The effect of expanding mesenchymal stem cells in media supplemented with lysate manufactured from fresh or expired platelet concentrates on chondrogenic differentiation

Salóme Ingólfsdóttir

Ritgerð til diplómaprófs
Háskóli Íslands
Læknadeild
Námsbraut í Lífeindafræði
Heilbrigðisvísindasvið

HÁSKÓLI ÍSLANDS

Áhrif þess að fjölga mesenchymal stofnfrumum í æti með viðbættu lýsati, útbúnu úr ferskum eða útrunnum blóðflögum, á brjósksérhæfingu þeirra.

Salóme Ingólfsdóttir

Ritgerð til diplómaprófs á meistarastigi í Lífeindafræði

Umsjónarkennari: Martha Ásdís Hjálmarsdóttir

Leiðbeinendur: Ólafur Eysteinn Sigurjónsson

Læknadeild

Námsbraut í Lífeindafræði

Heilbrigðisvísindasvið Háskóla Íslands

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Abstract in English

Mesenchymal stem cells (MSCs) are a group of heterogeneous cells that can be isolated from numerous tissues, differentiated into various cell lines and expanded extensively *in vitro*. Additionally, they possess anti-inflammatory properties, therefore providing great potential for stem cell tissue engineering and regenerative medicine. Traditionally, foetal bovine serum (FBS) is used for *in vitro* culturing of MSCs but recently, alternatives such as human platelet derived culture mediums have been explored due to the potential harmful effect of using animal serum.

In this study we expanded the MSCs in lysates produces from fresh and expired human platelet rich concentrates (HPLF and HPLO respectively) as well as FBS for comparison. Once expanded, chondrogenisis was induced by an aggregate pellet culture in a chondrogenic medium for duration of 31 days. At days 14 and 31, pellets were harvested for a histochemistry assay, glycosaminoclycan assay and a real time quantitative polymerase chain reaction assay.

The MSCs expanded in HPLF displayed more favourable chondrogenic properties in camparison to the other two treatment mediums. This could potentially be caused by growth factors and cytokines present in human platelets, which may be of better quality in the HPLF compared with HPLO.

Abstract in Icelandic

Mesenchymal stofnfrumur (MSC) eru hópur sundurleitra stofnfrumna sem hægt er að einangra úr ýmsum vefjum, sérhæfa yfir í mismunandi frumulínur og fjölga *in vitro.* Þær búa einnig yfir ónæmisbælandi áhrifum og þar af leiðandi eru miklar vonir bundar við notkun MSC í lækningaskyni. Hefð er fyrir því að nota kálfasermi við ræktum MSC en nýlega hefur möguleiki á notkun blóðflögulýsata unnum úr mennskum blóðflögum verið kannaður þar sem notkun kálfasermis býður upp á smithættu í læknisfræðilegri meðferð.

MSC var fjölgað í blóðflögulýsötum unnum úr ferskum blóðflögum annars vegar og útrunnum hins vegar en einnig var sett upp frumurækt í kálfasermi til samanburðar. Sett var upp svokölluð pellet rækt til þess að hvetja brjósksérhæfingu og tók hún 31 dag. Pelletar teknir úr rækt á degi 14 og 31 og var glycosaminoglycan brjóskpelletanna kannað ásamt svipgerðargreiningu og að lokum var sett upp rauntíma qPCR.

MSC sem ræktaðar voru í fersku blóðflögulýsati viðhélt bestum brjósksérhæfingareiginleikum Mesenchymal stofnfrumanna í samanburði við frumur ræktaðar í hinum lýsötunum. Möguleg skýring gæti verið að vaxtarþættir og cytokinar, sem eru til staðar í blóðflögulýsati unnu úr mennskum blóðflögum, varðveitast betur í ferska lýsatinu í samanburði við það útrunna.

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Abbreviations

BSA	Bovine Serum Albumin
DMEM/F12	Dulbecco's Modified Eagle's Medium with F12 Nutrient mixture
ECM	Extracellular Matrix
FBS	Foetal Bovine Serum
GAG	Glycosaminoglycan
HCl	Hydrochloric acid
HE	Haematoxylin and eosin
hESC	Human embryonic stem cell
HPL	Human platelet lysate
HPLF	Human platelet lysate fresh
HPLO	Human platelet lysate outdated
iPSC	Induced pluripotent stem cell
MSC	Mesenchymal stem cell
MSCGM	Mesenchymal stem cell growth medium
PBS	Phosphate buffered saline
PER	Papain extraction reagent
PRC	Platelet rich concentrate
qPCR	Quantitative polymerase chain reaction
TB	Toluidine blue
TGF-β3	Transforming growth factor beta

1 Introduction

Stem cell research is a fast expanding field that has generated great public interest due to its promising use in regenerative medicine and tissue engineering. One area of research involves the *in vitro* expansion of stem cells which is increasingly moving towards mimicking the *in vivo* microenvironment in which these cells proliferate. The traditional choice of culture medium for cellular expansion has been foetal bovine serum (FBS) which could present some immunological risks to the transplant recipient (2). Therefore, the search for a more appropriate culture medium has ascended with human platelet lysate (HPL) at the forefront. Recently the use of outdated human platelet lysate has been investigated and here we further explore that possibility and focus on its effect on chondrogenesis.

1.1 Stem cells

Stem cells are defined by their ability to self-renew and differentiate into any cell type of the tissue they originate from (3). They can divide symmetrically, resulting in two stem cells or asymmetrically, resulting in one stem cell and one progenitor cell, therefore allowing them to minimize the stem cell pool and meet the demand for new progenitor cells (4).They can be classified into a hierarchy based on their differentiation potential. Totipotent stem cells are at the top of the hierarchy and have the ability to develop into any type of host cell, including the cells of the placenta and umbilical cord. This unique property is only held from the moment an egg is fertilized until the embryo reaches the 4- to 8-cell stage (5). Pluripotent cells are able to differentiate into cells of all 3 germ layers, namely ectoderm, mesoderm and endoderm (6). These include embryonic stem cells, induced pluripotent stem cells and although some would classify Mesenchymal stem cells (MSCs) as pluripotent (7, 8), this is still under debate and most refer to MSCs as multipotent (6). Multipotent cells can differentiate into more than one cell lineage (9). These include MSCs (9) and Haematopoietic stem cells (HSCs).Unipotent cells can only differentiate into one cell type of the tissue that they originate from (6, 10).

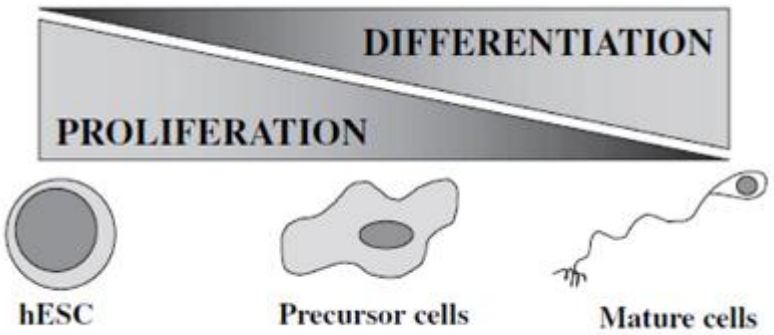

Figure 1: Stem cells have great proliferation potentials which gradually decrease as the cells commit further to differentiation. At the same time their differentiation potential increases. However the self-renewal ability is unique to stem cells. Adapted from Mountford *et. al.* (1).

1.1.1 Embryonic stem cells

Human embryonic stem cells (hESC) can be isolated from the inner cell mass of a blastocyst, usually on day 5 of the blastocysts development. (2, 9) These cells have the ability to go through indefinite cell divisions, a property

made possible by an active telomerase that reassembles the telomeres after mitosis. Generally, cells telomere will shorten with every cell cycle, therefore limiting the proliferation potential of the cell. Characterization of hESCs can be based on their ability to differentiate into cells of all three germ layers, therefore making them pluripotent (6). As stem cells continue to differentiate into precursor cells and finally committing to a specific cell line, they lose their ability to proliferate indefinitely whilst simultaneously increasing their differentiation potential (1) (Figure 1). Another unique property of the hESC is their ability to supply an unlimited number of diverse cell types, making them an attractive candidate for regenerative medicine. This is hampered by the moral controversy and legal restrictions placed on hESC research. A new *in vitro* manufactured stem cell with pluripotent abilities has recently emerged, called the induced pluripotent stem cell. The technique involves the reprogramming of somatic cells into an embryonic stem cell-like state. These cells could potentially substitute the use of hESC and simultaneously eradicate the moral controversy, but this debate is premature and more research is needed (11).

1.1.2 Somatic stem cells

A tissues ability to self-repair and self-renew is made possible by the presence of somatic stem cells or progenitor cells that reside within the tissue (12). These cells are believed by some to have a plasticity capability, the ability to generate tissue other than that from which they were isolated from, although this remains controversial (1). The somatic stem cell population can be categorized based on the germ layer they originate from. The ectodermal layer of an early embryo gives rise to the nervous system, skin, hair and mammary glands, while the endodermal layer gives rise to the intestine, pancreas, liver and lung. The middle layer, the mesoderm, gives rise to the circulatory system, muscle and mesenchyme (Figure 2). A fully formed foetus possesses a population of somatic stem cells that reside in niches within those tissues. Examples of somatic stem cells are neural stem cells from the ectoderm, the lung and crypt stem cells from the endoderm and the haematopoietic stem cells of the mesoderm (1). The haematopoietic stem cell has the potential to differentiate into all myeloid and lymphoid cell lines and can be derived from the bone marrow, umbilical cord blood and peripheral blood. Another example of a somatic stem cell that originates in the mesoderm is the MSC which will be the focus of this study (5).

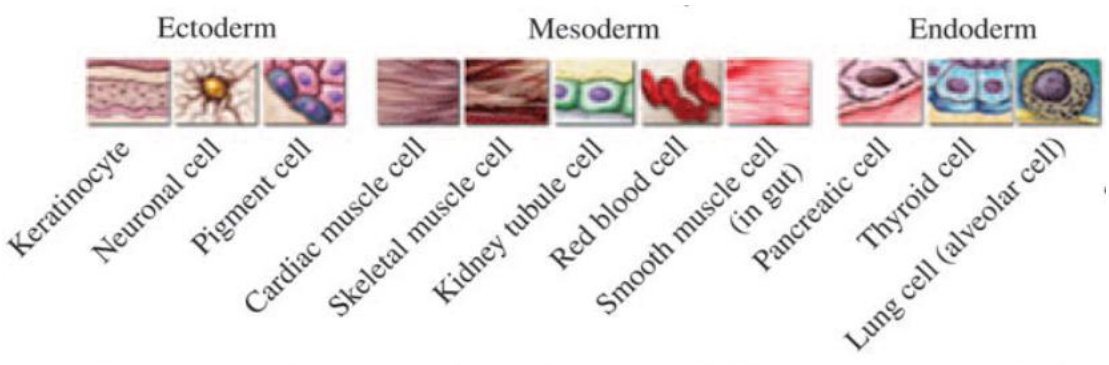

Figure 2: The three foetal germ layers and some of their respective cell lines. Adapted from Mountford *et. al*. (1)

1.1.3 Mesenchymal stem cells

Mesenchymal stem cells account for a large amount of clinical trials and published papers based on stem cell therapy because they are easy to isolate from numerous tissues, can differentiate into various cell lines and can be expanded extensively *in vitro* (9). In terms of morphology and expression of surface antigens they constitute a heterogeneous population. Three such populations have been described: small rapidly self-renewing cells, elongated fibroblastic-like, spindle shaped cells and slowly replicating large, cuboidal or flattened cells (13). The MSCs can give rise to bone, cartilage, adipose, connective tissue and the haematopoiesis-supportive stroma as well as playing a role in the formation of the haematopoietic stem cell niche (14), which is the bone marrow microenviroment in which stem cells reside and serves to protect the stem cells from depletion as well as protecting the host from amplified stem cell proliferation (15).

A definitive marker for the detection of MSCs does not exist, therefore the criteria for defining the cells has been based on various factors described by the international Society for Cellular Therapy as the following: When maintained in standard culture conditions, MSCs should be plastic adherent. They should express CD105, CD73 and CD90 but lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA DR surface molecules. They must differentiate into osteoblasts, adipocytes and chondrocytes *in vitro* (16, 17). The positively expressed surface molecules in question should be expressed by ≥ 95 % of the cellular culture and more than 98% of the culture should lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA DR surface molecules. This definition has provided researchers around the world with a guideline in defining MSCs, providing consistency to the field of MSC research (16).

MSCs can be sourced from various tissue stromas of the human body, including the bone marrow (BM), adipose tissue (AT), umbilical cord, placenta, kidney, liver, heart and spleen (18, 19). They are also found on the tissue side of every blood vessel in the body (20). MSCs are thought to be pericytes or at least have their niche in close proximity to them (21). It is believed that when injury occurs, MSCs get released from their niches on the vessel walls and start secreting various growth factors and immunomodulatory factors that may affect a regenerative microenviroment (21). The cause for their activation may be signals from the injured environment (22). The most commonly studied and sourced MSCs originate from BM or AT due to historical reasons and ease of access (2, 18). In the bone marrow MSCs represent only 1/10,000 to 1/100,000 mononuclear cells and from 1g of adipose tissue 5×10^3 MSCs can be isolated, which is 500 times more cells than from an equivalent amount of bone marrow. (2, 23-25). Mesenchymal stem cells sourced from adipose tissue and cultured in a commonly used hyaluronic acid scaffold, demonstrated less collagen II mRNA expression than bone marrow sourced MSCs (26), confirming a difference in MSCs in relation to the tissue from which they were obtained.

To differentiate cells *in vitro,* knowledge of the cellular biology is essential in order to induce the desired differentiation by applying the appropriate growth factors and environmental factors. *In vitro* differentiation of MSCs into chondrocytes is traditionally achieved by generating a micromass pellet

culture by enforced aggregation of 300,000 MSCs. This pellet is grown in a chondrogenic medium which contains growth factors and cytokines which mediate the chondrogenesis (27). These culture conditions are created to mimic the conditions in which chondrogenesis takes place *in vivo*. Additional culture conditions, such as hydrostatic pressure, have been experimented on, all of which with the common goal of simulating *in vivo* conditions for chondrogenesis (28). Other differentiation pathways include adipogenic and osteogenic differentiation, which along with chondrogenic differentiation are the most studied differentiation pathways of MSCs (29). Less studied pathways and therefore more controversial include MSC differentiation into skeletal muscle cells, neural cells (30) and cardyomyocites (31).

The immune modulatory properties of MSCs have evoked increased interest in recent years as they could prove to be valuable in therapies for various conditions such as inflammatory bowel disease, systemic lupus erythematous, collagen induced arthritis (32), graft vs. host disease as well as organ transplant (33). This immune modulation occurs as the MSC releases cytokines and growth factors that supress the inflammatory function of both the innate and adaptive immune system. It is believed that the *in vivo* origin of this immune modulatory effect stems from the fact that MSCs in the bone marrow (stromal cells) serve to protect the sensitive haematopoietic stem cell population from the immune system (34).

In terms of the effect on dendritic cells MSCs have been shown to inhibit the production of TNFα, an inflammatory cytokine, as well as stimulating the production of IL10, an anti-inflammatory cytokine. In the case of T-cells, the MSCs decrease the natural killer cell secretion of IFNγ and increase the production of anti-inflammatory cytokine IL4. T regulatory cells are anti-inflammatory cells that have shown to increase in the presence of MSCs. The overall effect shows immunosuppressive capabilities and is shown in figure 2 (35). Studies have also demonstrated that MSCs can hinder B cell proliferation as well as their capacity to produce antibodies (36). This immunosuppressive property has been shown to prolong skin and cardiac allografts in animal models and in clinical studies enrolling patients with severe acute graft versus host disease, the administration of MSCs resulted in a significant clinical response (37). In addition to this, MSC derived from the bone marrow express low levels of human leukocyte antigen major histocompatibility complex class I, a feature that could explain their immune privileged status in allogenic hosts (38). The allogenic escape mechanism may be valuable in therapies as it would be relatively easy to stock cultured allogenic human MSCs instead of having to culture autologous MSCs or MSCs from donors related to the patient (39). One in vitro study even suggested a stronger immunosuppressive effect of allogenic MSCs than of autologous MSCs (40). The homing ability of MSCs to a damaged tissue may present another feature, valuable in therapy and could be used for cartilage and bone repair (41).

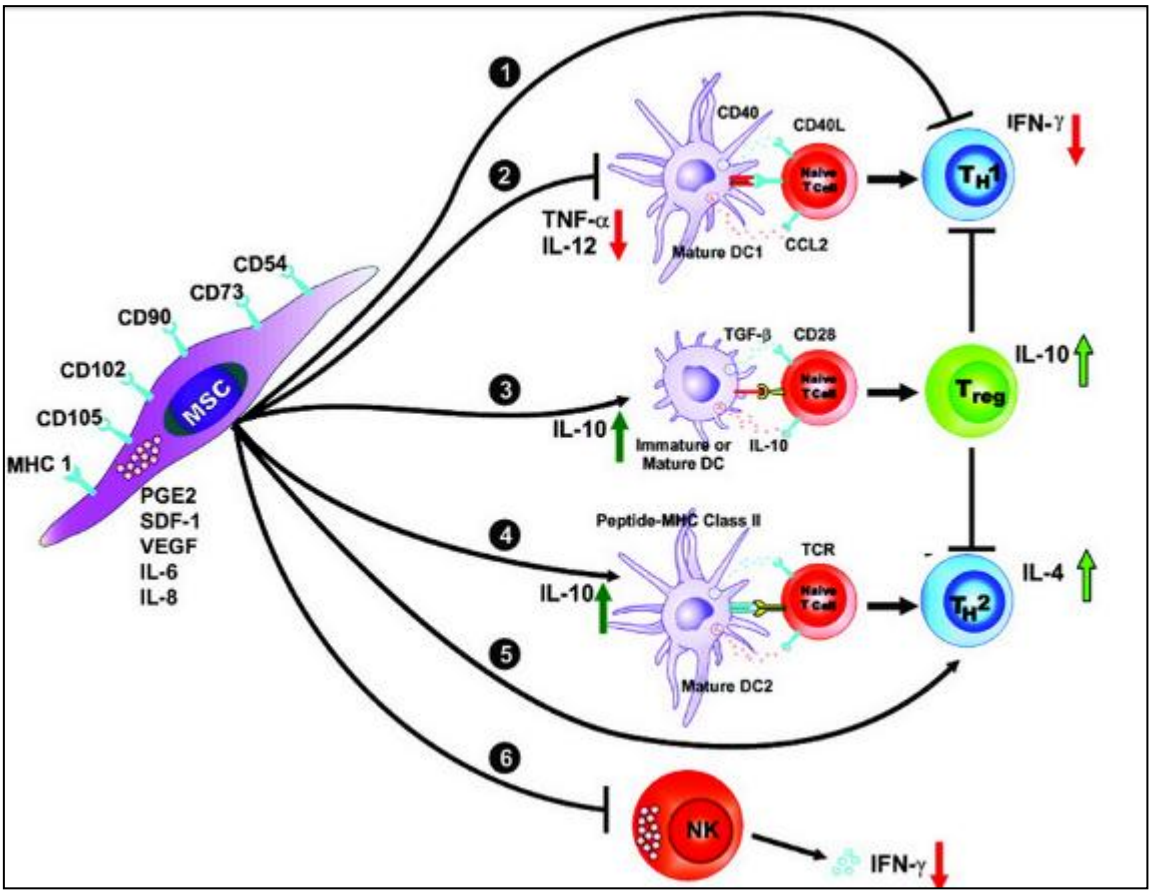

Figure 3: The anti-inflammatory effect MSCs offer by secreting growth factors and cytokines as well as using cell-cell interactions. Pathway 2-4 and 6 show how MSCs affect the innate immune response whereas pathways 1 and 5 show MSCs effect on the adaptive immune response (35).

1.2 Clinical Use

Stem cell therapy is based on the notion that undifferentiated stem cells can be delivered to an injured host where they will migrate to the site of the injury and start repairing the injury by differentiating into the desired cell type with the help of local signals (42). For years, stem cells have been used clinically for therapeutic purposes. This has for example been in the form of hematopoietic stem cell transplant in patients with leukemia and other cancers (42). Stem cells have also been used to generate new tissue *in vitro* for implantation such as in the case of cartilage repair, using MSCs. These implants have been performed using chondrocytes obtained from a non-weight bearing part of the patient's cartilage, causing injury to a healthy tissue. An attractive alternative is the use of MSCs for this purpose and clinical trials have already shown good results (43).

1.2.1 Tissue engineering

Tissue engineering aims to improve tissue function by the development of biological substitutes. It is composed of three main elements: A cellular component, capable of forming a functional matrix, a scaffold made up of biological or synthetic matter and growth factors and cytokines, capable of regulating the formation of a desired tissue (44).

In terms of cartilage tissue engineering the cellular component is the chondrocyte which can be produced by *in vitro* differentiation of MSCs. These cells can be either autologous, meaning cells harvested from the transplant recipient or allogeneic, meaning cells harvested from a donor. Autologous chondrocyte transplantation is a widely used repair technique which involves the harvesting of thin cartilage slices from the knee. The chondrocytes from these slices are expanded *in vitro* before being implanted to the injured site and secured with a periosteal patch. Allogeneic transplantation applies the same principle as autologous chondrocyte transplantation but uses cadaveric cartilage. Research has shown that the allogeneic transplant provokes an immune response that can gradually destroy the transplanted cartilage (33).

The choice of scaffold has to be based on the type of implant required and there are several patented scaffold options currently on the market. ChondroCelect® uses a chondrocyte subpopulation believed to possess molecular markers that predict the formation of hyaline-type tissue. A fibrin and polymer based scaffold of poly lactic glycolic acid (PLGA) and polydixanone is available through BioSeed® and showed good tissue integration in a recent clinical study (45). There are currently several other scaffold options available on the market for cartilage repair (33).

1.2.2 Regenerative medicine

Regenerative medicine aims to induce tissue self-healing with the help of an *in vitro* produced product that is then transplanted *in vivo* making it a less invasive alternative to tissue engineering. This was first attempted in 1997 by the integration of platelet rich plasma in fibrin glue for this purpose. Soon after followed the discovery that a small amount of stem cells from bone marrow origin were able to repair a tissue of the mesenchymal origin (46). MSCs present an attractive option for regenerative medicine as they can be easily isolated from BM aspirates, are easily cultured and show great plasticity along with unique immunomodolatory effects (47).

When cartilage injury occurs, the body's ability to self-heal is flawed and does not result in healthy cartilage but instead a fibrous cartilage that often causes more harm than good. This presents as a challenge for cartilage regenerative medicine. Another challenge is presented in the low number of chondrocytes that reside in cartilaginous tissue. A study using a three dimensional PLGA scaffold for MSCs showed promising results, giving rise to a smooth shiny hyaline-like cartilage at 4 and 12 weeks after the transplantation (48).

1.3 Cartilage

There are three cartilage varieties within the human body, fibrocartilage, elastic cartilage and hyaline cartilage. Here we will put emphasis on hyaline cartilage, which is present in joints and has a white, glassy appearance. It absorbs stress and distributes load, allowing smooth frictionless movement (15). The ear and nose comprise elastic cartilage which contains elastin in its extracellular matrix. Finally, fibrocartilage, present at the end of tendons and ligaments in apposition to bone, contains a higher proportion of collagen in its extracellular matrix than the other types (49). A high prevalence of hyaline

cartilage defects in the world and the painful disability such defects can cause, has put it at the forefront of majority of research looking at cartilage regeneration and tissue engineering (2).

Cartilage is an avascular, neuron derived connective tissue, a property, which minimises its ability to repair itself. It is believed by some that the size of the cartilage lesion has an effect on the tissues ability to self-repair and that lesions smaller than 3 mm in diameter have greater success in self repairing with hyaline like cartilage whereas larger defects are replaced with fibrous cartilage (33). It is composed of one type of cell, chondrocytes that make up only 1 to 5% of the tissue volume (50) and a vast extracellular matrix (ECM) component (2, 51). The ECM of human hyaline cartilage has the important role of undergoing cycles of deformation when put under stress and recovering when that load is removed (52). Homeostasis is achieved by a regulated interaction between the ECM and chondrocytes (53). Collagen type II accounts for 90-95% of the ECM collagen and has a high amount of bound carbohydrate groups which allow it to interact with water. Strength is provided by collagen types IX, XI and II which all form fibrils that interweave to form a mesh (49). Up to 80% of the wet weight of hyaline cartilage is composed of tissue fluid which contains gases, metabolites and a large amount of cations that have the role of balancing the negatively charged glycosaminoglycan (GAG) chains in the ECM. Proteoglycans, namely aggrecan, provide the cartilage with its osmotic properties which allow cartilage to resist compressive load (54). Oxygen and nutrients are exchanged between this tissue fluid and the synovial fluid (49). Aggrecan, GAG and collagen content of cartilage is directly proportional to the tissue's ability to withstand compressive forces, making them the most vital determinants of the repair tissue properties (50).

1.4 Expansion medium for MSCs

Before MSCs are differentiated *in vitro*, a monolayer expansion of the cells is required. This is traditionally done, using a growth medium containing 10% foetal bovine serum (FBS). The culture takes place in a plastic flask and is kept in a humid incubator at 37˚C, containing 5% CO_2 (27). Recent studies have explored the possibility of using humanized substitutes for MSC culture and differentiation such as umbilical cord blood serum, autologous and allogeneic human serum albumin, thrombin-activated platelet releasates, collagen-activated platelet releasates, autologous plasma-derived from bone marrow and platelet lysate to replace the traditionally used FBS for MSC culture (9, 55). These options are considered more appropriate as there are certain risks associated with the use of FBS in stem cell culture for the purpose of human stem cell treatment. These risk factors include the transmission of known and unknown pathogens and xenoimmunization against bovine antigens, along with the very low risk of prion disease transmission and zoonosis (29, 56). Nevertheless FBS has been used to culture MSC for clinical trials on humans (29).

1.4.1 Human platelet lysate

Human platelet lysate (HPL) is currently being explored as a potential MSC growth medium. Since Friedenstein et al. first described the MSC (57), foetal bovine serum has been used for this purpose but it may present certain risk factors such as FBS proteins being carried with transplanted cells, causing immune attacks and eventually transplantation failure (23).

Platelets are produced by fragmentation of the cytoplasm of megakaryocytes. A megakaryocyte matures by endomitotic synchronous replication whereas the DNA replicates in the absence of nuclear or cytoplasmic division. This results in the megakaryocyte developing into one of the largest cells of the human body before giving rise to 1000-5000 platelets. The differentiation from a stem cell to platelets averages approximately 10 days and the average platelet lifespan is 7-10 days. Platelets themselves are very small and discoid at about 3.0 x 0.5 µm in diameter and a mean volume of 7-11 fL (58). They contain a number of growth factors in α- and β- granules. When injury occurs, platelets migrate to the site of injury and promote healing by secreting the growth factors which include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), Epithelial growth factor (EGF) and insulin-like growth factor (IGF)(59, 60).

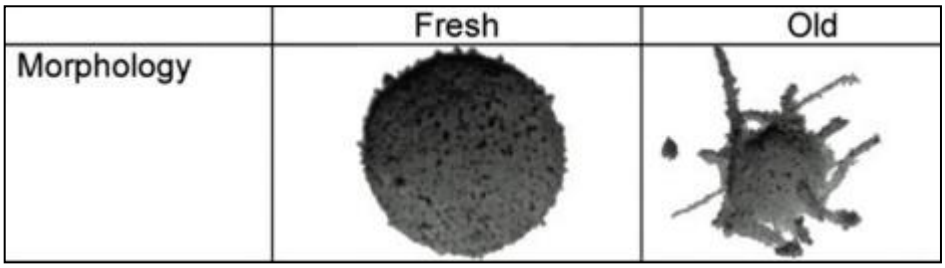

Figure 4: A fresh platelet displays a discoid morphology whilst an old one displays the spiny spheres shape (61).

Platelet rich plasma is formulated from anticoagulated blood and is full of growth factors shown in table 1. Its preparation involves the addition of citrate to whole blood to inhibit the clotting cascade by binding to the ionized calcium. PRP has been used for therapeutic purposes for over two decades with the purpose of speeding up the healing of tendon, ligament, muscle and bone after major operations and more recently, has been used to speed up the healing of sports injury (62). Platelets have a very short shelf life when it comes to storing them for blood transfusion. They can only be stored at room temperature and a transfer of fresher platelets has been shown to produce better prognostic outcome than a transfusion of old platelets. The platelet morphology varies greatly between fresh and old platelets, the fresh ones displaying a discoid shape whilst the old ones display a so called spiny spheres shape shown in figure 4. The spiny spheres shape is also apparent in activated platelets that have lost their integrity as a result of the breakup and reformation of actin filaments and centralization of microtubules and granules (61).

Table 1: Growth factors found in platelet rich plasma, their targets and function (62).

Growth factor	Target	Function
PD-EGF	**Blood vessel cells, outer skin cells, fibroblasts and other cell types**	**Cell growth, recruitment, differentiation, skin closure, cytokine secretion**
PDGF – A and B	**Fibroblasts, smooth muscle cells, chondrocytes, osteoblasts, mesenchymal stem cells**	**Potent cell growth, recruitment, blood vessel growth, granulation, growth factor secretion, matrix formation with BMPs (collagen and bone)**
TGF-β1	**Blood vessel tissue, outer skin cells, fibroblasts, monocytes**	**Blood vessel (±), collagen synthesis, growth inhibition, apoptosis, differentiation, activation**
IGF-I and II	**Bone, blood vessel, skin, other tissues, fibroblasts**	**Cell growth, differentiation, recruitment, collagen synthesis with PDGF**
VEGF	**Blood vessel cells**	**Cell growth, migration, new blood vessel growth, anti-apoptosis**
bFGF	**Blood vessels, smooth muscle, skin, fibroblasts, other cell types**	**Cell growth**

Studies have shown that HPL can be a more effective alternative to FBS in MSC culturing as well as eliminating the risks associated with using animal serum (56). It has also been shown that HPL cultured MSCs have comparable immunomodulatory capacaties to their FBS cultured counterparts (15). The potential use of outdated HPL in MSC culturing has not been fully explored to date.

2 Aim of study

The aim of this study was to analyse whether expansion of MSC in lysates manufactured from fresh and expired platelet concentrates may influence chondrogenic differentiation of MSCs. To answer that, chondrogenic differentiation was analysed by:

- Morphology and production of extracellular matrix
- Production of extracellular matrix protein glycosaminoglycan
- Gene expression analysis using qPCR

3 Materials and methods

3.1 Culture and chondrogenesis of MSCs

The isolation of MSCs is based on their ability to adhere to tissue culture plastic. A heterogeneous mixture of the original bone marrow cell suspension, including haematopoietic cell lineages is therefore seeded on the culture vessels allowing isolation of MSCs during medium changes and subsequent cellular passages, which will remove a vast majority of other cell types.

MSCs are cultured in a humidified atmosphere of 95% air and 5% CO2 at 37˚C. Colonies of spindle-shaped fibroblast-like cells will form and have to be subcultured in 7-10 days before becoming confluent. Inducing MSC to differentiate into chondrocytes is achieved by creating three dimensional culture conditions where MSCs are conformed into aggregates with high cell density and increased cell-cell interaction. This along with a defined culture medium containing TGF-β is necessary to achieve in vitro chondrogenic differentiation (52). The following chapters will describe the process involved from preparing the platelet rich concentration to the final stages of staining, glycosaminoglycan assay and real time quantitative polymerase chain reaction assay (Figure 5).

3.1.1 Platelet rich concentrate preparation

Platelet rich concentrate (PRC) preparation started with whole blood being drawn from a donor at the blood bank. A wide-bore siliconized needle was used to minimize the activation of platelets and clotting proteins and a blood collection pack containing citrate anticoagulant (Fenwal, Lake Zurich, IL, USA) was used. The blood was left at room temperature before separation into red cells, platelets and plasma. Separation was achieved by differential sedimentation which is accelerated by high force centrifugation. This process yields three products: plasma, red blood cells and a buffy coat, out of which the buffy coat was, pooled together with 4 other identically prepared buffy coat units all O^+. The pooled buffy coat units were further divided into a platelet concentrate and white cells by low force centrifugation. The platelet concentrate was finally divided into two equal units, one of which was stored at room temperature for 6 days before freezing at -80˚C and the other one placed in -80˚C freezer immediately, resulting in one fresh and one expired platelet rich concentrate.

The PRC was thawed in a waterbath at 37˚C, and transferred under the fume hood, dividing the content equally into 50 ml plastic tubes. The tubes were centrifuged at 5000 rpm for 20 minutes and transferred back under the fume hood. A filter was placed on top of a new 50 ml tube and the supernatant from the centrifuged PRC was poured through the filter using a pipette. From there the product was transferred into the top chamber of a filter, with pores measuring 0.40 µm in diameter and allowed to actively filter through into the bottom chamber using a vacuum. Next, the PRP was divided into 50 ml tubes from the filters bottom chamber and centrifuged at 5000 rpm for 20 minutes. Again, the PRC was transferred to the fume hood, the supernatant pipetted through a filter cap placed on a 50 ml tube, and from there into 15 ml tubes, at 10 ml per tube. The tubes were labelled with content, date and name, designating the term HPLF, to the fresh human platelet lysate and HPLO, to the expired human platelet lysate. Tubes were lastly placed in a tray and stored in a freezer.

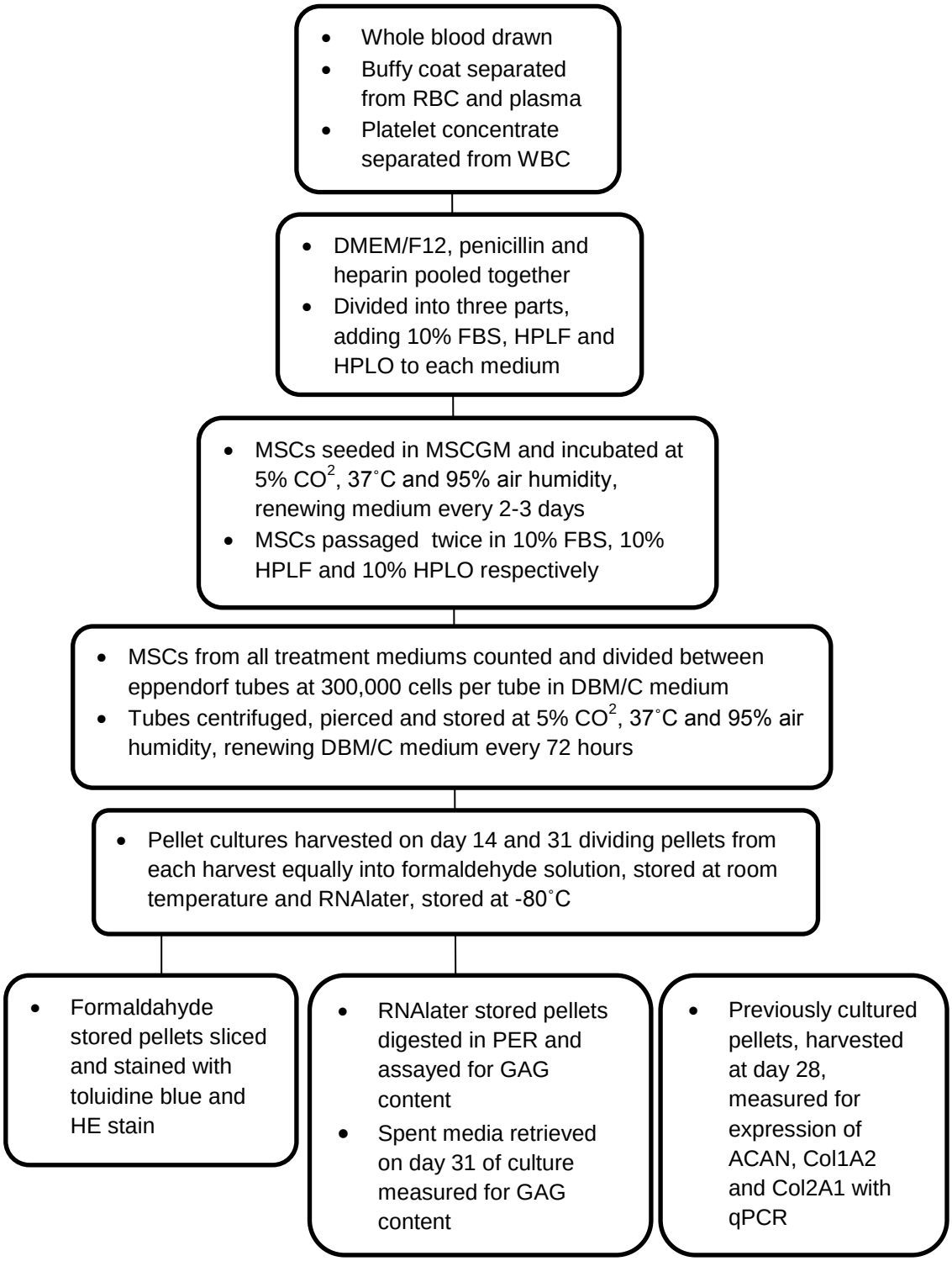

Figure 5: A flow chart showing the processes involved in this project from blood being drawn from a donor at the blood bank to the harvesting, staining, GAG assay and finally the qPCR performed using previously cultured pellets. RBC indicates red blood cells; WBC, white blood cell. DBM/C represents differentiation basal medium-chondrogenic.

3.1.2 Preparation of treatment mediums

Three different treatment medium compositions were prepared, containing 10% HPLO, 10% HPLF and 10% MSC Foetal bovine serum (FBS)(Gibco Invitrogen, Grand Island, NY, USA) respectively. The ingredients listed in table 2 were pooled before mixing well. The contents were divided into 50 ml plastic tubes (45 ml per tube) and centrifuged at 5000 rpm for 5 minutes. The supernatant was transferred into new plastic tubes and placed in a freezer.

Table 2: Composition of the three treatment mediums, applied for cellular expansion

Treatment medium ingredients	Volume (mL)
Dulbecco´s modified Eagle medium with F12 nutrient mixture (DMEM) (Invitrogen, Carlsbad, CA, USA)	**500**
HPLF, HPLO or FBS	**55**
Penicillin (Invitrogen)	**5**
Heparin (LEO Pharma AS, Oslo, Norway)	**2.4**

3.1.3 Seeding of MSCs

The mesenchymal stem cells (MSC) (Lonza, Basel, Switzerland) used for this project were aquired from a stem cell bank and had been passaged once in September 2010 at the blood bank, frozen at a concentration of 0.5 x 106 cells per ml in liquid nitrogen and labelled Donor 1. The chosen medium for passage 2 was Lonza's Mesenchymal stem cell growth medium (MSCGM), prepared by pouring MSCGM Single Quots into a 500 ml bottle of Mesenchymal stem cell basal medium. The Single Quots contained 50 ml of MCGS, 10 ml of L-Glutamine and 0.5 ml of GA-1000. Once prepared, the MSCGM was warmed up to 37˚C in an incubator. The MSCs were retrieved from the freezer and thawed carefully in a 37˚C water bath for no more than 1 minute. Cell suspension was gently pipetted into a 15 ml plastic tube and centrifuged at 1600 rpm for 5 minutes. Cells were transferred into a 75 cm^2 culture flask containing 22 ml of warm MSCGM, rocked gently and incubated at 5% CO2, 37˚C and 95% humidity. The cell density was evaluated on a daily basis and growth medium was renewed every 2-3 days by discarding of growth medium in culture flask and adding the same volume of fresh growth medium at 37˚C into the flask.

3.1.4 MSCs cultured in treatment mediums

Subculturing was performed when cell density had reached 80-90%. Growth medium was poured out of the culture flask and the remainder of the medium washed off with 5 ml of phosphate buffered saline (PBS) (Invitrogen). 5 ml of trypsin (Gibco Invitrogen) were inserted into culture flask before placing the flask in an incubator for 3-4 minutes, until the cells had been released from culture flask base, tabbing the bottom of the flask gently to release any remaining cells. The trypsin was neutralized by pouring 5 ml of MSCGM, containing 10% FBS into the flask. The cell suspension containing trypsin was centrifuged at 1200 rpm for 5 minutes and the supernatant carefully discarded off. The pellet was dissolved in 1 ml of appropriate growth medium and divided equally between 50 ml plastic tubes, containing 45 ml of 10% FBS, 10% HPLO and 10% HPLF at 37˚C respectively. Finally each growth

medium, containing cells, was transferred into 175 cm^2 culture flasks, which now contained cellular passage 3, and placed in an incubator at 5% CO_2, 37˚C and 95% humidity. Cell density was evaluated on a daily basis and growth medium replaced every 2-3 days. The final cellular passage was carried out following the same protocol as passage 3.

3.1.5 Pellet cultured MSCs

"Differentiation Basal Medium – Chondrogenic" from Lonza, with added Single Quots containing 1 ml dex., 2 ml ascorbate, 2 ml Its. + supplement, 2 ml pyruvate, 2 ml proline and 4 ml L-glutamine was termed incomplete medium. Complete medium contained 20 ml of incomplete medium with an additional 10 µl of previously prepared transforming growth factor beta (TGF-β3) (Lonza). The TGF-β3 was prepared by adding 40 ml of 4mM hydrochloric acid (HCl) to a plastic tube containing 40 µg of bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, MO, USA). TGF-β3, weighing 2 µg was dissolved in 100 µl of the HCl/BSA solvent. The product was divided into 10 eppendorf tubes and stored at -80˚C until needed.

The cells were counted before seeding. Firstly the cells were removed from the culture flasks and washed before transferring 20 µl of the cellular suspension, 30 µl of PBS and 50 µl of trypan blue (Gibco) into a small eppendorf tube. The sample was mixed well and a small amount transferred onto a haemocytometer for a cell count. In each case, cells covering 16 areas of the haemocytometer were counted. The total cellular count was divided by 16 and multiplied by 50,000, resulting in a cell per ml count of 3,600,000 for the MSCs expanded in 10% HPLF treatment medium. The cartilage pellets were seeded using approximately 330,000 cells per pellet. The correct amount of cells was achieved by dividing 3 ml of cellular suspension containing 3,600,000 cells per ml into two 15 ml plastic tubes. The tubes were centrifuged for 5 minutes at 150 g before carefully removing the supernatant and dissolving the pellet in 5 ml of incomplete medium. The cellular suspension was centrifuged again at 150 g for 5 minutes, supernatant discarded of and pellet dissolved, this time in 8ml of complete medium, resulting in a cellular suspension of 660,000 cells per ml. The suspension was divided equally between 32 eppendorf tubes, at 0.5 ml per tube, resulting in the desired 330,000 cells per pellet. The eppendorf tubes were centrifuged at 150 g for 5 minutes and subsequently pierced 3 times on the lid with a needle to allow airflow into the culture. The tubes were finally placed in an incubator at 5% CO_2, 95% humidity and 37˚C. The complete medium was freshly made and replaced every 72 hours for the duration of the culture. The same procedure was applied for MSCs expanded in 10% HPLO and 10% FBS treatment mediums, resulting in a cell per ml count of 3,700,000 and 2,800,000 respectively.

3.1.6 Havesting of MSC pellet culture

Harvesting was performed on two occasions, on day 14 and on day 31. On day 14, 5 pellets of each culture (HPLO, HPLF and FBS) were removed from culture tubes and placed in a new eppendorf tube. All remaining medium was removed before adding 0.5 ml of RNAlater® (Ambion Inc, Austin, TX, USA), a RNA stabilization solution, and placing in the freezer at -80˚C. Another 5 pellets of each culture were placed in a 15 ml plastic tube, removing all remaining medium before adding 0.5 ml of

paraformaldehyde. The tubes were wrapped in tin foil and stored at room temperature. The same procedure was performed on day 31 where the remainder of the pellets were divided equally between RNAlater® and paraformaldehyde storage options.

3.2 Histochemistry analysis

All processing and staining of samples was performed at the pathology laboratory at Landspítali Háskólasjúkrahús. The sample processing was performed in a histokinette which is an automatic, computer regulated device for dehydration of tissue. Dehydration was performed by soaking the cartilage samples in 95% ethanol (J.T.Baker, Deventer, Netherlands) and subsequently in 99% ethanol. They were cleared in xylene (J.T. Baker) and embedded in paraffin (Sakura Fintek Europe, Zoeterwoude, Netherlands) at 60˚C. The embedded sample was transferred into a metal container, placed on a hotplate where additional embedding wax was poured in to provide a cube of paraffin embedded cartilage sample, suitable for cutting. The paraffin cube was allowed to cool down and set on a cold plate before being placed in storage. Cutting was performed by an experienced Biomedical Scientist at the pathology laboratory. Each sample cube contained 5 cartilage pellets, from different growth mediums (HPLO, HPLF and FBS), harvested at either 14 days or 31 days. Each cube was cut into 20 slices, which were fixed onto microscope glass slides, placing two slices on each slide. The slides were labelled with the appropriate staining methods and air dried overnight.

3.2.1 Haematoxylin and eosin staining

All slides allocated for Haematoxylin and eosin (HE) staining were stained simultaneously, according to the protocol shown in table 3 to provide consistent results. Once the slides had been cleared the stained samples were covered with glue and a cover slide followed by air drying, after which the slides were ready for microscopic examination.

Table 3: Materials for Haematoxylin and eosin staining and the respective times the slides were submerged in them

Materials	Time (minutes)
Xylene (J.T.Baker, Deventer, Netherlands)	**5**
Air drying	**3**
Ethanol	**4**
Water	**0.5**
Haematoxylin stain (Thermo Scientific, Runcorn, UK)	**6**
Water	**0.5**
Acid	**3 dips**
Water	**0.5**
Base	**3 dips**
Eosin stain (Sigma, Steinheim, Germany)	**5**
Clearing from ethanol to xylene	**3**

3.2.2 Toluidine blue staining

All slides allocated for toluidine blue (TB) staining were stained simultaneously to provide consistent results and protocol in table 4 was followed. After the staining progress was completed, the samples were covered with glue and a cover slide, air dried and examined under a light microscope.

Table 4: Materials for toluidine blue staining and the respective times the slides were submerged in them

Materials	Time minutes
Xylene	**5**
Air drying	**3**
Ethanol	**4**
Water	**0.5**
Toluidine blue dye (Lipshaw MFG. Co, Detroit, MI, USA)	**5**
Water	**0.5**
95% Ethanol	**2 x 2**
100% Ethanol	**2 x 4**
Air drying	**3**
Xylene	**2**

3.3 Glycosaminoglycan assay

Cartilage samples, previously stored in a freezer submerged in RNAlater® were cleaned in PSB and transferred into eppendorf tubes with an addition of 0.5 ml of a papain extraction reagent (PER) and allowed to dissolve in a waterbath at 65˚C for 7 hours. The ingredients of the PER are listed in 5. The spent medium samples taken on day 31 had also been stored frozen at -80˚C and were thawed for the assay.

Table 5: Ingredients of papain extraction reagent. *(Carl Roth GmbH Co., Karlsruhe, Germany), **(Sigma-Aldrich, St. Louis, MO, USA)

Ingredients of papain extraction reagent	Amount
0.2 M sodium phosphate buffer pH 6.4 *	**100 ml**
0.1 M sodium acetate **	**0.82 g**
0.001 M Na_2EDTA **	**0.37 g**
0.005 M Cysteine HCl **	**80 mg**
crystallized papain suspension **	**80 µl**

All samples, standard and blanks were run in duplicates and a Blyscan kit (Biocolor Ltd., Carrickfergus, UK), containing all required ingredients, was applied. Deionised water and PER were

used as blanks and aliquots containing 1, 2, 3, 4 and 5 µg of the reference standard were prepared with an addition of each blank, totalling 100 µl respectively. The standards were used to prepare a calibration curve. A slope equation for the calibration curve was generated, converted and used to calculate the glycosaminoglycan content of the samples.

Aliquots of the dissolved cartilage samples containing 80µl with the addition of buffer to make up a total of 100 µl were put in eppendorf tubes. Aliquots of the spent medium samples of 50 µl with an additional buffer to make up a total of 100 µl were also put in eppendorf tubes. 1 ml of dye reagent was added to each tube and mixed well for 30 minutes. Tubes were subsequently spun at 12,000 rpm for 10 minutes in a microcentrifuge. The unbound dye was drained by carefully inverting the tubes. Dissociation reagent was added to each tube (0.5 ml) and the bound dye released using a vortex mixer. The bound dye was allowed to dissolve for 10 minutes before transferring 200 µl of each sample to individual wells of a 96 micro well plate (Appendix I). The microplate reader was set to 656 nm and absorbance measured against water for the reagent blanks, standards and test samples.

3.4 Quantitative PCR

Real time quantitative polymerase chain reaction (qPCR) is used to analyse gene expression in an affordable, reliable and easy way. It's a recent modification to PCR which was first introduced in 1992. qPCR provides a precise quantification of a specific nucleic acid in a complex mixture even when the starting amount of the mixture is at a very low concentration. The technique involves the use of fluorescent technology for monitoring the amplification of a target sequence in real time. The time it takes the amplified target to reach a threshold detection level relates with the amount of starting material present. It has been used for genotyping, quantifying viral load in patients, assessing gene copy numbers in cancer patients and most commonly to study gene expression levels by coupling it with reverse transcription (63).

In order to analyse the mRNA content, the cartilage pellets needed breaking down, a process that took place in a MP™ FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, CA, USA). Cartilage pellets previously cultured at the blood bank and frozen at -80˚C in RNAlater® were thawed and centrifuged for 10 minutes at 13200 rmp. RNAlater® was removed and 300 µl of lysis buffer (Qiagen, N.V. Venlo, Netherlands). added before transferring into a tube and placing into the homogenizer for 40 seconds at 4 m/sec. Tubes were finally centrifuged at 12000 rpm for 1 minute and 200 µl of supernatant subsequently placed into an eppendorf tube and centrifuged again at 12000 rpm for 10 minutes.

Supernatant was removed and 300 µl of lysis buffer added before placing into the RNA isolation BioRobot (Qiagen) in the sample tubes of an EZ-1 RNA Cell Mini Kit (Qiagen) The kit included a reagent cartridge, disposable tip holders, disposable filter tips, 2 ml sample tubes, 1.5 ml elution tubes, RLT buffer, RNase free DNase and RNase free water. The BioRobot process was as followed: EZ-1 RNA Cell Mini Kit products were placed in appropriate slot of BioRobot; the reagent cartridge containing magnetic beads was shaken; Puncture was made on an empty reagent cartridge and 10 µl of DNase added; Elution volume set at 50 µl and process initiated.

A High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster city, CA, USA) was used to synthesize cDNA from mRNA. All work took place on ice, keeping tubes submerged under ice between procedures. Once prepared, the solution (Table 6) was centrifuged and placed in thermal cycler set to the four following steps. Firstly, 10 min at 25˚C, secondly, 120 min at 37˚C, thirdly, 5 sec at 85˚C and finally, 4˚C until the mixture was removed and placed in -18˚C storage.

Table 6: Materials and quantities for cDNA synthesis solution

Materials	Volume (µl)
Nuclease free water	**3.2**
RT Buffer x 10	**2**
dNTP x 25 (100 mM)	**0.8**
RT random primers	**2**
RNase inhibitor	**1**
Multiscribe RTase	**1**
RNA	**10**

The gene expression of ACAN, Col1A2, Col2A1 and GAPDH (Table 7) was investigated by a real time quantitative PCR, performed in a 7500 Real Time PCR System from Applied Biosystems. A 48 microwell plate was prepared, inserting 10 µl Taqman master mix (Applied Biosystems), including 1 µl of the respective Taqman assay (Applied Biosystems) for each sample and 9 µl of 1:10 diluted cDNA samples. The microwell plate was sealed with adhesive foil and centrifuged at 1500 rpm for 2 min before commencing the assay. GAPDH was used as a reference gene to correct differences in gene expression and cDNA amount and all data were analysed using a $\Delta\Delta C_T$ method and presented as fold change compared to the pellets generated after being cultured in a 10% FBS supplemented treatment medium.

Table 7: List of primers used for qPCR

Protein	Target gene symbol	Primer for RT-PCR (TaqMan assay no., Applied Biosystems)
Aggrecan	**ACAN**	**Hs00153936_m1**
Type I Collagen	**Col1A2**	**Hs01028970_m1**
Type II Collagen	**Col2A1**	**Hs00156568_m1**

4 Results

To examine the effect of expanding MSCs in the three treatment mediums on the chondrogenic differentiation, three assays were performed. In addition we assessed the MSCs during the expansion stage, revealing that MSCs expanded in HPL supplemented treatment medium were much faster to proliferate than the MSCs cultured in FBS supplemented treatment medium. The three assays performed were the staining, GAG assay and qPCR. In short, the results from the staining processes revealed that pellets generated from HPLF cultured MSCs resulted in the more desirable hyaline like cartilage. The GAG results showed little difference between treatment mediums but again the HPLF cultured MSCs measured with the highest GAG content per pellet after 31 days of culture. The qPCR results did not back up the histochemistry and the GAG assay, but instead revealed that the MSCs expanded in the HPLO treatment medium had much greater expression of the desired Col2A1 gene. This indicates a possible error in the qPCR assay which would have to be repeated for confirmation.

4.1 Phenotype examination

Firstly, the MSCs were visually analysed during expansion face using the MSCGM media. This was done to confirm that the cells had the typical phenotype of MSC, spindle lime fibroblastic appearance that adhere to plastic. These cell lines had previously been analyzed for the expression and lack of expression of markers that are commonly used to define MSC as a population.

The visual examination confirmed a plastic adherent cellular population with a small spindle shaped appearance (Figure 6).

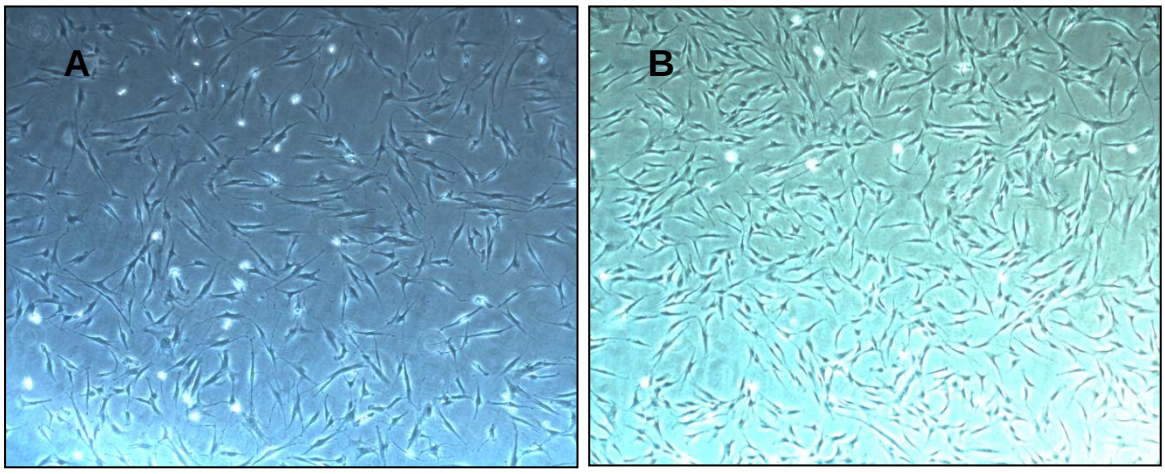

Figure 6: Microscopic examination performed in an inverted microscope (Leica Microsystems GmbH, Wetzlar, Germany) at 50x magnification displaying spindle shaped, plastic adherent MSCs expanded in MSCGM. A) Day one of cellular expansion displaying MSCs at approximately 20% confluence. B) Day two of cellular expansion displaying MSCs at approximately 40% confluence.

During cellular expansion in MSCGM and once the cells had been seeded in the 10%FBS, 10%HPLF and 10%HPLO supplemented treatment mediums, a visual examination was performed to assess confluence. Cells were passaged once they reached 90% confluence in order to prevent cell death or

spontaneous differentiation. It was observed during this assessment that cells cultured supplemented with 10%HPLF and 10% HPLO treatment mediums reached the 90% confluence stage on day 4 whereas cells cultured in 10%FBS supplemented treatment medium reached that stage on day 7. An expansion spurt was particularly noticeable a day after the treatment medium had been replaced (Figure 7). In short, the MSC proliferation was accelerated in the HPL supplemented treatment mediums compared with the FBS supplemented treatment mediums.

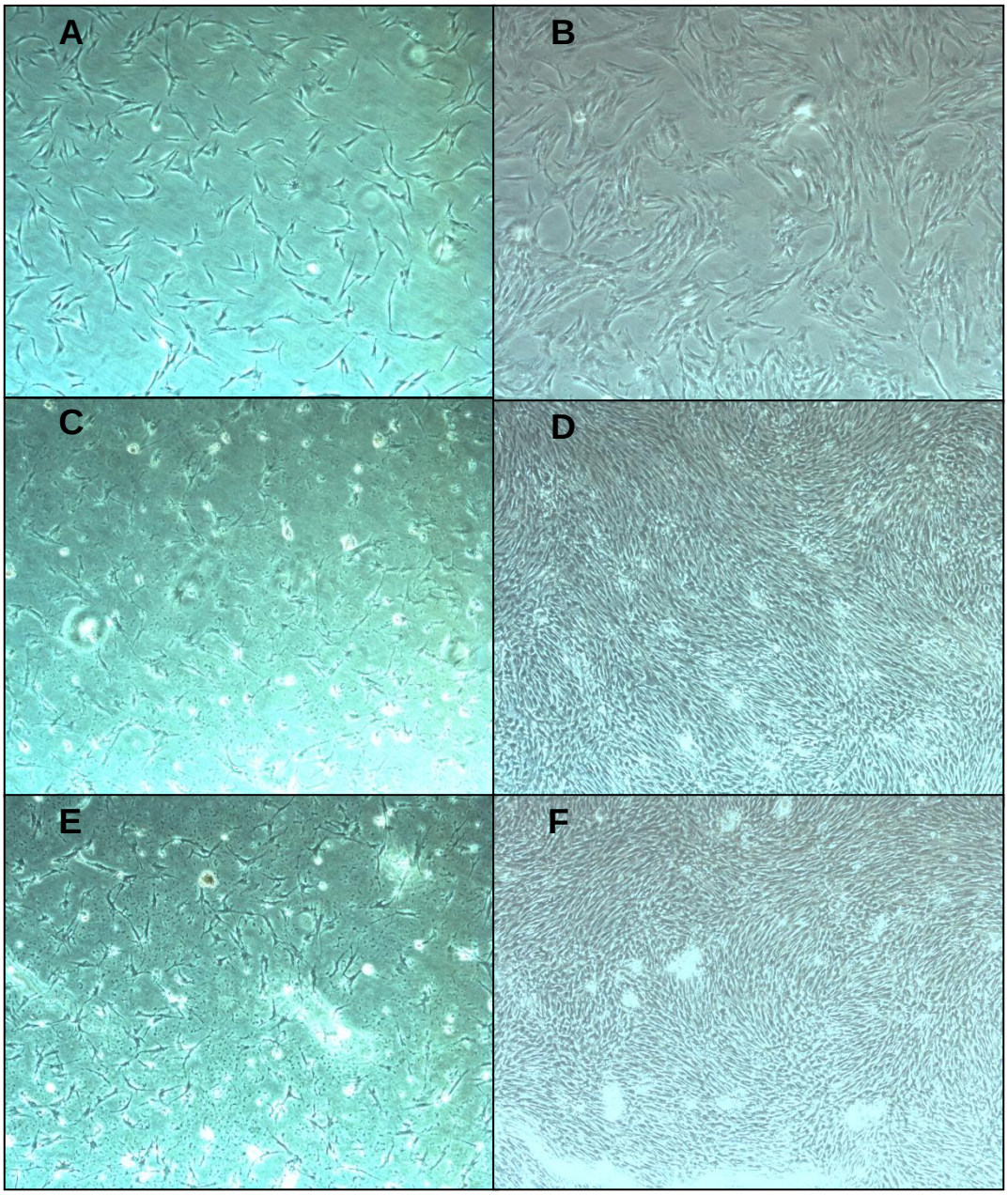

Figure 7: Microscopic examination performed in an inverted microscope at 50x magnification A) and B) MSC expansion in 10% FBS supplemented treatment medium on day three and four respectively. C) and D) MSC expansion in 10% HPLF supplemented treatment medium on day three and four respectively. E) and F) MSC expansion in 10% HPLO supplemented treatment medium on day three and four respectively.

4.2 Histological assessment

Histological assessment involved sectioning the cultured pellets to examine whether chondrogenesis had successfully produced a hyaline like cartilage. Cellular morphology and extracellular matrix structure were assessed by applying appropriate staining methods. All images were captured on an Olympus System Microscope model BX51 (Olympus Systems, Tokyo, Japan) and generated in Cell* imaging software (Olympus Soft Imaging Solutions GmBH, Münster, Germany) where the scale bars were added. The background contrast was adjusted in Photoshop CS5 (Adobe Systems Inc., Mountain View, CA, USA) and a table generated in Illustrator CS5 (Adobe Systems Inc.).

4.2.1 Haematoxylin and Eosin stain

Haematoxylin and Eosin (HE) staining is a commonly used staining method for nuclear and cytoplasmic staining. The Haematoxylin stains for the nuclei and eosin for the cytoplasm. The slides stained with HE staining method were microscopically examined and photographed at 100x and 200x magnification. The pellet culture harvested on day 14 (Figure 8) displays a more compact cellular association when cultured in 10% FBS supplemented treatment medium compared to the other mediums. Furthermore, cells cultured in 10% FBS supplemented treatment medium display elongated morphology at the edge of the pellet a feature that may also be observed to a lesser extent on the 10% HPLO supplemented treatment medium cultured pellet but not the 10% HPLF supplemented treatment medium pellet.

HE staining of pellets harvested on day 31 showed a more compacted cellular structure in pellets from the 10% FBS supplemented treatment medium and 10% HPLF supplemented treatment medium but a less compact cellular structure in pellets from 10% HPLO supplemented treatment medium when compared to the pellets harvested on day 14. Comparison between treatment mediums on day 31 showed that the 10% HPLF supplemented treatment medium had elongated cells on the pellet edge a feature that can be observed to a lesser extent on the 10% HPLO supplemented treatment medium pellet and even less on 10% FBS supplemented treatment medium. A sparse cellular density can be observed in HPLO pellet compared to the other two and a very compacted cellular density can be observed in the centre of the 10% HPLF supplemented treatment medium pellet (Figure 9).

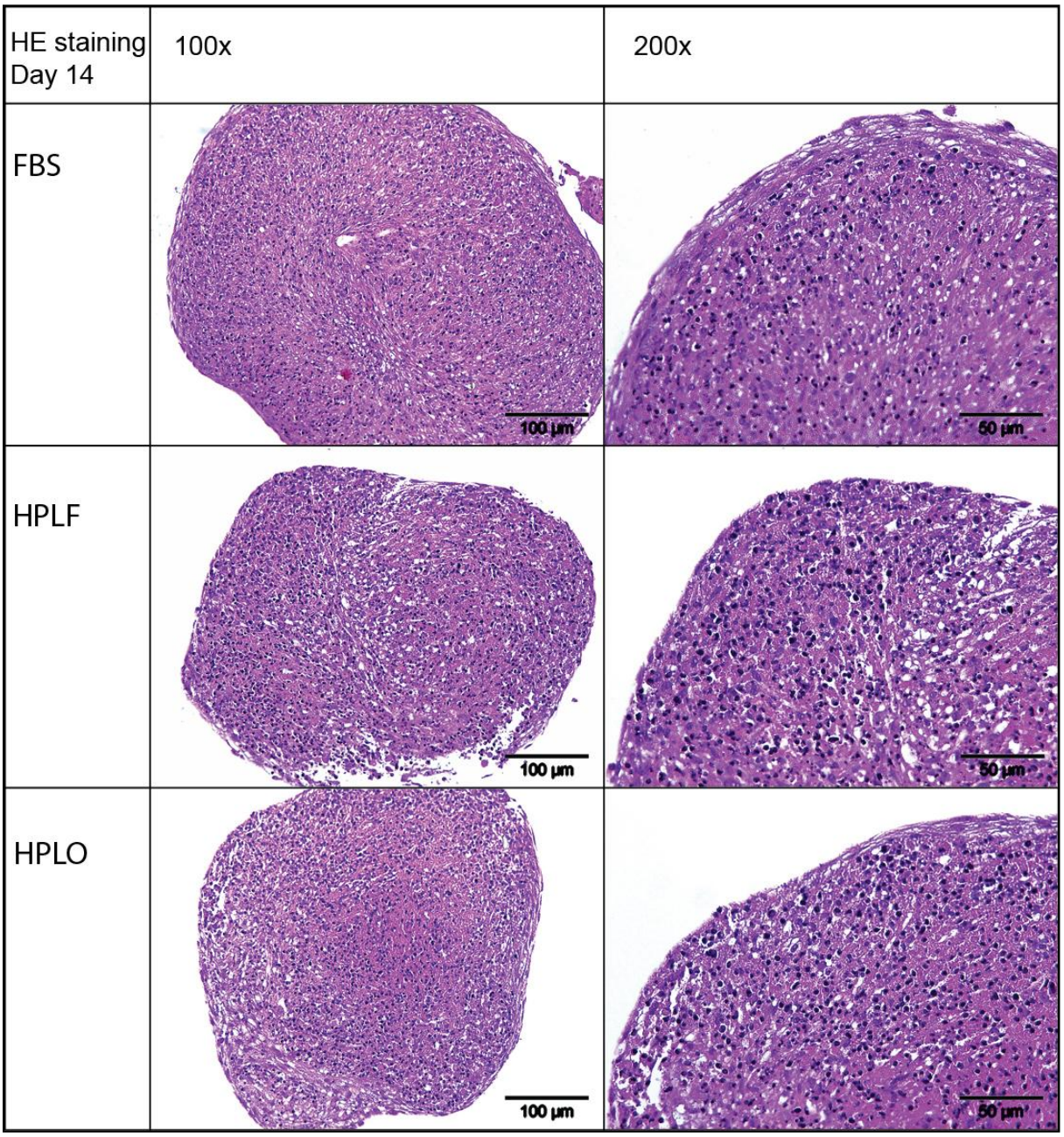

Figure 8: Transverse sections of MSC differentiated chondrogenic pellets stained with haemotoxilyn and eosin. The MSCs were expanded in treatment mediums, displayed to the left of the images. Harvesting took place on day 14 and the images were taken at 100x and 200x magnification. Scale bars display 100µm and 50 µm respectively.

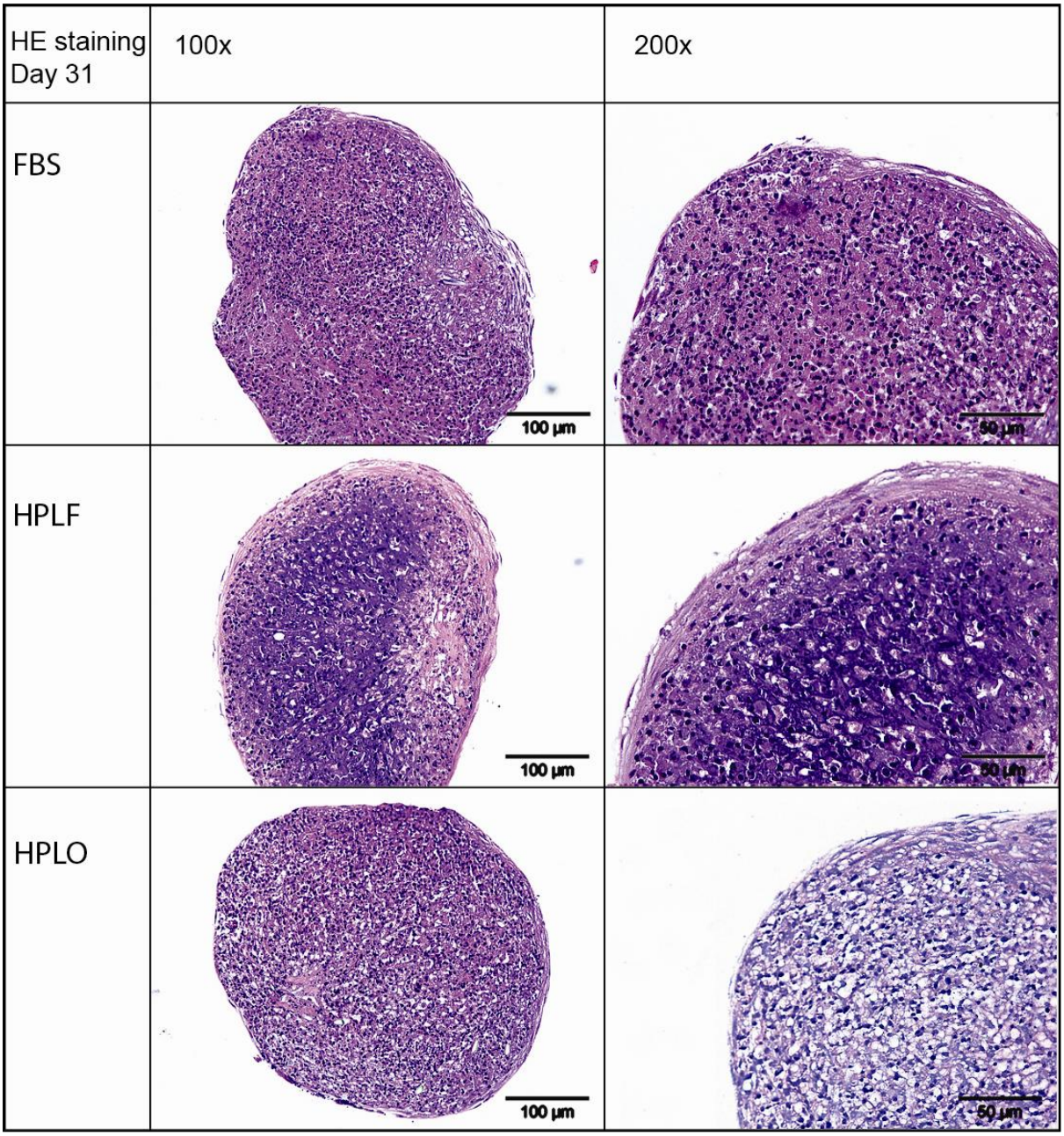

Figure 9: Transverse sections of MSC differentiated chondrogenic pellets stained with haemotoxilyn and eosin. The MSCs were expanded in treatment mediums, displayed to the left of the images. Harvesting took place on day 31 and the images were taken at 100x and 200x magnification. Scale bars display 100µm and 50 µm respectively.

4.2.2 Toluidine blue stain

Toluidine blue (TB) staining is most commonly used to analyse mast cells but may also be used to investigate cartilage tissue in terms of glycosaminoglycan and proteoglycan content, where the faded pink colour is and indicator of GAG and proteoglycan in the extracellular matrix.

The Toluidine blue staining performed on pellet culture harvested on day 14 revealed that the extracellular matrix production was greater in pellets previously cultured in 10% FBS supplemented treatment medium, compared with the other two treatment mediums (Figure 10). Toluidine blue staining was likewise performed on pellets harvested on day 31. The microscopic examination revealed that the pellets previously cultured in treatment medium supplemented with 10% HPLF had a densely packed glycosaminoglycan/proteoglycan area in the centre of the pellet whereas the both the FBS and HPLO supplemented treatment medium pellets had a more sparsely distributed area of GAG/proteoglycan content by the edge of the pellet (Figure 11).

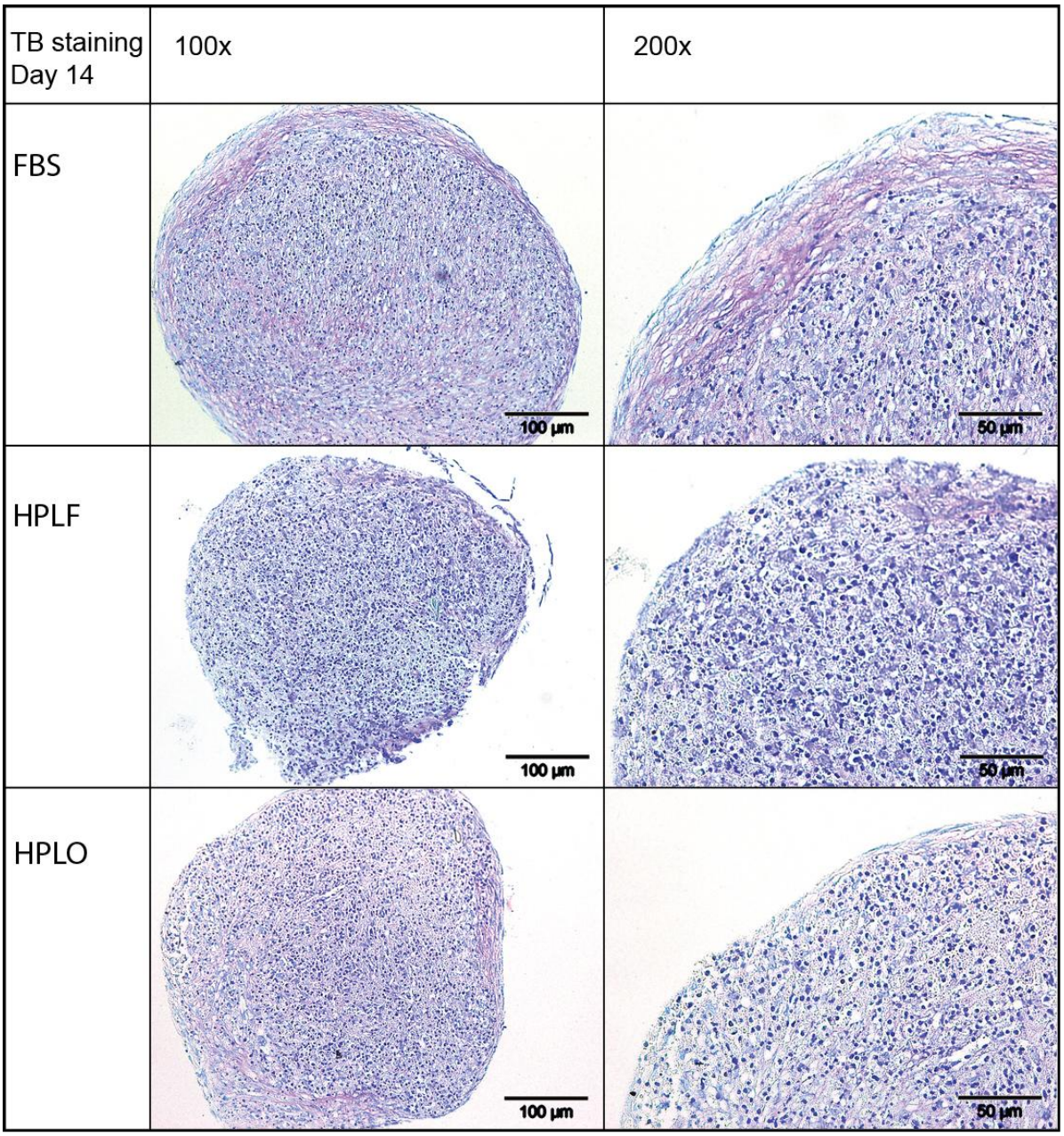

Figure 10: Transverse sections of MSC differentiated chondrogenic pellets stained with toluidine blue. The MSCs were expanded in treatment mediums, displayed to the left of the images. Harvesting took place on day 14 and the images were taken at 100x and 200x magnification. Scale bars display 100µm and 50 µm respectively.

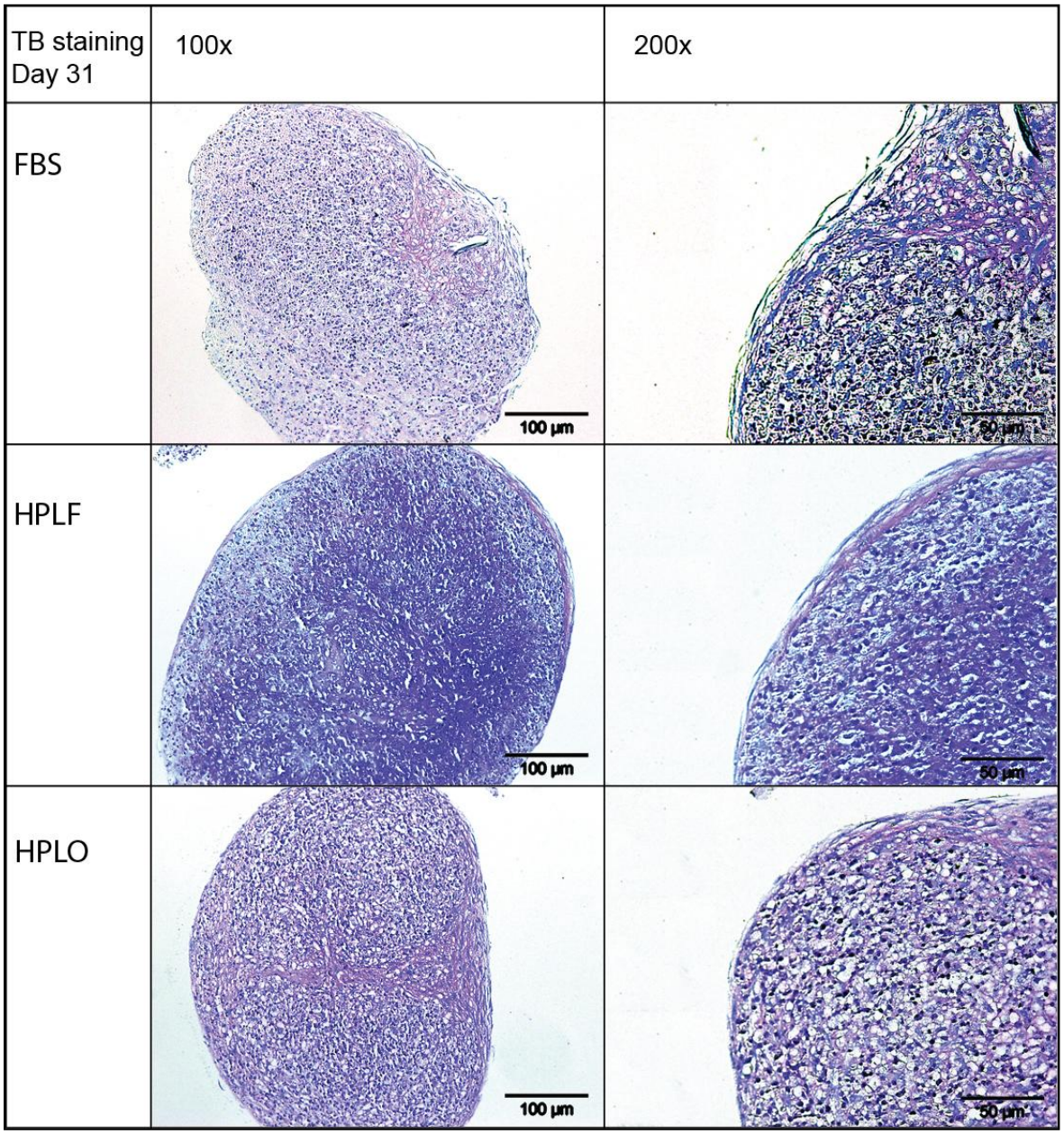

Figure 11: Transverse sections of MSC differentiated chondrogenic pellets stained with toluidine blue. The MSCs were expanded in treatment mediums, displayed to the left of the images. Harvesting took place on day 31 and the images were taken at 100x and 200x magnification. Scale bars display 100µm and 50 µm respectively.

4.3 Glycosaminoglycan content

The glycosaminoglycan content of the pellet culture was measured after 14 and 31 days of chondrogenisis. A sample of spent media, taken on day 31 of the chondrogenic pellet culture was also tested for glycosaminoclycan content. A Blyscan™ Sulfated Glycosaminoglycan Assay Kit (Biocolor) was purchased and manufacturer's instructions followed. The Blyscan assay is a quantitative dye binding method for the analysis of glycosaminoglycans (GAGs). The assay involves the use of dye label 1,9 dimethylmethylane blue which under certain conditions, accomplished during the assay (chapter 3.3), will bind to the sulphated polysaccharide component of proteoglycans or the protein free sulphated glycosaminoglycan chains. The absorbance can then be measured and compared to a standard curve, generated from a known GAG content in the reference standard and GAG content calculated based on that (64).

4.3.1 Pellet cultures

The glycosaminoglycan content in the papain buffer digested cartilage samples was measured by a colorimetric method with1,9 dimethylmethylene blue dye (Biocolor™). The measured absorption was used to read the GAG content in µg from a standard curve. The standard curve was generated using the known GAG content of the blyscan reference standard (Biocolor™). Two standard curves were generated, one for deionised water and another one for the PER buffer. Both the PER buffer and the deionised water generated similar curves (Appendix II).

Once the standard curves were generated, the GAG content for all pellet cultures from day 14 and 31 was read from the standard curves and presented as fold change setting FBS as a reference treatment medium. Figure 12 displays the results of the pellet culture harvested on day 14. The GAG content from pellet cultured in FBS treatment medium is slightly higher than the other two treatment mediums. A similar fold change plot was drawn for pellet culture harvested on day 31 (Figure 12), this time displaying the opposite results whereas the FBS treatment medium cultured pellet had less GAG content than the other two, out of which HPLF cultured pellets had the highest GAG content.

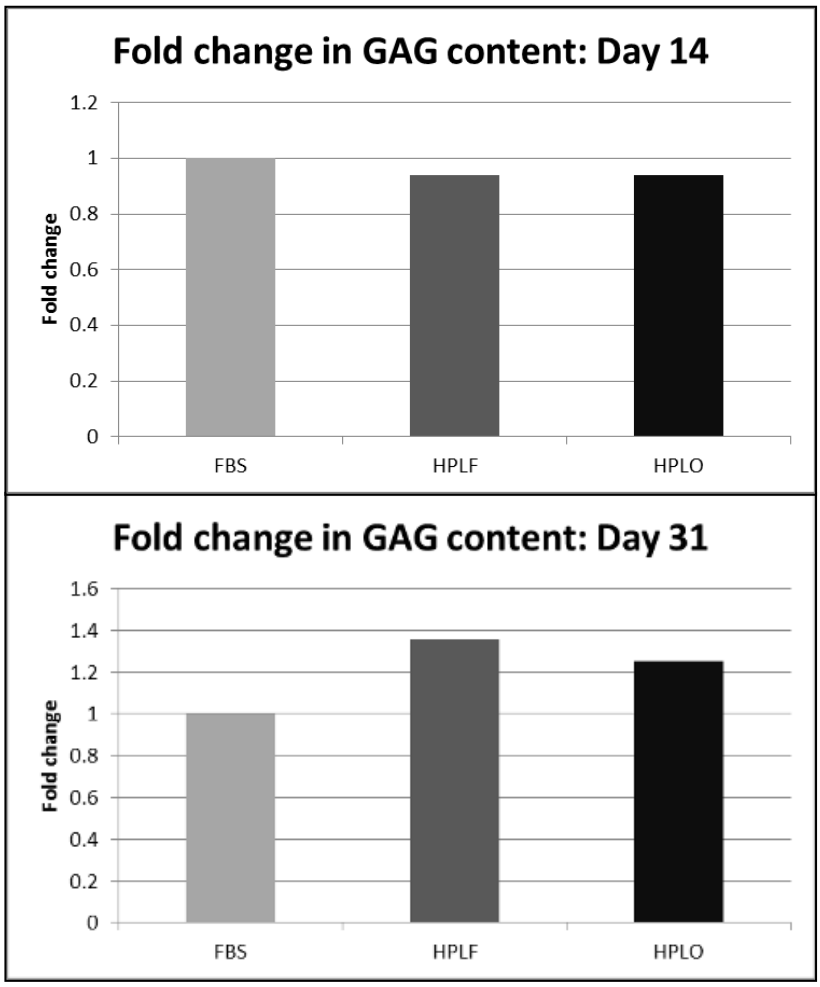

Figure 12: Fold change in GAG content on days 14 and 31 of pellet culture. Absorbance measurement of the test samples allowed for the GAG content to be read from the reference standard curve and the GAG content per pellet was calculated (µg GAG/pellet) and presented as a fold change, assigning 1 to the 10% FBS supplemented treatment medium expanded MSCs.

4.3.2 Spent media

The spent media from the pellet culture was collected on day 31 and frozen at -80˚C. The GAG content in the spent media was measured as per chapter 3.3. The gag content was calculated based on the standard curve displayed in appendix II. As table 8 shows, the sGAG content of the spent media came under the detection limit for all treatment mediums.

Table 8: Calculated GAG content of the spent medium samples collected on day 31.

Medium	Mean absorbance - blank	Calculated GAG µg
FBS	**0.085049**	**-0.43662**
HPLF	**0.201718**	**-0.02231**
HPLO	**0.138846**	**-0.24558**

4.4 Quantitative PCR

The real time qPCR analysis results were presented as a fold change calculated based on the cycle time of the target gene as shown in table 9.

Table 9: Fold change results for ACAN, Col1A2 and Col2A1 for the culture lysates.

Treatment Medium	ACAN	Col1A2	Col2A1
FBS	**1**	**1**	**1**
HPLF	**1.017955**	**0.952902**	**0.738155**
HPLO	**1.082923**	**0.792689**	**14.66027**

The results in table 10 were visually displayed in figure 13. The gene expression of ACAN does not significantly vary between the three treatment mediums, showing only a slight increase in gene expression from FBS to HPLO (Figure 13). Likewise there is no significant difference in the expression of the Col1A2 gene with only a slight decrease from FBS to HPLO (Figure 13). The Col2A1 on the other hand shows a significant increase in gene expression the case of HPLO which is more than 14 fold that of FBS (Figure 13).

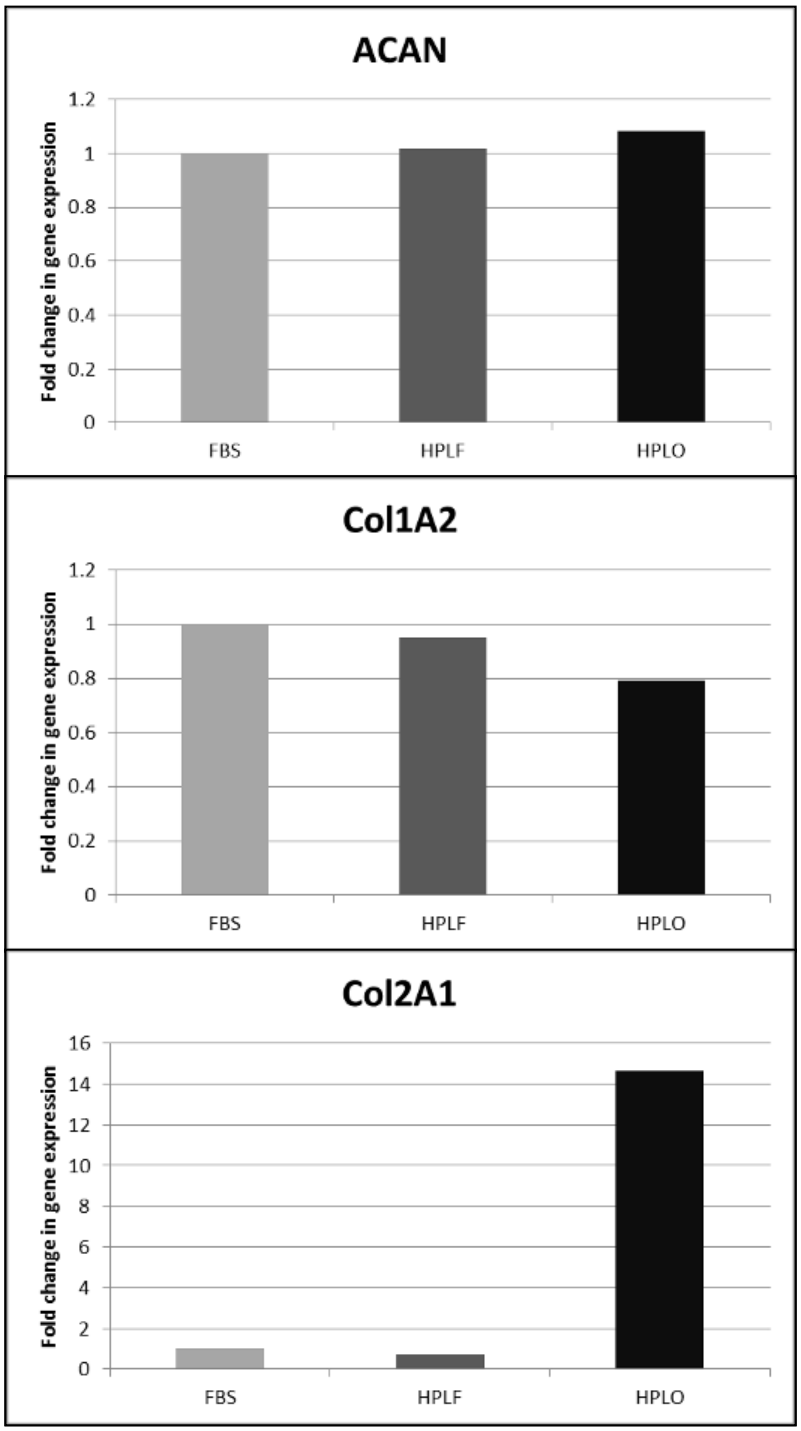

Figure 13: Fold change in gene expression for the ACAN, Col1A2 and Col2A1 genes in MSC pellet cultures harvested on day 28 and previously expanded in all three treatment mediums. Fold change was determined using real time qPCR and normalized by the expression of the reference gene GAPDH. The fold change of gene expression refers to increase/decrease of gene expression relative to that of pellets generated from MSC culture, expanded in 10% FBS supplemented treatment medium.

Discussion

With this study we aimed to analyse weather expansion of MSCs in 10% supplemented HPLO treatment medium would influence the chondrogenic differentiation of MSCs compared with a treatment medium supplemented with 10% HPLF. Our findings indicate that the use of 10% HPLF supplemented treatment medium is preferable in order to obtain the desired hyaline like cartilage. Histochemistry assays showed increased production of extracellular matrix in 10% HPLF supplemented treatment medium compared to the other two treatment mediums and the GAG content analysis showed consistent results to the histochemistry assay, where the 10% HPLF supplemented treatment medium contained more GAG on day 31 of pellet culture compared to the other two. The qPCR results were not consistent with the other two, revealing a vast increase in Col2A1 gene expression which indicates a possible error and will therefore have to be repeated.

We assessed the doubling time of the MSC expansion in the different treatment mediums and found that in agreement with other studies, the doubling time for MSCs cultured in HPL was significantly faster than that of MSCs cultured in FBS (65). Furthermore, we analysed the morphology in terms of cellular arrangement as well as the extracellular matrix components of a pellet culture generated after a MSC expansion in all three treatment mediums. In the case of both pellet cultures, harvested on days 14 and 31, the MCSs maintained their chondrogenic differentiation properties in the HPL supplemented treatment mediums in comparison to the FBS supplemented treatment medium, in agreement with other studies (66). In addition to this the pellet cultures harvested on day 31 seemed to have produced more extracellular matrix in the HPL supplemented mediums and in particular the HPLF supplemented medium which may indicate that the growth factors present in platelet lysates play an active role in the chondrogenic differentiation, a property which does not seem to be occurring in the HPLO supplemented medium. This difference could indicate that the expired HPL has lost some of the components that the fresh HPL possesses.

Glycosaminoglycan assay indicates that expanding MSC in media with different supplements does not influence the ability of the cells for chondrogenic differentiation. There were some indications that cells expanded in media containing 10% HPLF and HPLO may have higher GAG content after 31 days in culture; however this was not as apparent in the touidine blue staining. The likely difference in results may lie in the few number of experiments done and more experiments may lead to different results.

We assessed the extracellular matrix protein glycosaminoglycan (GAG), which is an important protein, involved in providing the cartilage with its ability to resist compressive force and stress. Our results showed that the GAG content in pellet culture harvested on day 14 was greater in the 10% FBS supplemented treatment medium compared with the other two, although the difference was very small. This was not the case for pellets harvested after 31 days as the GAG content was greater in pellets generated from MSCs, previously expanded in 10% HPLF treatment medium compared to 10% FBS and the GAG content in pellets from 10% HPLO measured halfway between the other two. This difference between the days the pellets were harvested is also coherent with the toluidine blue stained

cartilage pellets where the extracellular matrix production seems to increase more in HPLF than in the other two treatment mediums.

The qPCR was lastly performed to quantify the gene expression of two genes responsible for generating proteins (ACAN and Col2A1) essential to the integrity and structure of the cartilages extracellular matrix and one gene that indicates the formation of fibrous cartilage (Col1A2). The housekeeping gene GAPDH, was also measured for gene expression and used as a reference gene. Results showed a 14 fold increase in the expression of Col2A1, which is the gene that generates the desired Collagen II protein that is integral to the production of healthy hyaline like cartilage, in pellets generated from MSCs expanded in the 10% HPLO supplemented treatment medium compared to the 10% FBS supplemented treatment medium. This is most likely due to an error in the qPRC assay and the assay would have to be repeated at a later stage to confirm. There was no significant difference between any of the other measured gene expressions.

In agreement with previous studies, our results show that the use of HPL supplemented treatment medium, can in fact replace the previously used FBS and therefore increase the growth rate and prevent the adverse effect an animal serum can have on clinically applied stem cell transplants (65).

We have not demonstrated an advantage of using the HPLO supplemented treatment medium in comparison to the HPLF supplemented treatment medium and in fact the opposite seems to be the case when we look at the histochemistry results, where the HPLF supplemented treatment medium has produced a more desirable outcome. More research is needed though, as this could be caused by a number of variants such as donor variation.

The benefits of using treatment mediums supplemented with HPL in tissue engineering and regenerative medicine are indisputable as they eliminate the adverse effect of using an animal serum. The use of treatment mediums supplemented with expired HPL would be of further benefit as that could put to use some of the platelet rich plasma that are discarded of in blood banks around the world due to their limited shelf life (67). This study does not provide a definitive answer to weather the expired platelets can be used for this purpose. Future studies could take a closer look at this by setting up a similar research with increased statistical power acquired by the involvement of more MSC donors and possibly more units of platelet rich plasma.

Strengths and weaknesses

When it comes to researching somatic stem cells, appreciation of donor variation is essential. In this study we work with mesenchymal stem cells from two donors. One for the expansion process, pellet culture, staining and GAG content colorimetric assay, and a second one for the qPCR in which case we used previously cultured pellets from the blood bank. This weakens the comparison between the different assays, something that could later be approved by applying all assays on pellets, cultured from MSCs of one donor. Having said that, it would also be beneficial to work with many donors, where all donors could be assayed for the same components to generate more power for statistical analysis as well as the possibility for a comparison between the donors. This is restricted by factors

such as cost, and the ethical dilemma of performing bone marrow biopsies on healthy donors for stem cell research.

Another potential weakness is limited number of MSCs that can be generated by *in vitro* expansion. This is caused by the increased risk of infection in a prolonged expansion and also due to the fact that MSCs eventually lose their differentiation and proliferation potential (68). As well as limiting the number of MSCs generated, this also limits the number of differentiation conditions that can be set up *in vitro* and therefore the amount of different assays that can be performed on the final MSC product. Ideally one would expand MSCs from various donors and assay these cells in as many previously developed assays as possible, in one research using consistent protocols to provide a statistically powerful study, comparing the potential of MSCs from different donors in various differentiation circumstances.

The choice of reference gene for qPCR is another possible weakness in this study as some studies have suggested that the GAPDH housekeeping gene may not be the optimal reference gene. The RNA polymerase II reference gene has been suggested as a more accurate alternative by some (69) whilst others report that TBP, RPL13A and B2M display more accurate results (70). However, the use of GAPDH will perhaps put our study in a better context with previous studies since that is the traditional choice of reference gene.

Conclusion

This study has revealed that mesenchymal stem cells, expanded in HPL, maintain their chondrogenic differentiation properties, compared with those expanded in FBS. Furthermore, histochemistry reveals a slight advantage in terms of the production extracellular matrix components in the HPLF compared to the other two treatment mediums. This was backed up by the GAG results although the difference there was only slight, but not the qPCR results which contained a possible error and would therefore have to be repeated. Furthermore, expanding the MSCs in HPL shortens the culture time by increasing their growth rate. Finally we demonstrate that the expired platelet rich plasma cultured MSCs resulted in a poorer outcome after chondrogenisis was induced than the MSCs cultured in the fresh platelet rich plasma.

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Appendix I

Table 10: The distribution of standards, test samples and blanks in a 96 microwell plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Blank Water**	**Blank Water**	**Blank Buffer**	**Blank Buffer**	**80 µl HPLF14 20 µl Water**	**80 µl HPLF14 20 µl Water**	**80 µl HPLF14 20 µl Buffer**	**80 µl HPLF14 20 µl Buffer**	**50 µl HPLFS 50 µl Water**	**50 µl HPLFS 50 µl Water**	**50 µl HPLFS 50 µl Buffer**	**50 µl HPLFS 50 µl Buffer**
B	**10 µl Standard 90 µl Water**	**10 µl Standard 90 µl Water**	**10 µl Standard 90 µl Buffer**	**10 µl Standard 90 µl Buffer**	**80 µl HPLF31 20 µl Water**	**80 µl HPLF31 20 µl Water**	**80 µl HPLF31 20 µl Buffer**	**80 µl HPLF31 20 µl Buffer**	**50 µl HPLOS 50 µl Water**	**50 µl HPLOS 50 µl Water**	**50 µl HPLOS 50 µl Buffer**	**50 µl HPLOS 50 µl Buffer**
C	**20 µl Standard 80 µl Water**	**20 µl Standard 80 µl Water**	**20 µl Standard 80 µl Buffer**	**20 µl Standard 80 µl Buffer**	**80 µl HPLO14 20 µl Water**	**80 µl HPLO14 20 µl Water**	**80 µl HPLO14 20 µl Buffer**	**80 µl HPLO14 20 µl Buffer**	**50 µl FBSS 50 µl Water**	**50 µl FBSS 50 µl Water**	**50 µl FBSS 50 µl Buffer**	**50 µl FBSS 50 µl Buffer**
D	**30 µl Standard 70 µl Water**	**30 µl Standard 70 µl Water**	**30 µl Standard 70 µl Buffer**	**30 µl Standard 70 µl Buffer**	**80 µl HPLO31 20 µl Water**	**80 µl HPLO31 20 µl Water**	**80 µl HPLO31 20 µl Buffer**	**80 µl HPLO31 20 µl Buffer**				
E	**40 µl Standard 60 µl Water**	**40 µl Standard 60 µl Water**	**40 µl Standard 60 µl Buffer**	**40 µl Standard 60 µl Buffer**	**80 µl FBS1420 µl Water**	**80 µl FBS1420 µl Water**	**80 µl FBS1420 µl Buffer**	**80 µl FBS1420 µl Buffer**				
F	**50 µl Standard 50 µl Water**	**50 µl Standard 50 µl Water**	**50 µl Standard 50 µl Buffer**	**50 µl Standard 50 µl Buffer**	**80 µl FBS3120 µl Water**	**80 µl FBS3120 µl Water**	**80 µl FBS3120 µl Buffer**	**80 µl FBS3120 µl Buffer**				

Table 11: Measured absorbance for the blank, reference standard and digested pellet samples

	1	2	3	4	5	6	7	8
A	**0.1038**	**0.1034**	**0.0959**	**0.0997**	**1.5829**	**1.6143**	**1.7633**	**1.8323**
B	**0.5697**	**0.5779**	**0.5740**	**0.5813**	**2.1268**	**2.2017**	**2.3183**	**2.3781**
C	**0.8233**	**0.8078**	**0.8425**	**0.8575**	**1.5847**	**1.6572**	**1.8159**	**1.8539**
D	**1.0795**	**1.0737**	**1.1660**	**1.1744**	**1.9854**	**2.0657**	**2.2755**	**2.3512**
E	**1.3248**	**1.4009**	**1.4476**	**1.4240**	**1.6644**	**1.7291**	**1.8304**	**1.8865**
F	**1.5705**	**1.5051**	**1.6576**	**1.6402**	**1.6492**	**1.6630**	**1.8349**	**1.8726**

Table 12: Measured absorbance for spent media samples

	9	10	11	12
A	**0.3006**	**0.3105**	**0.2732**	**0.2826**
B	**0.2401**	**0.2452**	**0.2464**	**0.2535**
C	**0.1884**	**0.1893**	**0.2048**	**0.2096**

Appendix II

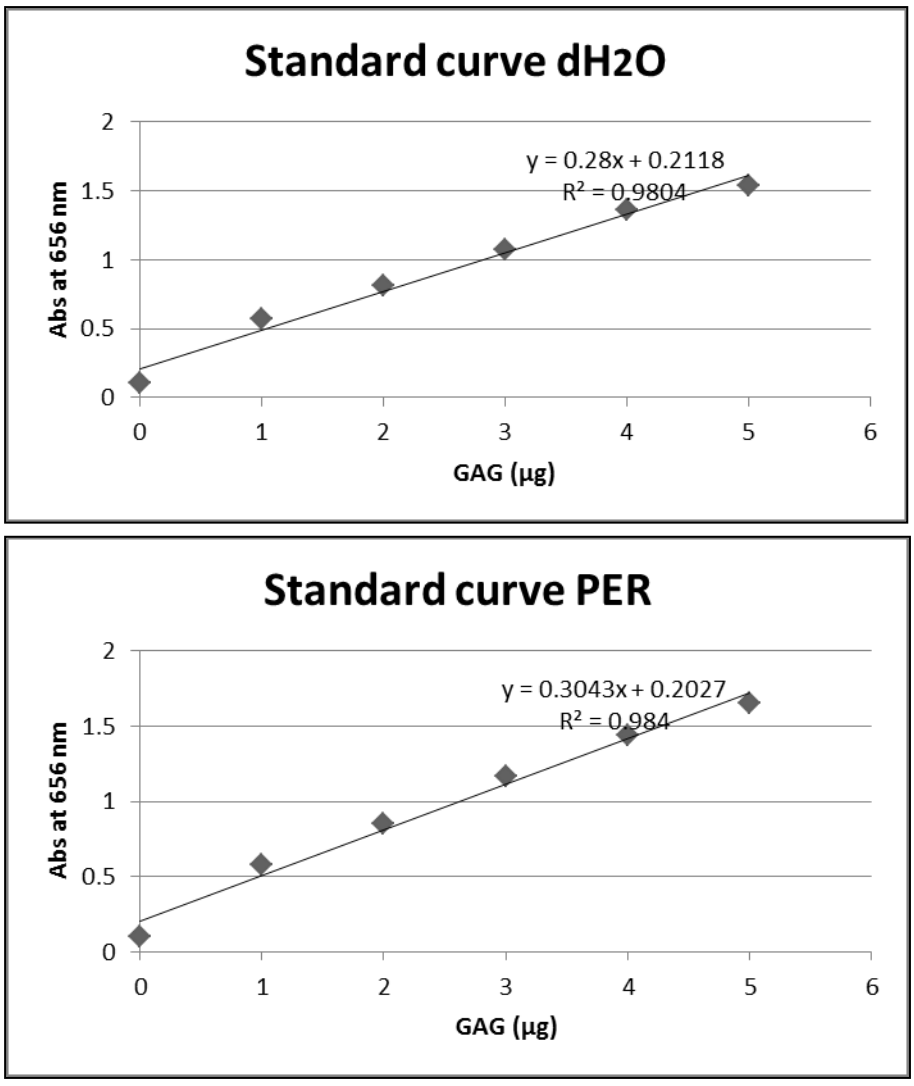

Figure 14: Standard curves, generated from the test samples formulated using the blyscan reference standard with known GAG content and measured for absorbance ad 656 nm