This issue might be investigated further by normalizing to cellular number, since mineralization is absent.

Thumb did not reach confluence until 1,5-2 weeks, and showed increased ALP-activity until the cell sheet detached from the well (around week 4) – also in this case most likely indicating a cellular increase rather than a continuously increased response to the treatment. Thumb did not show any noteworthy calcium deposition until week 5, where most of the remaining wells showed cellular detachment from the culture plastic, and does not seem to be an optimal positive control for osteogenic induction.

Comparing the first to the second round of experimental results, there are conflicting results. ALP-levels of hES-MP are continuously increasing in experiment 1, while it reaches a plateau in experiment 2, further indicating that the methodology was not completely optimized. In studies from de Peppo et al, who conducted osteogenic induction of hES-MP002.5 for six weeks, ALP-activity seemed to be increasing with time throughout the period of induction, and decreasing with increasing passage number[35]. The first round of experimental induction in this project would correlate better to these results.

Furthermore, de Peppo compared the differention potential of hES-MP002.5s and hMSCs, concluding that hMSCs showed significantly higher ALP-levels throughout the six weeks of induction, but that hES-MPs still showed superior capabilities of creating mineralized matrix[35]. These results indicate that even if ALP-levels are up-regulated in functional osteoblasts, it cannot single-handedly mirror a cell's capacity to function as an osteoblast.

There was no effort to try to optimize the osteogenic differentiation procedure in this project, but it could be of interest to modify the currently used additive concentration to reach even better results. Mostafa et al. suggest an optimal concentration of dexamethasone to be 100 nM when hMSCs are induced to maximize calcification, while 10 nM is to prefer if adipogenesis-related markers should remain at constant and low levels[36], however cross-differentiation does not seem to be an issue for hES-MP002.5. These concentrations should however be compared to the levels used in this project (1000 nM), which is higher than physiological levels of natural glucocortoids, and has been actually been shown to reduce osteogenesis[60].

5.3 Chondrogenic differentiation

The analysis methods used to evaluate chondrogenic differentiation were histological staining with Alcian Blue van Gieson and Safranin O and cartilage specific qPCR. The histological stains are widely used to detect GAG in cartilage ECM, and appeared to work flawlessly in this project (based on positive differentiation of Thumb). It was harder to obtain reliable results from the qPCR, since the extraction of RNA from pellet culture was problematic, and more samples should have been gathered to acquire significant results.

The initial round of differentiation was made in order to get some experience on pellet culture and hopefully an indication on how to proceed. Small adjustments were made to the original culturing protocol. One hypothesis was that an increased level of the growth factor TGF-β1 during pellet formation could help to "kick-start" chondrogenesis, as it would ease accessibility of TGF-β1 at the onset of culture. The second hypothesis was that a smaller pellet would enhance the differentiation since it would have better possibilities of mass transportation (as well, 200.000 cells are a common number for pellet culture), both by nutrients and active ingredients needed for differentiation. Thirdly, hypoxic conditions (5% O_2) instead of ordinary (21% O_2) during incubation were investigated, since several articles suggest that it could increase efficiency of differentiation[21,73]. However, the experiments were only able to confirm the previous concerns about the low ability for hES-MP002.5 to differentiate into chondrocytes. Hypoxic culture seemed to be the best option to continue with in the second round of experiments, even if it failed to generate observable GAG-expressing chondrocytes in the first round.

Under the second round of experiments, main focus was shifted to compare the chondrogenic potential of the different cell types in the project, both under regular and hypoxic conditions. However, in order to get some additional knowledge of the capacity of the primary cell type under investigation, hES-MP cells were supplemented either with growth factor TGF-β1 or TGF-β3[17] (both ChiPSC4-MP and Thumb were only treated with TGF-β1).

Results from the second experiments show that both hES-MP002.5 and ChiPSC4-MP fail to express large quantities of GAG irrespective of treatment. However, when TGF-β3 was administered under regular conditions, defined areas inside the pellets showed the same extracellular structure as Thumb; formation of lacunae-like spaces. In these areas compared to other regions of the pellets, a slightly more light-blue colour between the pinkish tones could be seen when stained with Alcian blue van Gieson, while Safranin O fails to stain an adjacent section. This could indicate a weak degree of chondrogenic differentiation. Chondrogenic qPCR data were inconclusive for all hES-MP002.5 cells due to the low amount of purified RNA. However, results from hypoxically cultured hES-MP002.5 were still generated with comparable Ct-values to those obtained from Thumb. These indicate that TGF-β3 administered cells expressed larger amount of ACAN than TGF-β1 administered cells do, which is in accordance with the literature that the experiment were based on (see section 2.4.2)[17]. Even if it is not possible to draw any direct conclusions from these results, it is recommended that future studies on chondrogenic differentiation should evaluate TGF-β3 and hypoxic culture more closely.

ChiPSC4-MP showed to be the most problematic cell type to culture in pellet format, since these pellets were less coherent early on during induction (i.e. loose connections between cells), and primarily under hypoxic conditions. hES-MP002.5 pellets were also less coherent during hypoxic culture, a fact that could be due to reduced ability of cell adhesion under low oxygen tension[52]. The reduction in pellet sizes of MP-cells was correlated to this, since cells tended to loose from the pellets under media changes. Conversely, Thumb cells were coherent early on in culture under both culture conditions. The condensed cell-nucleuses seen in MP-pellets under hypoxic conditions are an indicator of apoptosis[75]. The fact that cells in the MP-pellets show signs of apoptosis while Thumb does not, might connect to the fact that Thumb-cells have gone through a successful differentiation: genes that help cells cope under tight growth conditions and low-oxygen tension (normal for cartilage cells) could have been upregulated.

Thumb cells seem to behave much as native chondrocytes in hyaline cartilage, where matrix is deposited as to leave space for the cells to reside in. Thumb cells cultured in hypoxic incubator showed a substantial increase of deposited GAGs when compared to regular conditions. This is both supported by histological staining made, and qPCR data obtained at week 3 for the gene ACAN. qPCR data also suggest that Collagen type II is upregulated in hypoxic culture, which supports that fully functional chondrocytes are produced. The fact that the process of chondrogenesis is aided by low oxygen-tension is reasonable since cartilage is a non-vascularized tissue[3], and that chondrocytes therefore naturally must be adapted to reside and function under hypoxic conditions. It has previously been shown that hypoxic conditions supports redifferentiation of dedifferentiated chondrocytes[74].

In contrast, no greater difference could be seen between hypoxic and regular incubation conditions and pellet structure in the general case of MP-cells, indicating their low chondrogenic ability. From the small amount of useable chondrogenic qPCR-data, it appears as if the regulatory gene SOX9 in ChiPSC4-MP cells is expressed at lower levels in pellet culture than in 2-dimensional culture irrespective of incubation used, while the gene is upregulated in Thumb during the period of induction. If SOX9 actually is downregulated in response to the chondrogenic treatment, it would help to shed light over the cells default response towards the inductive treatment.

A question that is raised after viewing the results is where within the pellet culture chondrogenesis is initiated. From literature, sources propose that differentiation starts at the pellet periphery and is increased toward the centre with time[17, 50]. This is reasonable when considered that cells in the periphery of the pellet have quicker access to the inductive cues in the media than centrally positioned cells do. In the only successfully differentiated case in this project, Thumb, differentiation however seems to start from within, since the peripheral parts of the regularly cultured pellet do not show any GAG-deposition. This indicates that Thumb cells might initiate differentiation in pellet culture due to other cues than those supplemented in the inductive media. As the cell line Thumb stem from cartilaginous origin, it probably has a natural tendency to redifferentiate into the same lineage, a fact which has been seen in several other cases with articular chondrocytes[55]. This is also reinforced by qPCR data indicating that non-induced Thumb express high levels of Aggrecan, making it apparent that the cell type indeed is affected by its chondrogenic origin even if it dedifferentiates. To come to terms with the raised questions, a negative control of Thumb should also have been run where TGF-β administration would have been left out. In the case of a positive result, one might want to question the protocol in use rather than the non-responsive MP-cells examined.

As the ability for chondrogenic differentiation of the investigated MP-lines in this project seemed absent, an attempt to evaluate the possibility for the cells to respond to the signals given during induction was made. The most important signal for the artificial induction of chondrogenesis, given by the present literature, is the growth factor TGF-β, as the absence of it in control experiments fail to induce chondrogenesis of mesenchymal stem cells[40]. All isoforms of TGF-β (1, 2 and 3) bind to the membrane localized receptor TGF-β-RII, which relay the signal inwards through the cytosolic protein TGF-β-RI[56]. Thus, the presence of these proteins should be vital for cellular responsiveness to the induction medium, and the membrane localized receptor was chosen for investigation. However, no positive result could be detected for the presence of the membrane bound receptor II for TGF-β on any of the cells in the assay: hES-MP, ChiPSC4-MP, ChiPSC4 or Thumb. Thumb was included in the assay to serve as a positive control – however, made to do so without investigating its chondrogenic response if TGF-β1 was left out of the induction. Thus, it is not possible to determine from the results obtained if Thumb actually do not express TGFβRII to a measurable degree, or whether the antibody purchased was defective. The antibody purchased was raised against the full-length, human protein, why antibodies theoretically could have been raised against an epitope that on the cell is shielded by the membrane.

During literature research made subsequently of the TGFBRII-assay, an article of de Peppo et al was reviewed in detail, which demonstrates that hES-MP002.5 cells express TGFBRII to an extent comparable to MSCs[2]. Thus, unresponsiveness of hES-MP002.5 to common chondrogenic cues should not stem from the absence of TGF-β receptors and ability to respond to TGF-β signalling.

5.4 Adipogenic differentiation

The analysis methods used to evaluate adipogenic differentiation were histological staining with Oil red O and adipose specific qPCR. The histological stain are widely used to detect lipid accumulation in differentiated adipocytes, and appeared to work well in this project (based on positive differentiation of Thumb). The use of qPCR in the second round of experiments was of interest since it allowed for investigations of regulatory genes, and extraction was straight forward. However, several samples should have been gathered in order to acquire statistically significant results.

Initially, differentiation of hES-MP002.5 was performed according to the protocol used by Cellectis, which is comparable to published protocols commonly found. Some modifications were also made experiments in order to see if the differentiation potential could be positively reinforced. The use of BMP4 were the most promising,

since Rosiglitazone mainly had been recorded to augment an already present differential response, while BMP4 pretreatment had been seen to convert MDI-unresponsive mesenchymal like stem cells into preadipocytes[18].

Neither positive differentiation detected with Oil Red O, nor distinct visible morphological appearance correlated to adipogenic differentiation could be seen in hES-MP002.5 for any of the used treatments, indicating a low degree of multipotency. When compared to non-induced hES-MP002.5, growth rate seemed to be reduced, and cells were somewhat bigger with a less stringy appearance - much like osteogenically induced hES-MP002.5 looked. Alizarin Red staining was performed during the first round of experiments as concerns were raised towards the possibility of cross differentiation. In accordance with literature[54], a potential cross-differentiation of the adipogenically induced, pre-seeded hES-MP002.5s into mineralizing osteoblasts was revealed, regardless of BMP-4 supplementation. This is not unreasonable, since dexamethasone is used as an inductive cue in all mesodermal protocols. Perhaps the ease of osteogenesis stem from a commitment of hES-MP002.5 to the osteogenic lineage

The lack of responsiveness of hES-MP cells to the induction medium and BMP4-treatment could be due to numerous reasons, such as low expression of receptors that bind the active ingredients and relay the signals inwards, or that the hES-MP cells already are too far differentiated (i.e. have more resemblance to a tissue specific progenitor cell than a multipotent stem cell). Furthermore, in the literature-case of BMP4-treatment, the cell lineage used was of mouse-origin, which does not necessarily behave like human cells do. Another issue with hES-MP cells is that they proliferate particularly well, also when confluence is reached and differential induction is taking place. Adipogenic differentiation is a well-studied process in preadipocytes, where growth arrest followed by mitotic expansion are two essential steps in order for adipogenic differentiation to be initiated and expression of adipogenic proteins can take place[19]. Since the MP-cells do not behave in this way – but keeps growing until they detach from the culture material – it is doubtlessly hard to mimic the natural course of differentiation.

The secondary round of experiments included the differentiation of hES-MP, ChiPSC4-MP and Thumb. The original protocol was pretty much left unchanged, with the exception of Roziglitazone addition at 1 uM to ensure that the addition did not bring about any toxic consequences (10 uM was used at the first setup). No follow up was made on the use of BMP4 as no apparent difference in performance could be detected from previous experiments (however, possibilities for variation when pretreating cells with BMP4 could be of interest – for example a longer period of treatment, and passaging the cells after treatment to reduce osteogenic differentiation). A comparison of gelatin- and fibronectin-coated culture material was included on the basis that fibronectin coated culture plastic could improve adipogenic differentiation of adipogenic precursors[67].

Initially, all cell types seemed to benefit from gelatin coated material, since more cells adhered to the surface and full confluence was reached quickly, which is reasonable since both MP-cell types (hES-MP002.5 in passage 11 and ChiPSC4-MP in passage 8) were grown on gelatin in regular cell culture (for 3 and 7 passages, respectively), and possibly have become adapted to the material through the expression of adhesive proteins. However, no visible adipogenic response of the treatment could be seen in the case of hES-MP or ChiPSC4-MP on any of the chosen materials. Thumb did partly differentiate visually on both coatings, and from visual observation determined to have a slightly higher efficiency of differentiation on fibronectin. The obtained qPCR-data vaguely suggest the same thing for all genes considered, but more samples should have been run in order to generate conclusive results.

Lipid accumulation in Thumb could be detected from about day 10, appearing as light, spherical, small droplets; clearly within the borders of single cells. From thereon, the lipid content was increased over time until the experiment was terminated. Only a slight percentage of the present cells were detectable with lipid accumulation, and these differentiated cells grew regionalized in small “islands”, which could be due to a positive reinforcement from already differentiated cells[27]. Oil Red O staining of all other cell types did not reveal any results of the same

extent as Thumb did. At highest magnification (20x), all cell types showed small droplets stained red. However, this is most probably due to non-specific lipid content in the cells, such as vesicles, as it is most prominent in hES-MP control which also contains the largest quantity of cells.

With qPCR, two genes critical for adipogenic differentiation was investigated: C/EBPa and PPARg. All induced cell types seem to increase their C/EBPa-expression over the three weeks of induction, while the hES-MP control remaineds at a uniform level. Both hES-MP002.5 and ChiPSC4-MP seemed to respond in the same fashion as Thumb. However, Thumb displayed a considerable higher response than both MP-cell types were able to. Conversely, while Thumb showed a slight increase in expressional levels of PPARg, the expression seemed to be reduced in both MP-cell types when compared to non-induced cells. However, the same reduction in PPARg levels was seen in hES-MP002.5 control, which might implicate that the down regulation might be associated with an event such as cells reaching a high cell density, rather than the adipogenic induction media.

The final adipogenic gene investigated was GLUT4, a glucose transporter that is responsive to insulin which helps to regulate the energybalance of the adipocyte, thus vital for functionality of a fat cell. Neither hES-MP002.5 or ChiPSC4-MP showed a prominent increase of the gene during adipogenic induction, while Thumb seemed to experience a weekly increse with considerably elevated levels at the 3 week and final day of analysis. All of the qPCR results relates well to results obtained from microscopical inspection. As described in the literature review, C/EBPa and PPARg acts cooperatively to induce expression many adipocyte specific proteins such as GLUT4, thus correlating well to the postive differentiation seen of Thumb. PPARg-levels were on the other hand decreasing for MP-cells, and in combination with the very weak C/EBPa expression, it could help to explain the negative differential response, as vital gene expressions needed for adipocyte function probably would be lacking. Once again, it should be pointed out that these results only are indicative on a realtaionship, and that obtained mRNA-levels not per se correlate to a functional protein expression, as there are several layers of regulatory issues to pass, such as mRNA processing and degradation, protein assembly and posttranslational modifications.

5.5 Conclusive remarks

The explanation for the lack of response of hES-MP002.5 to chondrogenic and adipogenic induction could lie in the degree of differentiation of hES-MP002.5 to begin with – that a state more closely resembling natural osteoprogenitors rather than mesenchymal stem cell are reached. This is a hypothesis strengthen by the fact that even non-induced cells seem to increase expression of ALP at confluence, and a likely cross differentiation into osteoblasts (i.e. mineralization capacity) can be seen when cells are adipogenically induced. A potential commitment to the osteogenic lineage might block a competence for cross-differentiation into chondrogenic and adipogenic lineage *in vitro*. As an example, transcription factors Runx2, β-catenin and osterix essential for osteogenic differentiation is suggested to inhibit chondrogenic differentiation[25]. In de Peppos studies, hES-MP002.5 show a significantly larger expression of Runx2 compared to hMSCs at week 1 osteogenic induction, and actually decreases at week 2 and 3, a phenomenon which might reflect the originating levels of Runx2 expressed in hES-MP002.5[35]. I*n vivo* experiments of hES-MP002.5 demonstrate that it indeed is a multipotent cell type that has committed to the mesodermal lineage[1], but the complex inductive cues found under physiologic conditions may be too hard to simulate artificially. Parallel comparative genetic studies to hMSCs and adult osteoprogenitors could be made in order to determine the degree of commitment of hES-MP002.5.

The positive control used in the project (Thumb) is not an optimal control for the mesodermal potential experiments, since it is not a mesenchymal stem cell, even if it seems to have possibilities to differentiate into fat and bone. The optimal control would naturally have been to use human mesenchymal stem cells, since these are the cells that the hES-MPs and ChiPSC4-MPs are intended to mimic.

6 FUTURE WORK

Cellectis Stem Cells see a great interest in providing their customers with a broad range of products of high quality, and since hMSCs are a commonly used adult stem cell type in research as well as clinical applications, it would be of great value to offer mesenchymal stem cell like cells both derived from embryonic stem cells as well as induced pluripotent stem cells. As the work in this project failed to result in positive differentiation of hES-MP002.5 and the MP-line derived from ChiPSC4 into chondrogenic and adipogenic cells, more work has to be made in order to be able to provide multipotent mesenchymal progenitors. As experiments had to be initiated relatively early on during the project, suggestions on alternative methods of differentiation were found at later stages that could be of interest to assess.

For cartilage differentiation of hES-MP002.5, a suggestion is to perform a co-culture with articular chondrocytes[61]. Bigdeli et al. used such a co-culture to differentiate embryonic stem cells into chondrocytes, through a pellet culture of hESCs and irradiated chondrocytes for 14 days, which after a collagenase treatment resulted in proliferating cells (of embryonic stem cell origin) resembling mesenchymal stem cells with the potential to differentiate into chondrocytes in pellet culture with the sole use of soluble cues. This process could be mimicked when differentiating hES-MP002.5 in order to determine their chondrogenic potential, but would not be a technique recommended to use for differentiation purposes by customers. If responsive to exogenous cues from native chondrocytes under *in vitro* culture, hES-MP002.5 could still be of interest to further investigations as a progenitor cell type. The hypothesis is strengthened by the fact that hES-MP generates cartilaginous tissue when inplanted *in vivo*[1], an environment that could be seen as a complex kind of co-culture. An alternative use would be to create a chondrogenic progenitor cells directly from embryonic stem cells, as proposed in the article.

A method that could potentiate all efforts of mesenchymal differentiation procedures is to expand hES-MP002.5 under hypoxic conditions, before differentiation induction is initiated. This procedure has several times been shown to enhance the proliferation of hMSCs[62,63], which is in accordance with the fact that such stem cells *in vivo* reside in oxygen-deficient tissues and that low oxygen tension helps to maintain stem cell functions[64]. Furthermore, hypoxically pre-cultured adipose derived MSCs have been shown to improve adipogenic and osteogenic differentiation under normoxic conditions when compared to normoxic pre-culture[62], and other sources suggest that such pre-culture also augments chondrogenic differentiation of hMSCs[65]. Hypoxic culture might induce expression of genes that are essential for stem cell function in hES-MP002.5, and thus might help to increase stem cell function and restore differentiation potential.

As mentioned previously, it might be possible to obtain a cell type that is more prone to differentiate into the investigated mesodermal lineages investigated in this project by modifying the original protocol for MP-establishment. Two methods mentioned in the literature review suggest alternatives to the currently used protocols that manage to turn mouse and human iPS-cells into mesenchymal like cells, respectively (see section 2.4.4)[51,20]. In addition, an article describing a protocol yielding cells with mesenchymal like features (such as having osteogenic, chondrogenic and adipogenic differention potential) from both hES- and hiPS-cells suggest to grow pluripotent cells as single cells on a fibrillar collagen coating [66]. As such, there are several options available that can be used to modify the current protocol.

7 CONCLUSIONS

- *How well can Cellectis Stem Cells embryonic stem cell derived mesenchymal progenitor line hES-MP002.5, with the previously used protocols, be differentiated into:*
    - *Osteoblasts*
    - *Chondrocytes*
    - *Adipocytes*

With the previously used protocols, hES-MP002.5 was seen to be efficiently differentiated into what seems to be functional osteoblasts, that both show an upregulation of ALP-activity and clearly visible bone matrix deposition. However, hES-MP002.5 fails to yield any extra cellular matrix producing chondrocytes in pellet culture, nor to generate lipid accumulating cells in two dimensional culture, using the old protocol.

- *When and how does osteoblast differentiation from hES-MP002.5 start and proceed?*

With the examined treatment, hES-MP002.5 responds to the osteogenic induction medium within a few days when ALP-activity is under consideration, which was consequently increased until the third week of culture. However as the results were not normalized against cellular number, they might be misrepresentative. Functional bone deposition and mineralization could macroscopically be seen to take place 2.5-3 weeks after treatment was initiated, and Alizarin Red staining confirmed that the produced matrix was increasingly calcified from thereon to the end culture period.

- *Is it possible to enhance Cellectis Stem Cells protocols for differentiation of mesenchymal progenitors into chondrogenic and adipogenic lineages by changing additives/concentrations/procedures of differentiation?*

The methods examined were not able to enhance the degree of differentiation to a purposeful level.

Chondrogenic induction of hES-MP002.5 was attempted to be enhanced by using a smaller pellet size, increased TGF-β1 concentration during pellet formation, the replacement of TGF-β1 with the more potent isoform TGF-β3, and through hypoxic culture. However, no substantial amount of chondrogenic extracellular matrix could be detected at histological staining of pellet sections during these circumstances. If further chondrogenic experimentations are made with this or other MP-cell types, it is suggested that hypoxic conditions is used, since it is strengthened by vast quantities of literature and differentiation response from positive control, together with TGF-β3 supplementation, since partial ECM-deposition and histological resemblance to native hyaline cartilage was seen in hES-MP002.5, even if it was not positively stained for GAG-content.

Adipogenic induction of hES-MP002.5 was attempted to be enhanced by a BMP-4 pretreatment for lineage commitment, the addition of the PPARg-agonist Rosiglitazone at two different concentrations, and through culture in fibronectin- as compared to gelatin-coated wells. No positive lipid accumulation could be detected in any of the induced cells, neither through microscopic inspection or Oil Red O staining. Two regulatory genes were examined by qPCR; C/EBPa and PPARg when rosiglitation addition was investigated. C/EBPa seemed to be positively regulated throughout the induction period, but to a minor extent when compared to positive control. PPARg seemed to be down regulated during the period of induction – a fact that can help to shed light on the negative response to the differentiation treatment.

- *Is it possible to differentiate Cellectis Stem Cells induced human pluripotent stem cell line ChiPSC4 into a mesenchymal progenitor line? If so:*
    - *How well do the cell population resemble hES-MP002.5?If not, why so?*
    - *Does the cell population have the capability of differentiating into osteoblasts, chondrocytes and adipocytes in vitro with the protocols used to induce hES-MP002.5?*

A cell population, ChiPSC4-MP, with similar characteristics and morphological appearance to hES-MP002.5 can be established from ChiPSC4 with existing protocols for establishment of mesenchymal progenitors from embryonic stem cells. Morphologically, the ChiPSC4-MP cell population is heterogeneous and individual cells appear to be larger when compared to the small, elongated cells found in the exceptionally homogenous hES-MP002.5. Growth patterns and individual cell morphology are however mimicked to some extent. Furthermore, ChiPSC4-MP appear to grow more slowly than hES-MP0002.5 and have lesser proliferative potential: the two first batches stopped proliferating after 6-11 passages, while hES-MP002.5 have been shown to keep dividing beyond passage 30. Immunocytochemical assessment indicate that three individually established batches of ChiPSC4-MP displays the same epitopes as hES-MP002.5 do, and complete loss of pluripotent markers. Thus, compared to hES-MP002.5, both similarities and differences can be seen. A plausible reason for the different characteristics between the two MP-lines is the heterogeneity in the ChiPSC4-MP populations.

In mesenchymal studies of ChiPSC4-MP, differentiation into osteoblasts is potentially present – ALP-activity might be increased throughout the induction period, and a vague mineral deposition could be seen at week 4 of 6. Differentiation of ChiPSC4-MP into chondrogenic and adipogenic cells does however fail to occur, neither from original protocol or when modifications that seem to benefit differentiation of positive control are used. When qPCR data is examined, ChiPSC4-MP shows expressional patterns matching those of hES-MP002.5, but at a lower magnitude. The mesenchymal differentiation studies of ChiPSC4-MP in general point toward a similarity with hES-MP002.5.

- *In summary, what conclusions can be drawn from the work with hES-MP002.5?*

hES-MP002.5 should not be considered to be used as starting material for functional studies of cartilage and fat cells since a useful differential response into these cell types is absent, regardless if the previous or modified protocols of induction are used. The modified protocols assess the most commonly used additives and conditions that in published scientific journals has been shown to enhance the differentiation of mesenchymal stem cells and progenitor cell lines. Since no differential response could be seen of hES-MP002.5 to begin with, it is unlikely that further differentiation experiments with alternative modifications would yield greater improvements.

To overcome the issues of hES-MP002.5's lack of mesenchymal differentiation potential, an optimization or alteration of the protocol for mesenchymal progenitor establishment should be considered. This conclusion is strengthen by the fact that hES-MP002.5 and ChiPSC4-MP cells, established with the same protocol but from different sources, both seem carry the same strengths and weaknesses. A modification might yield a less differentiated cell type with greater potential for multipotency.

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9 APPENDIX

9.1 Chondogenic qPCR-data

Table 9. 1 *Summary of qPCR data results. Ct-values are an average of two technical replicates on one sample per group. 40 cycles were made. Bold notation indicates unreliable values.*

	CREBBP (Reference)	COL2A1		ACAN		SOX9	
Sample	**Ct**	**Ct**	**RQ**	**Ct**	**RQ**	**Ct**	**RQ**
Zerosample: hES-MP p8	25,227	**38,0225**	**7,71E-05**	**34,46**	**0,017**	26,4445	0,938
Zerosample: C-MP p8	24,9345	**35,3705**	**3,96E-04**	32,3735	0,06	**25,836**	**1,168**
Zerosample: Thumb p10	24,9435	34,369	7,98E-04	20,105	298,673	28,0725	0,249
hES-MP, TGF-B1, Regular, W3	30,4165	**37,675**	**0,004**	**35,226**	**0,372**	33,545	0,249
hES-MP, TGF-B1, Hypoxic, W3	27,302	**34,154**	0,005	32,099	0,375	30,7535	0,199
hES-MP, TGF-B3, Regular, W3	30,63	**-**	-	**-**	**-**	**33,9065**	**0,225**
hES-MP, TGF-B3, Hypoxy, W3	28,99	**35,541**	**0,006**	29,619	6,748	32,1065	0,252
C-MP, TGF-B1, Regular, W1	27,516	**37,6365**	**4,93E-04**	**34,857**	**0,064**	30,2595	0,326
C-MP, TGF-B1, Regular, W2	28,184	**37,799**	**7,00E-04**	**34,771**	**0,109**	32,5225	0,108
C-MP, TGF-B1, Regular, W3	29,2525	**38,906**	**6,81E-04**	**36**	**0,097**	**33,674**	**0,102**
C-MP, TGF-B1, Hypoxic, W1	26,515	**36,694**	**4,73E-04**	33,9365	0,061	29,259	0,326
C-MP, TGF-B1, Hypoxic, W2	31,247	**-**	-	**-**	**-**	**35,4185**	**0,121**
C-MP, TGF-B1, Hypoxic, W3	30,1315	**39,833**	**6,59E-04**	32,7225	1,732	34,838	0,084
Thumb, TGF-B1, Regular, W1	26,286	27,6535	0,213	20,794	469,715	26,034	2,599
Thumb, TGF-B1, Regular, W2	26,25	21,543	14,321	20,071	756,077	26,289	2,123
Thumb, TGF-B1, Regular, W3	26,477	21,7395	14,631	20,349	729,952	25,4335	4,497
Thumb, TGF-B1, Hypoxic, W1	27,6185	25,7935	1,943	20,503	1,45E+03	27,661	2,118
Thumb, TGF-B1, Hypoxic, W2	31,421	30,5385	1,011	26,0445	433,632	33,3535	0,572
Thumb, TGF-B1, Hypoxic, W3	27,0575	17,6625	369,171	19,111	2,58E+03	25,142	8,232
Calibrator #3	26,2475	25,3805	1	29,631	1	27,373	1

9.2 Adipogenic qPCR-data

Table 9. 2 *Summary of qPCR data results. Ct-values are an average of two technical replicates on one sample per group. 40 cycles were made. Bold notation indicates unreliable values.*

	CREBBP (Reference)	C/EBPa		PPARg		GLUT4	
Sample	**Ct**	**Ct**	**RQ**	**Ct**	**RQ**	**Ct**	**RQ**
Zerosample: hES-MP p8	25,187	**31,755**	0,005	27,2465	0,584	**35,6735**	0,004
Zerosample: ChiPSC4-MP p8	24,7105	31,263	0,005	26,6645	0,629	33,6965	0,01
Zerosample: Thumb p10	24,8555	27,344	0,087	27,199	0,48	34,3795	0,007
hES-MP, Gelatin, Week 1	26,085	**32,18**	0,007	29,2915	0,264	33,5825	0,029
hES-MP, Gelatin, Week 2	25,9755	31,252	0,013	29,084	0,282	34,0345	0,02
hES-MP, Gelatin, Week 3	26,1085	30,721	0,02	30,0545	0,158	34,0955	0,021
Control, Gelatin, Week 1	25,198	31,69	0,005	27,1815	0,616	34,312	0,01
Control, Gelatin, Week 2	25,4705	32,126	0,005	27,866	0,463	**34,962**	0,007

Control, Gelatin, Week 3	25,997	32,835	0,004	29,4015	0,23	**35,4955**	0,007
ChiPSC4-MP, Gelatin, Week 1	25,9335	32,51	0,005	29,5955	0,192	33,9365	0,021
ChiPSC4-MP, Gelatin, Week 2	26,2285	32,333	0,007	30,7085	0,109	34,464	0,018
ChiPSC4-MP, Gelatin, Week 3	26,692	32,566	0,008	31,7765	0,072	**34,891**	0,018
Thumb, Gelatin, Week 1	25,559	25,733	0,433	28,643	0,287	34,0865	0,014
Thumb, Gelatin, Week 2	26,1645	26,231	0,466	28,5945	0,452	33,552	0,032
Thumb, Gelatin, Week 3	26,4645	25,236	1,143	28,3955	0,639	32,14	0,103
Thumb, Fibronectin, Week 1	25,8755	26,196	0,391	28,5395	0,384	34,358	0,015
Thumb, Fibronectin, Week 2	26,095	26,127	0,478	28,3585	0,507	33,3595	0,034
Thumb, Fibronectin, Week 3	26,1785	24,274	1,827	27,71	0,842	31,281	0,154
Calibrator #3	26,0505	25,015	1	27,3345	1	28,452	1