The effects of outdated human platelet lysate on cartilage differentiation in mesenchymal stem cells and human embryonic derived mesenchymal progenitor cells

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HÁSKÓLI ÍSLANDS

Áhrif lýsata, unnin úr útrunnum blóðflögueiningum á brjósksérhæfingu mesenchymal stofnfrumna og mennskra fósturstofnfrumna sem eru sérhæfðar í mesenchymal stofnfrumur

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Ágrip

Brjósk er teygjanlegur bandvefur sem finnst á ýmsum stöðum í mannslíkamanum, til að mynda í liðum, eyrum, nefi, lungnapípum, hryggþófa og í brjóstkassa. Brjóskvefurinn er án æðakerfis og samanstendur af brjóskfrumum og utanfrumuefni, sem er ríkt af collageni, proteoglycans og elastín þráðum, meðal annars. Brjóskvefurinn er án æðakerfis, sem veldur takmarkaðri hæfni vefsins til viðgerðar. Brjóskfrumur eiga rætur sínar að rekja til mesenchymal stofnfrumna (MSCs). MSCs eru fjölhæfar frumur sem geta sérhæft sig yfir í bein-, brjósk- og fitufrumur. Einnig hafa þær hæfni til ónæmismótunar og því eru bundnar miklar vonir við notkun MSCs í læknisfræðilegar meðferðir. Erfiðlega hefur gengið að nota frumurnar í rannsóknum vegna meðal annars gjafabreytileika og hversu skammlífar þær eru. Nýlega tókst að sérhæfa fósturstofnfrumur yfir í mesenchymal forverafrumur (hES-MP) og sýnt hefur verið fram á að þær hafi svipaða eiginleika og MSCs hvað varðar útlit, svipgerðartjáningu og sérhæfingargetu. Þessir eiginleikar gera hES-MP frumur að góðum kosti fyrir til að mynda rannsóknir á brjóski. Kálfasermi (FBS) er mest notaða ræktunaríbætið fyrir ræktanir á stofnfrumum. Það telst hins vegar ekki fýsilegur kostur við læknisfræðilegar meðferðir vegna smithættu og siðferðislegra vandamála varðandi velferð dýra. Blóðflögulýsöt, unnin úr útrunnum blóðflögueiningum (HPLO), gætu mögulega gegnt hlutverki kálfasermis og hefur notkun þess við ræktum MSCs reynst vel.

MSCs og hES-MP frumur voru sérhæfðar í brjóskkúlur með þremur mismunandi brjósksérhæfingarætum. Þau voru 10% FBS æti, 10% HPLO æti og æti sem innihélt ekki ræktunaríbæti. Brjóskkúlurnar voru metnar eftir 7, 14, 28 og 35 daga í sérhæfingu með GAG prófi, vefjalitunum og RT-qPCR.

Niðurstöðurnar benda til þess að bæði MSC og hES-MP brjóskkúlurnar sem sérhæfðar voru í 10% HPLO, framleiða meira utanfrumuefni en brjóskkúlurnar úr öðrum ætum. Genatjáningin sýnir hins vegar að tjáning Aggrecan og COMP var lægri í MSC brjóskkúlum sem voru sérhæfðar í 10% HPLO en í brjóskkúlum ræktaðar í hinum tveimur ætunum. Auk þess var tjáning SOX9 lægri í hES-MP brjóskkúlum eftir sérhæfingu í 10% HPLO.

Mögulega er hægt að nota útrunnin blóðflögulýsöt sem ræktunaríbæti fyrir brjósksérhæfingu hES-MP og MSC frumna. Þó er nauðsynlegt að gera fleiri rannsóknir til að rannsaka hvað veldur þessari breyttu genatjáningu í brjóskkúlum sem hafa verið sérhæfðar í HPLO.

Abstract

Cartilage is a connective tissue found in various parts of the human body including the joints, ear, nose, bronchial tubes, intervertebral disc and the rib cage. Cartilage is avascular tissue comprised of chondrocytes and extracellular matrix (ECM) proteins including collagen, fibers, proteoglycans and elastin fibers. Since cartilage tissue is avascular, it has limited repair ability and a slow growth rate leading to limited repair capabilities. Chondrocytes are derived from mesenchymal stem cells (MSCs), which are multipotent cells that can differentiate into osteocytes, adipocytes and chondrocytes. Additionally, MSCs have been shown to have trophic effect on their surroundings, anti-inflammatory effect and immunomodulatory effect. This has made MSCs an interesting candidate for use in regenerative medicine and tissue engineering. Using MSCs in experimental models has been problematic due to their limited expansion capacity and variation between donors. Recently, mesenchymal progenitors were derived from human embryonic stem cells (hES-MP). It has been shown that hES-MP cells have similar characteristics as MSCs, regarding their morphology, phenotype and differentiation potential, making hES-MP cells a good candidate for analysis of e.g. chondrocyte differentiation. Fetal bovine serum (FBS) is the most used supplementary media for stem cell expansion. However, this supplement may not be the optimal choice of cells that are to be used in regenerative medicine e.g. due to contamination risks and animal welfare ethical issues. Lysates made from outdated human platelets (HPLO) could possibly substitute FBS as a supplementary media and it has been tested on MSCs with good results.

In this thesis MSCs and hES-MP cells were differentiated to chondrocyte micromass pellets structures with three different types of chondrogenic differentiation media containing either 10% FBS, 10% HPLO or without additional supplements. These chondrocytic pellets were then evaluated after 7, 14, 28 and 35 days of differentiation with GAG assay, histological stainings and RT-qPCR.

Results show that both MSC and hES-MP chondrocytic pellets, differentiated with 10% HPLO, produce more extracellular matrix than pellets differentiated from other media. However, the gene expression shows that the expression of Aggrecan and COMP was lower in MSC pellets differentiated with 10% than pellets from other media and the expression of SOX9 was lower in hES-MP pellets differentiated with 10% HPLO.

There is a possibility of using HPLO as a supplementary media for cartilage differentiation in MSCs and hES-MP cells. It is however necessary to determine what factors are causing this changed gene expression in chondrocytic pellets differentiated with HPLO.

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Formulas

Abbreviations

ACI	Autologous chondrocyte implantation
ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ASCs	Adult stem cells
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
CD	Cluster of differentiation
CFU-F	Colony-forming unit fibroblasts
COMP	Cartilage oligomeric matrix protein
CSCs	Cancer stem cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GDF	Growth differentiation factor
hES-MP	Human embryonic stem cell derived mesenchymal progenitor
HLA	Human leukocyte antigen
HPL	Human platelet lysate
HPLF	Human platelet lysate fresh
HPLO	Human platelet lysate outdated
hrbFGF	human recombinant basic fibroblast growth factor
IGF	Insulin-like growth factor
iPSCs	Induced pluripotent stem cells
ISCT	The International Society of Cellular Therapy
MAP	Mitogen-activated protein
MMP	Matrix metalloprotease
MSCs	Mesenchymal stem cells
N.S.	No Supplement
OCS	Open Canalicular System
PC	Platelet concentrates
PDGF	Platelet-derived growth factor

PEG	Polyethylene glycol
PER	Papain extraction reagent
PLA	Polylactic acid
PLGA	poly [lactic-co-glycolic acid]
PLT	Human platelets
PRP	Platelet rich plasma
SLRP	Small leucine-rich repeat proteoglycan
TGF-β	Transforming growth factor-β
TSP5	Thrombospondin-5

1 Introduction

1.1 Cartilage

Cartilage is a connective tissue that has a good ratio of elasticity to strength. Cartilage is located where it is important to have both flexibility, support and protection from compression and is a fundamental part of the musculoskeletal system, respiratory tract, nose and ears (Rubbelke, 1999; Salter, 1998). A disperesed amount of chondrocytes reside in carilage and they implement maintenance function and matrix-generation. They develop extracellular matrix (ECM), which makes up approximately 95% of the cartilage content and is the working factor of the tissue (Kheir & Shaw, 2009; Zuscik et al., 2008). ECM gets the elastic and durable properties from elastic protein fibres and collagen that also aid in shock absorbance and resistance to forces that cause compression, e.g. weight bearing forces (Rubbelke, 1999). Cartilage is categorized into three types based on different chondrocyte morphology and proliferation in each type.

Hyaline cartilage is the most common type of cartilage in the body and is present in for example the developing skeleton, articular surfaces of synovial joints and bone growth plates. It is a tough, elastic, flexible and semi-transparent tissue consisting of cartilage cells and ECM (Kheir et al., 2009; Salter, 1998). The chondrocytes in hyaline cartilage may be either round or ovoid in shape and most of them are located in gaps of the extracellular matrix. Articular cartilage and the growth plate are types of hyaline cartilage (Salter, 1998).

Fibrocartilage is present at the ends of ligaments and tendons in apposition to bone. Furthermore, the cells in this type are similar to fibrocytes or inactive mesenchymal cells in both function and morphology (Salter, 1998; Temenoff & Mikos, 2000).

Elastic cartilage is present in the larynx and ear. The chondrocytes in this type of cartilage have a round to oval shape, similar to chondrocytes in hyaline cartilage. What separates elastic cartilage from hyaline cartilage is the presence of an elastic material, called elastin, in the ECM (Salter, 1998; Temenoff et al., 2000).

Most cartilage in the body is avascular and is devoid of neurons and lymphatics. Cartilage is enclosed by a thick fibrous membrane called the perichondrium, except at synovial surfaces and osseous junctions (Kheir et al., 2009; Salter, 1998). Small deformities in mature hyaline cartilage are regenerated by migration of chondrocytes that are situated in the ECM but large deformities are healed by the development of fibrocartilage that is biomechanically insufficient compared to native cartilage (Danišovič et al., 2012). Due to lack of vascularisation, nutrient delivery hampers tissue repair. This gives cartilage very low regenerative capacity unlike other types of connective tissues where vascularisation is present, e.g. bone (Rubbelke, 1999).

1.1.1 Formation of cartilage

During embryonic development, mesenchymal stem cells (MSCs) give rise to two distinct types of cartilage during embryonic development. First, there is the permanent hyaline cartilage which derives entirely from MSCs at the distal ends of developing bones. After primary condensation, stable chondrocytes generate from stem cells differentiation and these chondrocytes then synthesise the ECM of articular cartilage (Pelttari et al., 2008). The second type of cartilage that evolves from MSCs is the transient cartilage. Chondrocytes form a transitional cartilaginous model of the skeleton before skeletal bone formation. This model is subsequently replaced by mineralised bone via a mechanism called endochondral ossification. During this process, the chondrocytes cease to proliferate, become hypertrophic and start expressing collagen type X, which is a hypertrophy marker. After additional differentiation, the chondrocytes begin to calcify the surrounding matrix and either transdifferentiate to osteoblasts, or undergo apoptosis (Freyria & Mallein-Gerin, 2012; Pelttari et al., 2008). Following vascularisation, matrix mineralisation takes place which starts migration of bone-depositing osteoblasts and matrix-degrading osteoclasts.

Another mechanism of bone formation exists, called intramembranous ossification, where the bone is synthesized directly without any intermediate cartilage phase. However, the skeleton is mainly formed through endochondral ossification (Shapiro, 2008).

1.1.2 Hyaline cartilage

1.1.2.1 Chondrocytes

Approximately 1% of the volume of hyaline cartilage is nucleated cells, the chondrocytes. Even though the number of chondrocytes are low, they are crucial for maintenance and production of the ECM in hyaline cartilage since they replace degraded matrix molecules to maintain the mechanical properties and structure of cartilage tissue (Kheir et al., 2009; Temenoff et al., 2000). Chondrocytes are surrounded by a thin pericellular matrix, called the chondron, which supplies the chondrocytes with hydrodynamic protection. Every chondron consists of a chondrocyte which is connected with high concentration of proteoglycans, biglycans, hyaluronan and matrix glycoproteins that surrounds the cell (Kheir et al., 2009). Chondrocytes originate from pluripotent mesenchymal stem cells during chondrogenesis. After differentiation, the function of chondrocytes is the combination, maintenance and deposition of ECM molecules such as collagen type II (Imabayashi et al., 2003; Pelttari et al., 2008).

S100 proteins are a family calcium-binding proteins with a low molecular weight (Yammani, 2012). These proteins have intracellular functions that involve e.g. calcium homeostasis, cell survival and differentiation. They also have extracellular functions that involve e.g. interacting with cell surface receptors (Yammani, 2012). S100 proteins are located in chondrocytes but very little is known about their specific role in cartilage. However, S100 histological staining can be used to determine the quantity of chondrocytes in tissue (Yammani, 2012).

1.1.2.2 Extracellular matrix

The extracellular matrix (ECM), sometimes referred to as the cartilage matrix, is a substantial element in cartilage. The ECM is composed of water, collagens (mostly types II, IX, and XI), proteoglycans, glycoproteins and non-collagenous proteins (Fox et al., 2009). Together, collagens and proteoglycans compose 90% of the dry weight in normal tissue (Fox et al., 2009; Freyria et al., 2012; Salter, 1998). All of these components work together and bind water within the ECM. This is important in order to preserve its uncommon mechanical features (Fox et al., 2009).

The ECM is categorised into four distinctive zones. These zones are; the superficial tangential zone, the middle zone, the deep zone and the calcified zone (Figure 1) (Fox et al., 2009; Freyria et al., 2012). The superficial zones' main function involves protecting and maintaining the deeper layers and it is approximately 10-20% of total cartilage density (Fox et al., 2009). Chondrocytes in the superficial layer are numerous and their shape is flat. They synthesize high levels of collagen and low levels of proteoglycans, which causes this zone to have the highest water content (Bhosale & Richardson, 2008; Fox et al., 2009; García-Carvajal et al., 2013). Due to its contact with synovial fluid, a flexible property of cartilage exists and enables it to withstand tensile and compressive forces that are inflicted by movement (Fox et al., 2009). The middle (transitional) zone is beneath the superficial zone and is 40-60% of total cartilage density. This zone provides a functional and structural link between the superficial and deep zones and it contains spherical chondrocytes at low density. This zone contains also proteoglycans and thicker collagen fibrils than the superficial zone (Fox et al., 2009; García-Carvajal et al., 2013). The deep zone is approximately 30% of total cartilage density and it has the highest volume of proteoglycans and the lowest amount of water. The deep zone provides the most resistance to compressive forces given the high proteoglycan content. The chondrocytes are most often in columnar orientation and parallel to the collagen fibres (Fox et al., 2009; García-Carvajal et al., 2013). The calcified zone is distinguished from the deep zone with the tide mark, which is visible with basic dyes, such as toluidine blue (Bhosale et al., 2008; Fox et al., 2009). The chondrocytes in this zone are few in numbers and hypertrophic. The main role of this zone is securing cartilage to bone by attaching the collagen fibrils of the deep zone to the subchondral bone (Fox et al., 2009).

The ECM is not only divided into zones based on different structure and composition. It can also be divided into three distinct regions based on formation and proximity to chondrocytes (Fox et al., 2009; García-Carvajal et al., 2013). These regions are pericellular, territorial and interterritorial matrixes (Figure 1). The pericellular matrix is a thin layer close to the cell membrane and is rich of proteoglycans, glycoproteins and non-collagenous proteins. This region could possibly play a role in initiating signal transduction within the cartilage (Bhosale et al., 2008; Fox et al., 2009). The territorial matrix consists mostly of fine collagen fibrils, which forms somewhat of a basketlike network around the cells. It surrounds either one or more chondrocytes as well as their pericellular matrix. The main function of this region is presumably to protect the cartilage cells against mechanical stresses (Bhosale et al., 2008; Fox et al., 2009). The interterritorial matrix is the largest matrix region and is composed of random bundles of large collagen fibrils. High amount of proteoglycans is present in the interterritorial matrix.

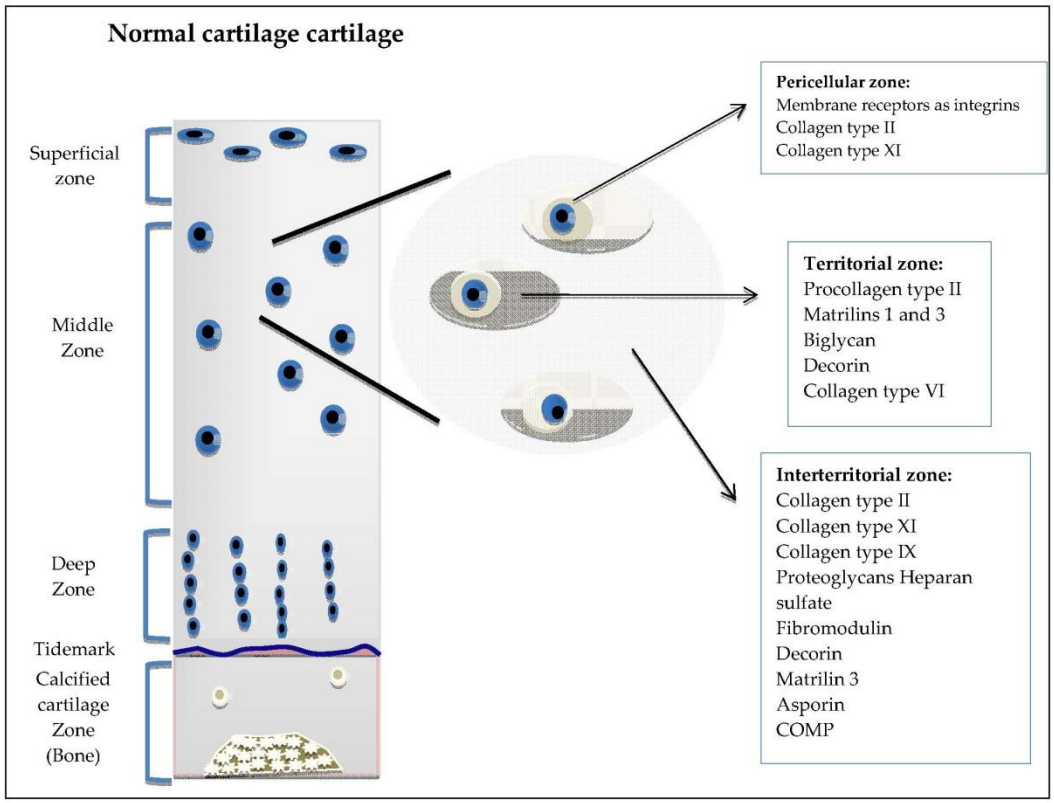

Figure 1 Schematic diagram of normal articular cartilage

The arrangement of chondrocytes in cartilage is separated into superficial, middle and deep zones. A tidemark subdivides the calcified zone from the other zones. The extracellular matrix is divided into three regions depending on their distance from the chondrocytes. Those regions are the pericellular, territorial and interterritorial matrixes. Each region has a unique molecular composition (García-Carvajal et al., 2013).

1.1.2.2.1 Collagens

The extracellular matrix is rich of collagens and these structural macromolecules make up about 50-60% of the dry weight in articular cartilage (Fox et al., 2009; Martel-Pelletier et al., 2008). Collagen fibrils form a specialised network that provides structure and shape to the tissue, with their strength and tensile stiffness being crucial properties (Martel-Pelletier et al., 2008).

Type II Collagen is the most abundant collagen in cartilage and it comprises approximately 90-95% of the collagen in the ECM (Fox et al., 2009). Type II consists of three identical polypeptide chains, αI (II) chains, which tangle and form a triple helix for most of their length (Martel-Pelletier et al., 2008). Type II collagen is synthesised in a procollagen form that includes propeptides. These propeptides are necessary for a suitable fibril assembly, where they are removed after secretion and formation of fibril takes place (Salter, 1998). These type II collagen fibrils are composed of tropocollagen molecules and they are held stable by hydroxypyridinium crosslinks, which are formed after secretion (Salter, 1998). The triple helix region is immune to degradation by almost all proteases. However, the helix can be cleaved by collagenases such as the metalloprotease 13 (MMP-13) (Martel-Pelletier et al., 2008). The main function of type II collagen is to form a fibril network where

proteoglycans reside (Kheir et al., 2009). Collagen type I is composed of two α1 (I) and one α2 (I) chains (Dalgleish, 1997). It is one of the most heavily studied collagen and is the major collagen of tendons, skin and many interstitial connective tissues (Gelse et al., 2003). However, this type is only a minor component of the ECM in cartilage. It helps along with other minor collagens to stabilise and shape the type II collagen fibril network (Fox et al., 2009).

Other collagen types (VI, IX, X, XI, III, XII and XIV) are triple helical proteins and reside in the ECM, similar to collagen type I and II (Kadler et al., 2008). Each type has a specific function in the cartilage structure, which is described in table 1.

Collagen fibers can be stained with a Masson Trichrome staining, which is primarily used to display muscle and collagen in normal tissue. In addition, it is the stain of choice when histological changes have to be analysed in e.g. connective tissue diseases and tumours (Jones, 2010). The Masson Trichrome staining does not however distinguish between collagen types, making it hard to determine what type of collagen is present in the dyed tissue.

Table 1 Function of collagen in hyaline cartilage

Abundant of collagen types function within the hyaline cartilage (Kheir et al., 2009).

Collagen Type	Function
II	Form the network of fibrils within which the proteoglycans are contained
VI	Found mainly at the edge of the chondrocyte where it helps to attach these cells to the matrix framework
IX	Facilitates fibril interaction with the matric proteoglycan molecules
X	Organizes the collagen fibrils into a three dimensional hexagonal lattice
XI	Regulation of fibrils size
III, XII & XIV	Function in article cartilage unknown

1.1.2.2.2 Proteoglycans

Proteoglycans form about 5-10% of the wet tissue weight of cartilage and represent the second largest part of the solid phase in articular cartilage (Martel-Pelletier et al., 2008). Imperative types of proteoglycans that are present in cartilage include glycosaminoglycans (GAGs), Aggrecan and small leucine-rich repeat proteoglycans (SLRPs).

GAGs are carbohydrate molecules that engage in both physiological and pathological processes through regulation of various proteins (Gandhi & Mancera, 2008). GAGs are negatively charged polysaccharides and are categorised into two groups, the non-sulphated and sulphated GAGs (Gandhi et al., 2008). Three types of GAGs are present in the ECM of hyaline cartilage. These types are hyaluronan/hyaluronic acid, which is a non-sulphated GAG, and chondroitin sulphate and keratin sulphate, which are sulphated GAGs. All GAG chains may attach to a core protein with the exception

of hyaluronic acid. When a core protein is bound by GAGs, this complex forms a proteoglycan monomer e.g. Aggrecan (Kheir et al., 2009).

Aggrecan is a significant proteoglycan of cartilage. It is a proteoglycan monomer that binds multiple keratin sulphate and chondroitin sulphate GAG molecules. The Aggrecan core protein has three globular regions; G1, G2 and G3 that contain cysteine residues, which engages in the formation of disulphide bonds (Gandhi et al., 2008; Roughley, 2006; Salter, 1998). A high content of Aggrecan in hyaline cartilages is one of its main characteristics. Aggrecan forms proteoglycan aggregates with the Link protein and Hyaluronan. These aggregates provide the osmotic properties needed to withstand compressive factors and account for the swollen appearance of the cartilage (Roughley, 2006).

Similar to other types of connective tissue, cartilage has a variety of small leucine-rich repeat proteoglycans (SLRPs). Biglycan, decorin, lumican and fibromodulin are major SLRPs in cartilage and they help balance its metabolism and preserve the stability of the tissue (Roughley, 2006).

1.1.2.2.3 Growth factors

Growth factors are vital polypeptides that contribute to cellular proliferation, signalling and differentiation (Fortier et al., 2011). These factors are required for the regulation of homeostasis and in cartilage they contribute to ECM maintenance and development of articular cartilage (Martel-Pelletier et al., 2008). Growth factors are found in limited amount in a healthy adult cartilage where their main goal is to maintain the optimal ECM quality (Martel-Pelletier et al., 2008). In addition, these growth factors are indispensible in the chondrogenic differentiation process of cells such as mesenchymal stem cells (MSCs) (Danišovič et al., 2012). Several growth factors that can be found in cartilage and their effects on MSCs are listed in table 2 (Danišovič et al., 2012).

The transforming growth factor-β (TGF-β) superfamily consists of growth factors that are the most investigated molecules in the field of cartilage tissue engineering and development (Danišovič et al., 2012). There are more than 30 members that belong to the TGF-β superfamily, including TGF-βs, growth and differentiation factors (GDFs) and bone morphogenetic proteins (BMPs) (Wu & Hill, 2009). The members of the TGF-β superfamily are all structurally related homo- and heterodimers connected together with a single disulphide bond. As for their function, they are indispensable for differentiation, cell proliferation and apoptosis, as well as the control of ECM degradation and synthesis (Blaney Davidson et al., 2007; Danišovič et al., 2012).

There are three isotypes of TGF-β in mammals; TGF-β1, -β2 and -β3 (Blaney Davidson et al., 2007). TGF-β1 increases ECM synthesis and stimulates the synthetic activity of chondrocytes (Danišovič et al., 2012; Elisseeff et al., 2001). TGF-β1 increases proliferation and the production of ECM. When MSCs are treated with TGF-β1 it causes up-regulation of collagen type II and aggrecan, and down-regulation of collagen type I (Danišovič et al., 2012). All three isoforms are expressed by muscle and Schwann cells, but TGF-β2 is the only isoform that is differentially regulated during the muscle development (Heupel et al., 2008). TGF-β3 promotes the production of cartilaginous ECM similar to TGF-β1 and the treatment of MSCs with TGF-β3 causes increased synthesis of sulphated GAGs (Danišovič et al., 2012).

Table 2 Specific growth factors and their effect on MSCs

Growth factor	Effects on MSCs	References
TGF-β1	Increases proliferation and cartilaginous ECM productions, down-regulates collagen type I gene expression	(Blaney Davidson et al., 2007; Danisovic et al., 2007; Havlas et al., 2011)
TGF-β3	Increases cartilaginous ECM production	(Mohan et al., 2009; Thorpe et al., 2010)
BMP-2	Increases proliferation and cartilaginous ECM production, down-regulates collagen type I gene expression	(Blaney Davidson et al., 2007; Gobbi & Bathan, 2009; Sekiya et al., 2005)
BMP-4	Accelerates the progression of cartilage differentiation to maturation.	(Hatakeyama et al., 2004; Miljkovic et al., 2008)
BMP-7	Inhibits cell proliferation, induces chondrogenic differentiation, additive effect on chondrogenesis with TGF-β3 and IGF-1	(An et al., 2010; Bai et al., 2011; Shintani & Hunziker, 2007)
GDF-5	Increases cartilaginous ECM production	(Feng et al., 2008; Kou & Ikegawa, 2004)
IGF-1	Increases proliferation and cartilaginous ECM production, additive effect on chondrogenesis with TGF-β1 and BMP-7	(An et al., 2010; Miyakoshi et al., 2005; Schmidt et al., 2006)
FGF-2	Increases proliferation, increases proteoglycan production	(Ge et al., 2006; Park & Na, 2008)
FGF-18	Inhibits cell proliferation, induces chondrogenic differentiation	(Davidson et al., 2005; Yamaoka et al., 2010)

Other growth factors that belong to the TGF-β superfamily are bone morphogenetic proteins (BMPs). These factors are homodimeric molecules and their function is important in the chondrogenesis and osteogenesis processes *in vivo* and *in vitro* (Danišovič et al., 2012; Martel-Pelletier et al., 2008). Various BMPs (BMP-2, -4 and -7) can be found in low amounts in healthy human cartilage where they are believed to have roles in tissue repair and the maintenance of joint stability (Martel-Pelletier et al., 2008).

Growth differentiation factors (GDFs) also belong to the TGF-β family and several proteins are a part of GDFs. However, GDF-5 is the only member that has relevance to cartilage tissue development. GDF-5 heightens the expression of Sox-9, a transcription factor which has a role in chondrocyte maturation and differentiation (Danišovič et al., 2012). Sox-9 is an early marker for cell condensing and is necessary for the expression of cartilage-specific matrix proteins, such as collagen type II (Goldring et al., 2006).

Other growth factors, such as insulin-like growth factor 1 (IGF-1) and the fibroblast growth factor (FGF) family, are also important growth factors that reside in cartilage. IGF-1 is a primary anabolic growth factor in articular cartilage that is fundamental for proteoglycan synthesis and cartilage

homeostasis for example. FGFs are heparin-binding polypeptides which have a role in proliferation and differentiation (Danišovič et al., 2012). The various proteins and growth factors present in cartilage matrix play important roles in the maintenance of equilibrium and proper function of cartilage.

1.1.2.2.4 Cartilage oligomeric matrix protein

Cartilage oligomeric matrix protein (COMP), also accepted as thrombospondin-5 (TSP5), is a 524-kDa pentameric ECM glycoprotein and belongs to the thrombospondin gene family (Gagarina et al., 2008; Haleem-Smith et al., 2012; Hecht et al., 2005). COMP has shown to tether molecules such as collagen types I, II and IX and aggrecan for example (Gagarina et al., 2008). COMP is not only found in cartilage but also in bone, blood vessels and other tissues (Haleem-Smith et al., 2012). This protein is expressed not only in mature cartilage but also in developing cartilage and it has been shown that COMP is mostly located between chondrocytes in the interterritorial matrix (Gagarina et al., 2008; Murphy et al., 1999). Today, the role of COMP in healthy cartilage is unclear but a mutation in the COMP gene causes heritable diseases such as pseudoachondroplasia, which implies that COMP is important for normal cartilage function (Haleem-Smith et al., 2012).

1.2 Stem cells

1.2.1 Definition and characterization

Stem cells can be found in nearly all multicellular organisms and they act as the fundamental element in both regeneration and growth (Burgess, 2013). Self-renewal and their ability to differentiate are properties that define them as stem cells. Both of these functions are accomplished by a process of non-symmetrical division, where one daughter cell differentiates and the other stays undifferentiated (Burgess, 2013; Sánchez et al., 2012). They not only play a pivotal role in the development during embryogenesis, but they are also indispensable in the human adult body as the main source of cellular replenishment for essentially all differentiated mature cell types (Burgess, 2013). Stem cells can be categorised into five groups based on their differentiation potential or potency (Table 3).

Totipotent cells, the most undifferentiated cells, are generated by the fusion of an egg and a sperm cell. The fertilized oocyte and the cells created from the first two divisions are referred to as totipotent as they can differentiate into both embryonic and extraembryonic tissues. Thus, totipotent cells can assemble into a complete, viable organism (Hima Bindu & B., 2011; Kolios & Moodley, 2012).

Pluripotent cells are the descendants of totipotent cells and they have the ability to differentiate into every cell that arises from the three germ layers; ectoderm, endoderm and mesoderm. The self-renewal and differentiation potential to make any differentiated cell in the human body are what characterises these cells. Embryonic stem cells (ESCs) are pluripotent stem cells that are derived from the inner cell mass of the blastocyst (Hima Bindu et al., 2011; Kolios et al., 2012).

Multipotent cells are stem cells that can be found in almost all tissues and have the ability to differentiate into cells from a single germ layer. Mesenchymal stem cells (MSCs) are an example of

multipotent cells due to their ability to differentiate to a variety of tissues derived from mesoderm, such as bone marrow, bone, adipose tissue and cartilage (Hima Bindu et al., 2011; Kolios et al., 2012).

Oligopotent cells can differentiate into two or more cell types that belong to their residing tissue or organ. Hematopoietic stem cells are a good example of oligopotent stem cells due to their ability to differentiate into both lymphoid and myeloid lineages (Hima Bindu et al., 2011; Kolios et al., 2012).

Unipotent cells have the capability to differentiate and self-renew, but only to one specific cell type and form one lineage. Muscle stem cells are an example of unipotent cells. Their differentiation potential limits them to only give rise to mature muscle cells and no other (Hima Bindu et al., 2011; Kolios et al., 2012).

Table 3 Stem cell types and their differentiation potential

Stem cells are categorized into five types based on their differentiation potential.

Stem cell types	Differentiation potential	An example
Totipotent stem cells	Undifferentiated cells that can differentiate into all cell types	Fertilized egg (Zygote)
Pluripotent stem cells	Have the ability to differentiate to every cell that arises from the three germ layers	Embryonic stem cells
Multipotent stem cells	Have the ability to differentiated into cells form a single germ layer	Mesenchymal stem cells
Oligopotent stem cells	Can differentiate into cell types that belong to their residing organ or tissue	Hematopoietic stem cells
Unipotent stem cells	Can only differentiate into one specific cell type	Muscle stem cells

Stem cells are also classified based on their origin. Those groups are embryonic stem cell, adult stem cells, induced pluripotent stem cells and cancer stem cells as will be discussed below.

1.2.1.1 Embryonic stem cells

Thomson and colleagues were the first to derive human embryonic stem cell (hESC) lines from the inner cell mass (ICM) of human blastocysts developed *in vitro* (Thomson et al., 1998). The inner cell mass is destined to give rise to all tissues of the body and ESCs are derived from these cells (Friel et al., 2005).

hESCs have the ability to differentiate into any cell of the body. Thus, they are pluripotent (Table 3). These cells give rise to all three embryonic germ layers; ectoderm, endoderm and mesoderm (Hima Bindu et al., 2011; Li & Xie, 2005). hESCs proliferate slowly in culture, their morphology is described as flattened two-dimensional colonies and they can proliferate *in vitro* for 4 to 5 months but still maintain their differentiation potential (Hadjantonakis et al., 2013; Thomson et al., 1998). hESCs maintain their normal karyotype during a long time of proliferation due to their unlimited self-renewal

potential (Mountford, 2008). The main cause of general replicative senescence in cells is telomere shortening due to deterioration at the ends of individual chromosomes, which forms a cellular replicative clock (Mountford, 2008). This explains why somatic stem cells do not have an unlimited proliferation potential. hESCs have a high expression of the telomerase enzyme that repairs telomeres at chromosome ends, which allows them to divide continuously and evade the replicative clock (Mountford, 2008).

1.2.1.2 Adult stem cells

Adult stem cells (ASCs) are also referred to as somatic stem cells. They have the ability to divide and create another identical cell (Symmetric cell division) or divide and create a cell that differentiates (Asymmetric cell division). Most of ASCs are lineage-restricted and are derived from adult tissues, such as bone marrow, central nervous system and adipose tissue (Burgess, 2013; Hima Bindu et al., 2011; Kolios et al., 2012).

ASCs reside within adult tissues in a supportive microenvironment called the stem cell niche. The niche is important for both the maintenance of the stem cell pool as well as for stem cell differentiation (Burgess, 2013). By supporting the stem cell self-renewal process, the stem cell niche promotes the maintenance of homeostasis and tissue regeneration (Kiefer, 2011). Thus the stem cell nice has a specialized role, to maintain stem cells and keeping their microenvironment stable by building a specific reserve for stem cells with signaling molecules and cell adhesion (Kiefer, 2011).

1.2.1.3 Induced pluripotent stem cells

Induced pluripotent stem cells (iPSCs) are produced by encouraging specialised cells to express genes that are expressed in embryonic stem cells and control cell functions and potency (Takahashi & Yamanaka, 2006). Even though iPSCs have similar characteristics as ESCs, they are not identical. These cells are however not adult stem cells. Instead they are reprogrammed cells with pluripotent abilities (Hima Bindu et al., 2011). iPSCs can possibly be an indispensible tool in regenerative medicine due to the possible ability to send them back to earlier undifferentiated stages and thus creating a perfect match to the cell donor and likely avoid rejection issues (Kolios et al., 2012).

1.2.1.4 Cancer stem cells

It is well known that accumulation of mutations and epigenetic changes are responsible for the transformation of normal cells to tumour cells. Cancer stem cells (CSCs) represent a minor population of cells within a tumour that has the ability both to produce more cancer stem cells through self-renewal as well as to create tumour cells that differentiate. (Soltysova et al., 2005). CSCs thus divide by asymmetric cell division (Yu et al., 2012). It is possible that CSCs originate from the transformation of normal stem cells in tissues. CSCs are tumorigenic and have the ability to form a tumour in an animal host after transplantation. These cells are thought to be an important target for innovative cancer drug discovery (Yu et al., 2012).

1.3 Mesenchymal stem cells

1.3.1 Definition and characterization

Mesenchymal stem cells (MSCs) were first described in the 1970s by Friedenstein and his colleagues (Friedenstein et al., 1976). They showed that the bone marrow contained a small population of plastic-adherent stromal cells in addition to hematopoietic stem cells (Williams & Hare, 2011). These stromal like cells, defined as colony-forming unit fibroblasts (CFU-F), were spindle-shaped and clonogenic in culture conditions (Friedenstein et al., 1976). CFU-F derived stromal cells can serve as feeder layers for the expansion of hematopoietic stem cells and may differentiate into osteoblasts, chondrocytes and adipocytes both *in vitro* and *in vivo* (Salem, 2009; Uccelli et al., 2008). Further studies established the capability of these expanded cells, derived from single CFU-Fs, to proliferate while preserving their ability to differentiate to osteoblasts, adipocytes and chondrocytes *in vitro* (Nombela-Arrieta et al., 2011; Pittenger et al., 1999).

Only 0,001-0,01% of the total population of nucleated cells in the bone marrow are MSCs (Pittenger et al., 1999). However, even though MSCs are only a small fraction of the cells, they can be isolated, expanded with high productivity and then induced to differentiate under specific culture conditions (Barry & Murphy, 2004). Despite years of research, the definition of MSCs is largely derived from *in vitro* experiments. Due to the lack of specific markers to identify MSCs, the location and the *in vivo* role of the innate cells within their tissue of origin are not known (Augello et al., 2010b). There have been speculations whether the behaviour and phenotype of MSCs differ between *in vivo* and *in vitro* conditions. It has been reported that the phenotype of these cells can change during *ex vivo* manipulations. That could result in acquiring new markers and losing the expression of others (Augello et al., 2010b; Jones et al., 2002).

The International Society for Cellular Therapy (ISCT) has published minimum criteria for defining multipotent MSC in standard cultivation (Dominici et al., 2006; Salem, 2009; Williams et al., 2011). This criteria is as follows:

1) They are plastic-adherent under standard culture conditions.
2) They are express CD73, CD105 and CD90, but do not express the hematopoietic cell surface markers CD34, CD11a, CD19, CD45 and HLA-DR.
3) They should be able to differentiate into osteocytes, adipocytes and chondrocytes *in vitro* under specific conditions.

MSCs express moderate amounts of human leukocyte antigen (HLA) major histocompatibility complex class I molecules but do not express major histocompatibility complex class II molecules (HLA-DR), B7, CD40 ligand and co-stimulatory molecules such as CD80 and CD86 (Sensebe et al., 2010; Williams et al., 2011). This uncommon immunophenotype together with their ability to target immune cells and secrete soluble factors, give MSCs a great tolerance as an allogeneic transplant (Williams et al., 2011). MSCs cause an immunomodulatory effect by interacting with both adaptive and innate immune cells. The innate immune cells, such as neutrophils, natural killer cells and macrophages, are responsible for the non-antigen-specific immune defense mechanisms. MSCs

suppress both innate (neutrophils, natural killer cells, dendritic cells) and adaptive (T- and B cells) immune cells (Williams et al., 2011). These findings have increased the clinical interest in MSCs as allogeneic transplant products and as “universal donor cells”, especially in the field of regenerative medicine and for the induction of tolerance in allogeneic transplantation (Kode et al., 2009).

As stated before, stem cells have usually been defined by their self-renewal and multipotency capabilities. Whether MSCs are in fact stem cells or rather multipotent precursor cells, has therefore been debated (Williams et al., 2011). MSCs can be isolated from various tissues. However, MSCs that have been isolated from separate tissues have different gene expression, differentiation capabilities and they have demonstrated a heterogeneous morphology. Various terms have been used to describe their appearance, such as fibroblastoid cells, spindle shaped, flattened cells, very small round cells, giant fat cells and blanket cells (Pevsner-fischer et al., 2011; Williams et al., 2011). More research is needed in order to further understand the biological differences between MSCs from different tissue sources and the characteristics of these tissue-specific MSCs (Williams et al., 2011).

1.3.1.1 Characterization with surface markers

Each cell type in the human body has specific proteins on their surface that have the ability to interact selectively with other signalling molecules and receptors in their environment. These surface proteins are called cell surface markers (Health, 2009). Cell surface markers have been used to identify cells in addition with morphological and molecular indication of function, such as enzyme expression. However, all stem cells express different surface markers and since they have not differentiated to a specific function, the identification of these cells relies heavily on these surface markers (Prentice, 2003).

MSCs comprise a heterogeneous population of cells in terms of their morphology and expression of surface markers. They express various surface markers, but there are no distinctive surface markers that can selectively identify MSCs. This makes it problematic to assess their actual numbers or to identify their specific locations (Caplan & Bruder, 2001; Docheva et al., 2008; Wong, 2011).

Expressions of CD (Cluster of Differentiation) markers in MSCs have been examined in much detail due to the lack of distinctive markers for MSC characterization. Their phenotypic characterization varies between studies. Mafi et al. examined 29 studies that had been focusing on MSCs expression of cell surface markers (Mafi et al., 2011). Their conclusion was that markers such as CD105, CD73, CD90 and CD29 were among the most common positive surface markers on mesenchymal cells in these studies and surface markers such as CD45 and HLA-DR were most commonly negative (Mafi et al., 2011). Some of the CD markers used to characterize MSCs are displayed in table 4.

Table 4 Cluster of differentiation molecules that can been used to characterize MSCs

These CD antigens can be used to define and characterize MSC. MSC express CD29, CD90, CD73 and CD105 but not CD45 (Murphy et al., 2012).

CD antigen	Cellular expression	Molecular weight(kDA)	Function
CD29	Leukocytes	130	Integrin β-1 subunit, associates with CD49a in VLA-1 integrin
CD90	CD34+ prothymocytes (human) Thymocytes, T cells (mouse)	18	Unknown
CD45	All haematopoietic cells	184-240 (multiple isoforms)	Tyrosine phosphatase, augments signaling through antigen receptor of B and T cells, multiple isoforms result from alternative splicing
CD105	Endothelial cells, activated monocytes and macrophages, bone marrow cell subsets	90 homodimer	Binds TGF-β
CD73	B-cell subsets, T-cell subsets	69	Ecto-5'-nucleotidase, dephosphorylates nucleotides to allow nucleoside uptake

1.3.2 MSCs differentiation

As stated before, MSCs are defined by their ability to differentiate into osteocytes, chondrocytes and adipocytes (Augello & De Bari, 2010a). Differentiation processes, such as chondrogenesis, are activated by incubating MSCs with certain stimulants and morphogens (Kassem et al., 2008).

1.3.2.1 Osteogenic differentiation

To differentiate MSCs to an osteogenic lineage, MSCs need to be stimulated with ascorbic acid, dexamethasone and β-glycerol-phosphate. When the cells are cultured in monolayer in the presence of these stimulants, it results in an increase of alkaline phosphatase (ALP) activity and calcium-rich mineralized extracellular matrix (Augello et al., 2010a; Barry et al., 2004).

1.3.2.2 Chondrogenic differentiation

Chondrogenic differentiation is promoted by cultivating MSCs in a three-dimensional culture format, such as in pellet culture systems, and in a serum-free medium in addition of a member of the TGF-β family (Augello et al., 2010a; Barry et al., 2004). TGF-β1, -β2 and -β3 have the capacity to induce chondrogenesis but TGF-β2 and -β3 are most effective (Barry et al., 2004). When MSCs are cultivated with TGF-β3, they start to lose their fibroblastic morphology and start to produce numerous cartilage specific components such as GAGs, Aggrecan, type II collagen, COMP and more factors that belong to a normal articular cartilage matrix (Barry et al., 2001; Barry et al., 2004).

1.3.2.3 Adipogenic differentiation

MSCs are differentiated to adipogenic lineage by cultivating them in a monolayer with the stimulants dexamethasone, insulin, isobutyl methyl xanthine and indomethacin. The differentiation is acknowledged with oil red O staining to detect lipid vacuoles (Augello et al., 2010a).

1.3.2.4 Regulation of Differentiation

Two pathways, the Wnt canonical pathway and the TGF-β superfamily pathway, play a role in regulation of MSCs differentiation (Figure 2). Wnt glycoproteins are soluble glycoproteins that intertwine with receptor complexes formed by Lrp5/6 proteins to induce a chain reaction of intracellular events that finally regulate cell proliferation and differentiation (Williams et al., 2011). The Wnt is crucial in skeletogenesis by inducing osteoblast proliferation and discourage chondrocyte formation with β-catenin, which is imperative in transducing Wnt signaling to the nucleus (Williams et al., 2011).

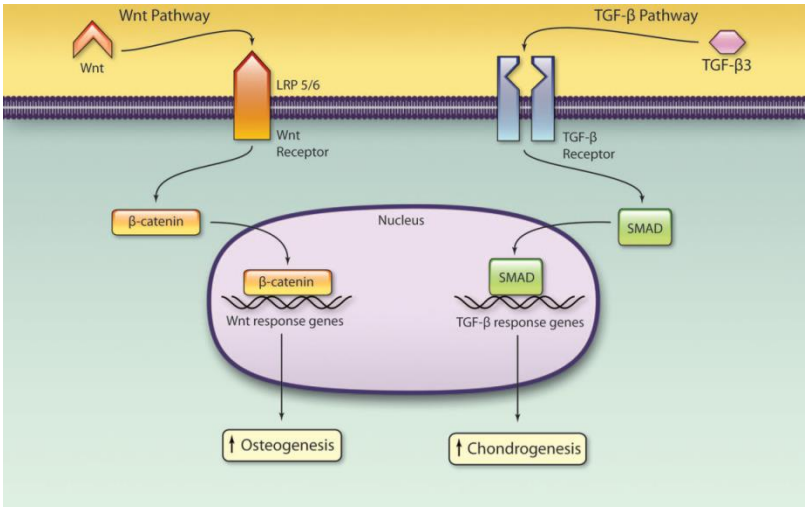

Figure 2 Molecular regulation of MSCs differentiation

Wnt and TGF-β pathways regulate differentiation of MSCs by inducing intracellular signaling (Williams et al., 2011).

Activation of the TGF-β pathway affects skeletal tissue growth and regulation of MSCs differentiation into chondrocytes. TGF-β3 causes up-regulation of gene expression in MSCs to promote chondrogenic differentiation. This is achieved via several intracellular cascades such as SMAD proteins, p38 and mitogen-activated protein (MAP) (Williams et al., 2011). Other molecules, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), have also been shown to regulate MSCs differentiation (Williams et al., 2011).

1.4 Human embryonic stem cells derived from mesenchymal progenitors

1.4.1 Definition

hES-MP^{TM}002.5 cells (human embryonic stem cell-derived mesenchymal progenitor) are human embryonic stem cells that have been induced towards mesenchymal–like state and are thought to have high resemblance to adult human mesenchymal stem cells (Cellartis-AB).

These cells are established and characterized at Cellartis AB, Gothenburg, Sweden. hESC in an undifferentiated state are removed from their supporting layers and cultured in a supplemented medium with fetal bovine serum (FBS) and human recombinant basic fibroblast growth factor (hrbFGF). To initiate derivation, they are passaged enzymatically as a single cell suspension and expanded until they become homogenous for hES-MP morphology (Karlsson et al., 2009).

1.4.2 Characteristics

Both hESCs and MSCs have raised high expectations for usage in cell therapy and tissue engineering due to their unique qualities. However, both cell types have limitations regarding usage in regenerative medicine. Transplantation of undifferentiated hESCs may lead to tumour formation (teratoma) in the recipient and MSCs do not maintain their replicative ability after extended *ex vivo* expansion, which limits cell production for tissue engineering purposes (de Peppo, G. M. et al., 2010; Karlsson et al., 2009). hES-MP cells do not have these limitations.

hES-MP cell lines are easy to culture and have an excellent proliferative capacity as these cell lines could expand for 16-20 passages before a decrease in proliferation was detected (Karlsson et al., 2009). These cells also have an elongated spindle-shaped cell morphology with branching pseudopodia, which is similar to MSCs morphology and significantly different from those of hESCs (Karlsson et al., 2009). Karlsson et al. also showed that hES-MP cells do not express markers typically found expressed in hESCs, such as Oct-4, Nanog, SSEA-3 and SSEA-4. Instead, they express early mesodermal markers, such as Desmin, Vimentin, CD105 and CD10, which is similar to MSCs expression (Gunnarsdottir, 2013; Karlsson et al., 2009).

Guiseppe Maria de Peppo et al. looked at gene expression in hES-MP cell lines, including SA002.5. They found that hES-MP cells repress genes such as Nanog and Sox-2 in hES-MP derivation, which provides evidence for a lineage commitment detected in hES-MP cells compared with hESCs. In addition, there is evidence for a decreased chance of teratoma formation in contrast to hESCs due to their induced expression of NRF2F and p53 associated genes LTPP2 and TFAP2A (de Peppo, Giuseppe Maria et al., 2010). p53 is an important gene for tumour development and is inactivated in many tumours. NRF2F induces transcription of p53 when it binds to the p53 promoter (de Peppo, Giuseppe Maria et al., 2010).

Li et al. demonstrated that hES-MP002.5 cells possess similar functions compared to BM-MSCs, aside from the immune-modulatory effects. The hES-MP cells in this study remained non-immunosuppressive after interferon-γ treatment, which was previously shown to increase HLA-DR

expression and to enhance immune-suppressive activity of fetal MSCs (Li et al., 2013). So they concluded that hES-MP002.5 cells are an appealing unlimited source for stroma transplantations when immune suppression is either not potentially dangerous or required in clinical situations (Li et al., 2013).

Thus, hES-MP cell lines are lineage restricted, do not form teratomas *in vivo*, display high proliferation ability and possess analogous functional and biological properties to MSCs (de Peppo, G. M. et al., 2010; de Peppo, Giuseppe Maria et al., 2010; Li et al., 2013). hES-MP cells can also be derived under xeno-free conditions, unlike other hESC-derived mesodermal progenitors. This feature is essential for using stem cells in clinical conditions (de Peppo, G. M. et al., 2010; Karlsson et al., 2009).

1.5 Regenerative medicine

Regenerative medicine is a term used to describe biomedical methods that utilize cells, e.g. stem cells, to heal the body. These biomedical methods revolve around replacing or regenerating human cells, organs, tissues to establish normal function (Bouros & Laurent, 2012; Riazi et al., 2009). Regenerative medicine can be categorized into two fields, cellular therapy and tissue engineering. The main difference between those two fields is the use of scaffolds in tissue engineering but not in cellular therapy (Ikada, 2006). With improved life expectancy, the prevalence of diseases, such as cystic fibrosis and chronic lung diseases e.g., associated with aging have greatly increased. Dysfunction or deficit of specific cell types is the primary reason for many of these diseases (Bouros et al., 2012). Stem cells are great contributors to regenerative medicine due to their self-renewal and differentiation potential, as stated before. Stem cells are already in use for clinical purposes. For example, HSCs have successfully been used for more than 40 years in bone marrow transplantations to treat blood disorders such as leukaemia (Teo & Vallier, 2010) and still represent the only standard of care therapy using stem cells.

Stem cells and their use in clinical therapies has been a promising field for some time. Scientists believe that stem cells from human embryos could hold the key to understanding and even controlling human development (Mountford, 2008; Robertson, 2010). The use of hESCs in clinical application is complicated by at least three issues; ethical issues, immunological rejection by the transplant recipient and possible teratoma formation (Riazi et al., 2009). Immunological rejection of donor cells by the transplant recipient is possible after hESC transplantation. Studies have shown that hESCs can express high levels of MHC class I (Drukker et al., 2002). Thus, grafts with hESCs might suffer from similar acute and chronic rejection problems as other grafts (Mountford, 2008; Prockop et al., 2008). In addition, their unrestricted growth potential could cause teratomas after transplantation due to random differentiation (Mountford, 2008; Prockop et al., 2008). The ethical issues concerning hESCs involve the cell source, as hESCs are derived from early embryos (Robertson, 2010). Some people believe that all embryos, even though they are destined to be destroyed according to tissue banking regulations due to excess or other reasons, should be kept alive and strongly disapprove of their use

for scientific purposes (Mountford, 2008; Robertson, 2010). Notably, these ethical issues may be avoided with progress in the field of iPSCs (Okita et al., 2007).

MSCs have also made a huge impact on stem cell research in the recent years. They have gained increased interest, not only due to their multilineage differentiation potential and the ability to self-renew, but also because they are easier to deal with compared to hESCs (Wong, 2011). As stated before, MSCs are immune privileged cells as they have the ability to evade the immune surveillance for a long time, due to their low levels of HLA class I and II. This may be an important asset for their use in transplantation in regenerative medicine (Kode et al., 2009; Wong, 2011). MSCs may also be of help after musculoskeletal trauma working through paracrine mechanisms. This causes adjustment in the regenerative environment via immunomodulatory and anti-inflammatory mechanisms (Murphy et al., 2013). However, some disadvantages are associated with the use of MSCs in regenerative medicine. Donors have to undergo invasive procedures, such as bone marrow aspiration. The MSCs are subsequently isolated via incompletely developed procedures that can result in a high degree of heterogeneity (de Peppo, G. M. et al., 2010; Ho et al., 2008). Additionally, MSCs lose their replicative capability after extended *ex vivo* expansion, which limits cell production for tissue engineering (de Peppo, G. M. et al., 2010). Extended *ex vivo* cultivation on MSCs has also been associated with karyotypic instability and uncontrolled malignant transformation (de Peppo, G. M. et al., 2010).

Even though ESCs and MSCs each have limitations, these cells are both important for regenerative medicine and continued search for solutions is essential when looking at the ultimate objective, which is to treat various diseases that plague mankind (Bongso & Lee, 2011).

1.5.1 Cartilage tissue engineering

The purpose of tissue engineering is to create biological substitutes that improve or replace the tissue and its original function (Hillel et al., 2010; Moreira-Teixeira et al., 2011). Tissue engineering can be implemented by three crucial elements: 1) cellular components that have the ability to compose a functional matrix, 2) a scaffold made from biologic or synthetic material that contributes to cellular support and finally, 3) cytokines and growth factors that regulate the formation of a desired tissue (Hillel et al., 2010).

The degeneration of cartilage tissue has major impact on humans as it can cause disabilities due to joint disorders, such as osteoarthritis or trauma (Keeney et al., 2011). The intrinsic biology of the cartilaginous tissue limits its ability to self-regenerate. The normal mechanism of tissue repair does not apply due to lack of vascular system and nerves, as stated before, and this makes mature joint cartilage unable to repair itself adequately after tissue damage (Moreira-Teixeira et al., 2011; Toh et al., 2011). These complications have created the need for cartilage tissue engineering (Moreira-Teixeira et al., 2011).

The fundamental part of cartilage tissue engineering is the use of chondrocytes or MSCs (Moreira-Teixeira et al., 2011). MSCs have been used successfully in clinical trials for various diseases such as spinal cord injuries (Syková et al., 2006) and cartilage lesions (Hui et al., 2005).

The first cell type to be used for cartilage repair was autologous chondrocytes and the autologous chondrocyte implantation (ACI) was first described in 1994 (Brittberg et al., 1994). This procedure involves harvesting thin cartilage slices through an arthroscopic procedure, and the chondrocytes from these slices undergo expansion *in vitro*. The chondrocytes are then implanted and secured under a cover of periosteum within the injured site (Iwasa et al., 2009; Keeney et al., 2011; Revell & Athanasiou, 2008). However, limitations such as donor site morbidity and the loss of phenotype during expansion causes problems. Other cell types have also been researched as alternative cell sources for cartilage repair and they are listed in table 5 (Keeney et al., 2011).

Table 5 Comparison of various cell sources for cartilage tissue engineering

	Cell types	Advantages	Disadvantages
Differentiated cells	Adult chondrocytes	• Autologous • Cartilage tissue specific	• Limited availability • Invasive harvest procedure • Donor site morbidity • Dedifferentiate in vitro
	Neonatal chondrocytes	• High proliferation rate • High collagen II gene expression and GAG production • Non-immunologenic	• Limited availability
	Fetal chondrocytes	• High potential for cartilage synthesis	• Limited availability
Multipotent stem cells	Synovial-derived stem cells	• Autologous	• Retain some fibroblastic characteristic after induction
	ASCs	• Autologous • Minimally invasive harvest • Abundance	• May be less robust in chondrogenesis compared with BM-MSCs • High total collagen synthesis but low of collagen type II
	BM-MSCs	• Autologous • High collagen II	• Hypertrophic phenotype
Pluripotent stem cells	ESCs	• Unlimited self-renewal	• Ethical controversy • Potential tumorgenicity
	iPSCs	• No ethical controversy • Patient-specific • Unlimited self-renewal	• Safety concerns

Scientists have begun to use three-dimensional scaffolds for cartilage repair (Iwasa et al., 2009). Chondrocytes reside in the body within a complex three-dimensional environment. When these cells are isolated and cultured in two-dimensional culture, they lose their differentiated phenotype (Vinatier et al., 2009). However, chondrocytes can dedifferentiate and thus reverse this process when relocated into a three-dimensional environment, i.e. they withhold their differentiated phenotype in three-dimensional scaffolds (Iwasa et al., 2009; Vinatier et al., 2009). In addition, these scaffolds can act as a certain barrier to protect the graft from fibrous repair (Iwasa et al., 2009).

Certain biomaterials have been used to culture chondrocytes in a three-dimensional setting and for implantations. The biomaterials that are used as scaffolds can be categorized into three types; protein-based, polysaccharide-based and synthetic. An overview of these types and material can be found in table 6 (Vinatier et al., 2009). However, the use of these materials in clinical therapies is limited, primarily due to immunological reactions and the risk of disease transmission (Iwasa et al., 2009). Growth factors also play a pivotal role, in addition to an appropriate scaffold, where they promote specific differentiation pathways and maintenance of the chondrocyte phenotype (Vinatier et al., 2009).

Table 6 Various biomaterials used as scaffolds for cartilage tissue engineering

Type	Material
Protein-based	Collagen Fibrin
Polysaccharide-based	Alginate Chitosan Hyaluronic acid Cellulose
Synthetic	PLGA (poly[lactic-co-glycolic acid]) PLA (polylactic acid) PEG (polyethylene glycol)

Tissue-engineering methods with scaffolds are an up and coming field in cartilage treatment. Iwasa et. al. studied various scaffold techniques and their conclusion was that even though these procedures reduce morbidity and surgical time in contrast to ACI, none of them were considered superior to ACI (Iwasa et al., 2009). Cell-based techniques in combination with growth factors, scaffolds and possibly gene therapy is a promising approach in cartilage tissue engineering (Iwasa et al., 2009).

1.6 Platelet lysates

1.6.1 Platelet biology and structure

Platelets are fragments derived from megakaryocytes, which are produced in the bone marrow. Megakaryocytes differentiate from hematopoietic stem cells through massive cell organization that leads to formation of pro-platelets that are released into the circulation where shear forces trigger their release (Harmening & Moroff, 2005; Italiano Jr & Hartwig, 2007).

Platelets are the second most numerous cells in the blood stream with the concentration of 150.000 to 350.000 platelets per µl in normal circumstances, following red blood cells. Their shapes has been described as discoid with an average diameter of 2 to 4 µm, have a mean volume of 7-11 femtoliters and they usually circulate in the bloodstream for 10 days (Harmening et al., 2005; Harrison, 2005; Kickler, 2006). The platelet has three distinct zones (Figure 3). First, there is the

peripheral zone. It consists of the platelet membrane, surface-connecting channels (Also known as the Open Canalicular System (OCS)). It transfers secretory products of platelet granules) and glycocalyx (Harmening et al., 2009). The glycocalyx is an important part of the platelet structure. It is not only a barrier that keeps internal contents from the exterior, it senses changes in the vasculature where the hemostatic response occurs (White, 2007). Second, there is the Sol-gel/structural zone (also called cytoskeleton). Microtubules (coat the platelet, which maintains this distinctive discoid shape), microfilaments (actin and myosin) and submembranous filaments characterize the sol-gel zone and are located within the matrix of platelets (Harmening et al., 2009). Third, there is the organelle zone, where metabolic processes of the platelet take place (Harmening et al., 2009).

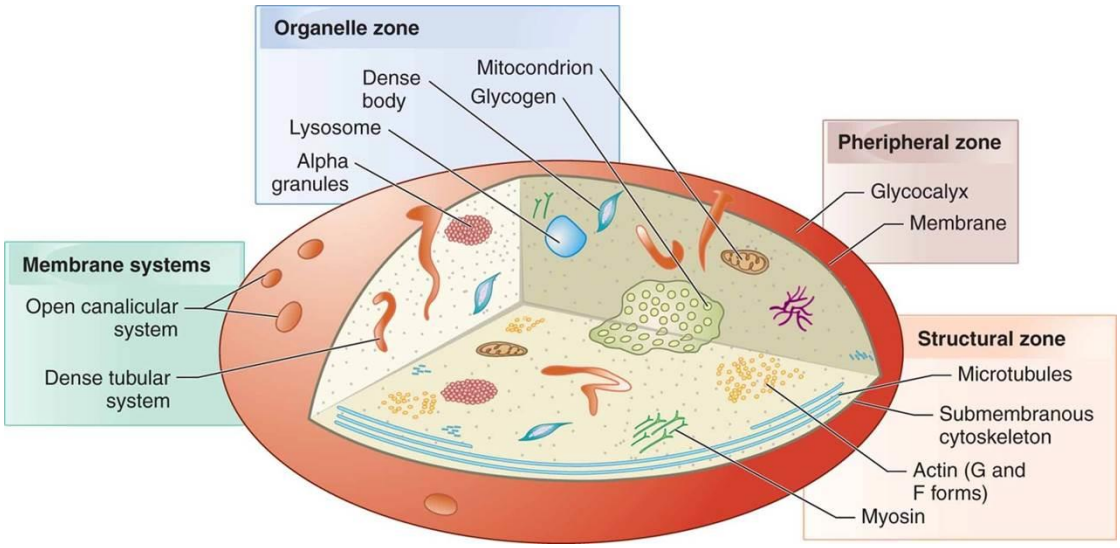

Figure 3 The structural zones of platelets

The platelet structure can be divided into the peripheral zone, structural zone and the organelle zone (K, 2013).

Platelets do not possess a Golgi body or rough endoplasmic reticulum, like other cells. However, they have mitochondria and cytoplasmic granules. Platelets contain three types of granules, within the organelle zone, that are morphologically diverse (Harmening et al., 2009; White, 2007). First, there are α granules, which are the most common of the platelet organelles (Usually 40 to 80 α granules per platelet). They contain numerous molecules that can be categorized into groups based on their biological function such as adhesion molecules, growth factors and chemokines (Table 7) (Reed, 2007). Second, there are dense bodies (δ granules) that are more or less acidic with a pH of 6.1. They contain small molecules such as adenosine diphosphate (ADP), serotonin and lysosomal membrane proteins such as CD63 (LAMP-3) (Reed, 2007). Last, lysosomes contain lysosomal membrane proteins like dense bodies plus acid hydrolases and cathepsins (Reed, 2007).

Table 7 Categorization of α-granules content

Platelets contain numerous molecules that can be grouped based on their biological function. These molecules are numerous so only examples are given in this table (Reed, 2007).

Adhesion molecules	P-Selectin, von Willebrand factor, thrombospondin, fibrinogen, integrin αIIbβ3, integrin αvβ3, fibronectin
Chemokines	Platelet factor 4 and its variant (CXCL4), β-thromboglobulin, CCL3, CCL5, CCL7, CCL17, CXCL1, CXCL5, CVCL8
Coagulation pathway	Factor V, multimerin, factor VIII
Fibrinolytic pathway	α_2-Macroglobulin, plasminogen, plasminogen activator inhibitor 1
Growth and angiogenesis	bFGF, EGF, HGF, IGF, TGF-β, VEGF-A, VEGF-C, PDGF
Immunological molecules	β1H Globulin, factor D, c1 inhibitor, IgG
Other proteins	Albumin, α1-antitrypsin, Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectin protease nexin-II (amyloid beta-protein precursor

1.6.2 Platelet function

Platelets are engaged in many physiological processes such as hemostasis, vessel constriction and repair, host defence and thrombosis (Harrison, 2005). Their small size and shape allows them also to attach to the edge of vessels, where they constantly monitor the stability of the vasculature (Harrison, 2005).

The main function of platelets however is within the hemostatic system, where platelets form a clot that stops bleeding after vascular injury and tissue trauma (Davi & Patrono, 2007). Hemostasis is a pivotal process that keeps a complete balance between bleeding and clotting in the human body. This process is sensitive to any imbalance in the interactions of five factors; blood vessels, fibrinolysis, platelets, serine protease inhibitors and coagulation proteins (Harmening et al., 2009). Hemostasis is categorized into primary and secondary hemostasis. Primary hemostasis involves a vascular injury response that forms a platelet plug at the side of damage. This platelet plug adheres to the endothelial wall and restricts the bleeding. Secondary hemostasis is the response from coagulation proteins to produce fibrin from fibrinogen. This response stabilizes the platelet plug. This plug dissolves due to fibrinolysis, when fibrinogen and fibrin are digested by the enzyme plasmin. Hemostasis is completed when platelet-derived growth factor (PDGF) is released and promotes repair to the vasculature (Harmening et al., 2009).

1.6.3 Platelet lysates as a media supplement

Basal media for cell culture is usually supplemented with serum. Supplementary serum in culture media is supposed to provide several factors such as transport proteins, trace elements, minerals and lipids (Rauch et al., 2011; Vis et al., 2010). In addition, it should promote differentiated function, maintain pH with stabilizing and detoxifying factors and hold hormonal factors that vitalise cell growth and proliferation (Rauch et al., 2011). Fetal bovine serum (FBS) is most frequently used as supplementary serum in stem cell culture and especially for MSCs (Vis et al., 2010). FBS is an excellent supplement due to its low gamma-globulin content, but gamma-globulin is known to have suppressive effects on proliferation and growth (Rauch et al., 2011). FBS is however not a desirable supplement in cell therapy. Animal serums are possible transmitters of bacteria, prions, viruses, fungi and infectious diseases that transmit between animals and humans (Fekete et al., 2012; Tekkatte et al., 2011). In addition, FBS can cause unexpected characteristics in cell growth and there are ethical issues around the harvest of FBS, but the serum is harvested from bovine fetuses from pregnant cows before slaughter (Rauch et al., 2011; Tekkatte et al., 2011). Therefore, scientists have been searching for an alternative for FBS.

MSCs require special serum-specific growth factors for cultivation. This is a major disadvantage in finding a replacement for FBS (Tekkatte et al., 2011). In addition, the FBS substitute also needs to obtain qualities such as low cost, easy access, no possibility of contamination and have the ability to be preserved for a longer time (Tekkatte et al., 2011). Several substitutes have the potential of replacing FBS as a supplementary media for MSCs culture, such as human serum, umbilical cord blood serum and human platelet lysate (Tekkatte et al., 2011).

Human platelets (PLT), present in PLT lysate, contain components such as PLT-derived growth factors (PDGF), transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (Schallmoser et al., 2007). PLTs are mitogenic for MSCs, chondrocytes and bone cells (Schallmoser et al., 2007). This abundance of growth factors is essential for the expansion of MSCs and therefore could platelet lysate possibly be a valuable substitute for FBS (Pérez-Ilzarbe et al., 2009; Rauch et al., 2011). Human platelet lysate (HPL) is produced from platelet concentrates/platelet rich plasma (PC/PRP). Studies have already demonstrated the possibility of MSCs expansion with platelet lysates. Pérez-Ilzarbe et al. for example concluded that the immunophenotype, differentiation and growth potential of MSCs did not vary between FBS and platelet lysate (Pérez-Ilzarbe et al., 2009). HPL has also been reported to accelerate the expansion rate of MSCs and spontaneously induce expression of osteoblastic genes (Chevallier et al., 2010).

HPL can be produced either as fresh (HPLF) or outdated (HPLO). HPLF is produced by freezing PC immediately after sampling and HPLO is produced by keeping PC in room temperature for several days before freezing. The description of a HPL preparation protocol can be found in chapter 2.1. Our group has also demonstrated that lysates from outdated platelet concentrates can act as a substitute for FBS and be used for MSCs culture (Jonsdottir-Buch et al., 2013).

These findings make HPL an attractive choice as an alternative for FBS in cell culture. The production cost for HPL, even in large amounts, is relative low and no ethical issues are linked to the

HPL process. HPL may therefore be a great asset to the fields of cellular therapy and tissue engineering in the future (Johansson et al., 2003; Rauch et al., 2011).

Our group mainly focuses on using HPLO as supplementary media. The PCs collected from healthy donors can only be stored for 5-7 days before transfusion. Most of the PCs are used but there are always some bags that are discarded after 5-7 days. Instead of discarding the expired units, they are stored in -80°C until they are used for platelet lysate production. Therefore, instead of competing with the Blood Bank and patients for PCs, we use the ones who are not suitable for transfusion. This also prevents donor variation because several bags are pooled during the platelet lysate production.

1.6.3.1 Pathogen inactivated platelet lysate

The Blood Bank recently changed their policy on platelet production: All PCs are now pathogen inactivated with the INTERCEPT Blood System for Platelets from Cerus. This system is based on Helinx technology where amotosalen-HCl targets nucleic acids of DNA or RNA and enters their helical region (Janetzko et al., 2004; McCullough et al., 2004). When the integrated amotosalen molecules are activated by long-wavelength ultraviolet light (UVA) at 320-400 nm, they react with the pyrimidine bases of the nucleic acids and form intra- and internucleic acid strand cross-links (Janetzko et al., 2004; McCullough et al., 2004). This causes replication restriction of RNA or DNA, which reduces the levels of many bacteria and viruses within the platelet components to such a degree that they are not likely to be transmittable (McCullough et al., 2004). One of the additional benefits in using the INTERCEPT system is that the amotosalen and the UVA light applied to the platelets reduces non-specific reactions, which results in protein conservation (Irsch et al., 2010).

Objectives

The aim of this study was to determine the impact of *pathogen-inactivated lysates produced from outdated platelets as a media supplement* for the culture and chondrogenic differentiation of hES-MP cells and MSCs. The effects on extracellular components, morphology and gene expression of these was evaluated. The use of platelet lysates as a media supplement will also be compared to FBS or no supplement at all. In addition, the different cell types will be compared.

These objectives were analysed by the following methods:

1. Expansion and chondrogenic differentiation of hES-MP cells and MSCs
2. Measuring the GAG content in the chondrocytic pellets with a GAG assay
3. Histologic staining of chondrocytic pellets with Hematoxylin and eosin, Masson Trichrome and S100
4. Examining the gene expression of chondrogenic and extracellular markers, SOX9, Collagen I, COMP and Aggrecan in chondrocytic pellets

2 Material and methods

2.1 Preparation of human platelet lysates

Human platelet lysates (HPLs) were produced from platelet rich concentrates (PRCs). PRCs were produced with a routine procedure and then obtained from the blood-processing department of the Blood Bank Reykjavik, Iceland. As soon as they became outdated, they were stored at -80°C until HPL preparation (Figure 4). In this study, 12 units of PRCs were used for the HPL preparation, 8 PRCs from buffy coats and 4 PRCs from apheresis with total of 68 healthy Icelandic blood donors representing these 12 units of PRCs. All PCRs had been pathogen inactivated with the INTERCEPT system (Cerus, Amersfoort, Netherlands) according to manufacturer's instructions by trained personnel of the Blood Bank. After being frozen at -80°C, each PRC was completely thawed in a 37°C waterbath. After thawing, the PRCs were returned to -80°C. This was repeated three times. After thawing the PRCs for the third time, the PRCs were opened under sterile operating procedures, the content of all PRCs were pooled and then poured into 50 ml tubes (BD Biosciences, Bedford, MA, USA). The tubes were then platelet depleted with centrifugation for 20 minutes at 4975 x g. The supernatant was then transferred to empty 50 ml tubes through 40 µm cell strainers (BD Biosciences) but the pellet, consisting of platelet fragments, was discarded. Following a second centrifugation step for 20 minutes at 4975 x g, the supernatant was again transferred to empty 50 ml tubes through a cell strainer. The resulting HPLO (human platelet lysate outdated) was finally stored at -20°C until use for MSCs or hES-MP expansion.

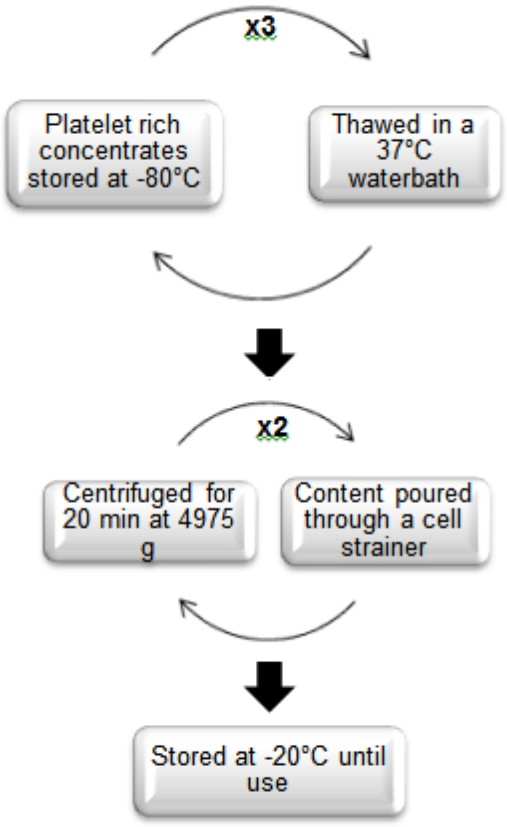

Figure 4 Preparation of human platelet lysates

Platelet rich concentrates (PRCs) undergo a specific process to produce human platelet lysates.

2.2 Cell expansion

hES-MP cells (Cellartis AB, Gothenburg, Sweden) and MSCs from two donors (D3 and D6) were seeded and expanded in two different supplemented media (10% HPLO and 10% FBS) until sufficient

amount of cells was available for either analysis or differentiation. MSCs were purchased from Lonza and had been screened for HIV-I, hepatitis B and C viruses (Lonza, Walkersville, MD, USA).

2.2.1 Seeding cells

Expansion media were prepared by adding 0,1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) the basal medium (DMEM F12+glutamax growth medium (Gibco)) and then either MSC screened FBS (Gibco) or HPLO cell culture supplement was added to a 10% concentration. Unmodified FBS was added to the expansion medium that was subsequently stored at 4-6°C. Before supplementing the expansion media with 10% HPLO, the HPLO was removed from -20°C storage and centrifuged at 4975 x g for 5 minutes. The supernatant was added to DMEM F12+glutamax growth medium and the expansion medium was made 10% concentrated. The 10% HPLO medium was sterile filtered by using a Stericup™ Sterile Vacuum Filter Unit (Millipore, Billerica, Massachusetts, USA) and applying a -0.9 bar pressure. Expansion media (10% HPLO and 10% FBS) were brought to 37°C prior to use.

Cell expansion flasks (BD Biosciences) for hES-MP cells were coated with 0,1% gelatin (SIGMA-ALDRICH® Taufkirchen, Germany) for 30 minutes at 4-6°C before seeding. There was no need for coating expansion flasks for MSCs due to their plasticity ability. Cryovials that contained either 0,5 x 10^6 MSC or hES-MP cells were removed from -180°C N_2 storage and thawed in 37°C water bath. Cells were added to 5 ml of warmed media (either 10% HPLO or 10% FBS) and centrifuged at 545 x g for 5 minutes. After centrifugation, supernatant was poured off and the cell pellet was re-suspended gently in 1 ml of warmed medium. Gelatin was poured off and warmed medium was added to all flasks. The cell suspension was then added to the flasks. All cells were incubated in 5% CO_2 incubator at 95% humidity and 37°C. This is referred to as standard culture conditions hereafter.

2.2.2 Cell subculturing and harvesting

Cells were subcultured when they had reached 85-90% growth confluency. Expansion media were warmed to 37°C as well as 0,25% Trypsin-EDTA (Gibco). Medium was poured out of the flasks and phosphate buffered saline (Gibco, PBS) added into the flasks. The cell culture surface was gently washed with PBS that was then disposed of into a waste disposal. Trypsin was pipetted into the flasks, allowed to completely cover the cell culture surface, and incubated at 37°C and 5% CO_2 for 3-5 minutes or until the cells were released from the flasks. Once the cells were released, warmed media was added into the flasks to neutralize the trypsin and release remaining cells. The content of the flasks were pipetted into a plastic tube and centrifuged at 509 x g for five minutes. Supernatant was poured into a waste disposal and the cell pellets re-suspended in warmed media. Cells were counted in a hemocytometer (Assistent, Munich, Germany) and the cell count per mL was determined. Cells were then either seeded into new expansion flasks or harvested. The cells were expanded in a standard culture conditions.

2.3 Cell counting

To acquire the right amount of cells for subculturing and any analysis, cell counting and calculation is necessary. After resuspending the cells in 1 ml of medium, 20 µl of sample was mixed with 50 µl of Trypan blue (Gibco) for staining, after diluting it with 30 µl of PBS. Stained samples were counted two times with an improved Neubauer hemocytometer in an inverted microscope (Leica Microsystems, Wetzlar, Germany) at 10x magnification (Figure 5) where cells are only counted in the white squares. Cell count per ml was then determined by using the formula; Cells/ml = $Cells_{Mean} * 5 * 10^4$

$Cells_{mean}$ symbolizes the mean amount of cells counted in all four white squares, five is the dilution factor and 10^4 is the conversion factor to convert the result to mL

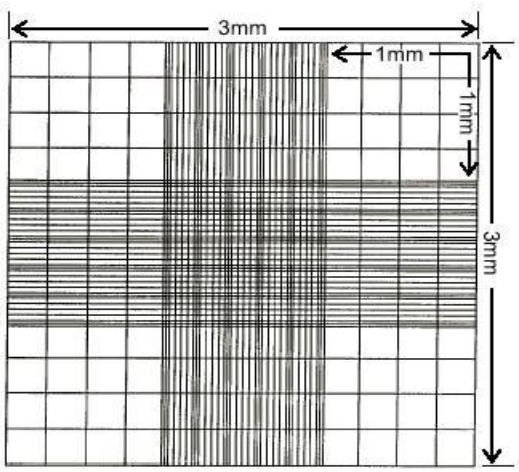

Figure 5 An improved Neubauer hemocytometer

Cells were stained with Trypan blue and counted two times in all four white squares at the end of each passage (Integrated Publishing).

2.4 Immunophenotyping of MSCs and hES-MP cells

hES-MP cells and MSCs that had been expanded in either 10% HPLO or 10% FBS were analysed with flow cytometry (FACS) in triplicates.

Cells were harvested as described above and counted. The required amount of cells for FACS analysis was approx. 100.000 cells. However, 150.000 hES-MP cells, from passage 3 (P3), from both HPLO and FBS expansion were harvested as well as 100.000 MSCs from D6 (P7) from both expansions. hES-MP cells from passage 3 (P3) and MSC (P7) from both media were stained for CD73, CD45, CD90, CD29, HLA-DR (BD Biosciences) and CD105 (Immunotech, Prague, CZ) according to manufactured instructions. Afterwards, the vials were centrifuged at 4°C for five minutes at 500 g and 500 µl of 0,5% paraformaldehyde in PBS was then added to the stained cells. The cells were then ready to be analysed. Each sample was vortexed for couple of seconds to mix the sample before it was measured in FacsCalibur (BD Biosciences).

2.5 Cartilage differentiation

2.5.1 Preparation of cartilage differentiation media

Three types of an in-house formula for chondrogenic media were prepared. These media contained either 10% HPLO, 10% FBS or no additional supplement. The content and preparation of these media are described in table 8.

Table 8 Differentiation media content

The ingredients needed to make 50 ml of each chondrogenic differentiation medium. DMEM-H is the basal medium.

Ingredients	10% HPLO (50 ml)	10% FBS (50 mL)	Without supplement (50 ml)
Dexamethasone (0,1 µm)	50 µl	50 µl	50 µl
Ascorbic acid (50 mM)	50 µl	50 µl	50 µl
Sodium Pyruvate (1mM)	0,5 ml	0,5 ml	0,5 ml
Proline (40 µg/ml)	0,5 ml	0,5 ml	0,5 ml
ITS+	0,5 ml	0,5 ml	0,5 ml
Penicillin/Streptomycin	0,5 ml	0,5 ml	0,5 ml
TGF-β3 (10 ng/µl)	5 µl	5 µl	5 µl
DMEM-H	X	42,9 ml	47,9 ml
FBS	X	5 ml	X
10% HPLO (HPLO + DMEM-H)	48,4 ml	X	X

TGF-β3 was diluted by dissolving 100 µg of TGF-β3 in 1 ml of 7,5 mM citric acid. The 1 ml of dissolved TGF-β3 was added to 9 ml of 1% BSA in PBS and then divided to 10x1 ml aliquots, making each 1 ml 10 µg/ml concentrated. Each 1 ml was then divided in 5 µl aliquots.

2.5.2 Seeding for cartilage differentiation

The three in-house formulas were used to differentiate MSCs (D3 and D6) and hES-MP cells to chondrocytic pellets. After the cells were expanded (in either 10% HPLO or 10% FBS) and trypsinated (Chapter 2.2.2), they were counted and 250.000 cells were seeded to each micro-tube, which contained 0,5 ml of complete chondrogenic media. When the cells had been added to the complete differentiation media, the micro-tubes were centrifuged for 5 minutes at 152 x g to concentrate the cells at the bottom. Tubes were punctured with a sterile needle to facilitate gas exchange before they were incubated at standard culture condition. The differentiation medium was replaced every 2-3 days.

2.5.3 Harvesting of chondrocytic pellets

When the chondrocytic pellets had reached 7, 14, 28 and 35 days in culture, they were harvested. For each time point, two pellets were harvested and placed in 1 ml 10% formalin for histological staining (Chapter 2.10). Five to six pellets were harvested and placed in 0,5 ml papain solution for GAG assay

(Chapter 2.9) and six to seven pellets were harvested and placed in 1 ml Trizol (Invitrogen) and stored at -80°C prior to RNA isolation (Chapter 2.8.1) and then later RT-qPCR (Chapter 2.8.3).

2.6 Osteogenic differentiation

MSC and hES-MP cells were differentiated towards an osteogenic lineage to demonstrate whether hES-MP cells have similar differentiation potential as MSCs. Cells were seeded at approximately 3800 cells/cm^2 of MSC (D6) and hES-MP cells (expanded in 10% HPLO or 10% FBS) were seeded in 12-well plates in sextuplicates, after being trypsinated (Described in chapter 2.2.2). The hMSC Osteogenic Differentiation BulletKit™ was purchased from Lonza and used to differentiate the cells to an osteogenic lineage. This kit contained basal medium and a SingleQuots kit. When the ingredients in the SingleQuots kit (Table 9) were added to the basal medium, the osteogenic differentiation medium was complete.

Table 9 Content of the Osteogenic SingleQuots kit

List of ingredients, added to the basal differentiation medium.

1,0 mL	**Dexamethasone**
2,0 mL	Ascorbate acid
20 mL	MCGS
2,0 mL	B-Glycerolphosphate
2,0 mL	Penicillin/Streptomycin
4,0 mL	L-Glutamine

2.7 Alkaline phosphatase (ALP) analysis

The alkaline phosphatase (ALP) activity in osteogenic cultures was monitored by performing an ALP activity assay after 0, 7, 14, 21 and 28 days in osteogenic differentiation. To obtain day 0, cells were expanded in 10% FBS or 10% HPLO for 7 days.

Cells were washed three times with PBS and 0,02% Tritonx100 (Sigma) in PBS was added to remove the cells from the surface. Cells were scraped loose from the surface with a pipette tip. When the cells had been scraped off, they were transferred to microcentrifuge tubes and vortexed strenuously for 30 seconds. Tubes were then centrifuged at 15700 x g at 4°C for 15 minutes. Meanwhile, a p-nitrophenyl phosphate (pNPP) solution was prepared by dissolving a pNPP and buffer tablets (Sigma) in dH_2O. After centrifugation, supernatant was transferred to new micro-tubes filled up with an equal amount of pNPP solution before incubated for 30 or 45 minutes in an incubator at 37°C. The solutions were then transferred into a 96-well plate in triplicates and the optical density was

measured in Multiskan® spectrum spectrometer (Thermo Scientific, Vantaa, Finland) at 400 nm. ALP was then converted from p-nitrophenyl phosphate to nMol/min by using formula 1.

Formula 1 ALP activity

The following formula was used to convert p-nitrophenyl phosphate into p-Nitrophenol as nMol/min. The measure optical density (A) was divided by 18,8 (the extinction coefficient) and divided by the time of incubation (30 or 40 minutes). The amount was then multiplied by 1000 to obtain the concentration in nMol/min.

$$nMol/_{min} = \frac{A/_{18,8}}{(\mathbf{30\ or\ 45})} * 1000$$

2.8 Gene expression analysis

2.8.1 RNA isolation

Chondrocytic pellets were harvested and stored at -80°C in 1 ml Trizol (Invitrogen) until RNA was isolated from samples. Before the RNA isolation procedure was executed, the pellets had to be disassociated in a homogenizer. Pellets were transferred in Trizol to gentleMACS M tubes (Miltenyi Biotec, Bergish Gladbach, DE), which are specialised tube for the gentleMACS Dissociator (Miltenyi Biotec), where the pellets were homogenized for future steps.

When samples had been homogenized, they were incubated for 5 minutes at room temperature to enable complete dissociation of the nucleoprotein complex. 0,2 ml of chloroform (Invitrogen) was added to each sample and tubes were vortexed for 15 seconds. Samples were incubated for 2-3 minutes at room temperature before centrifuging them at 12.000 x g for 15 minutes at 4°C. During the centrifugation, the samples separated into a lower red phenol-chloroform phase, named interphase, and a colourless upper aqueous phase, where RNA remains exclusively. The aqueous phases were removed and placed in another tube and 0.5 ml of 100% Isopropanol was added to the aqueous phase of each sample. Samples were incubated at room temperature for 10 minutes and then centrifuged at 12.000 x g for 10 minutes at 4°C.

Supernatant was aspirated of the RNA pellets that had formed during the centrifugation and the pellets washed with 1 ml of 75% ethanol, vortexed briefly, and centrifuged at 7.500 x g for 5 minutes at 4°C. Afterwards, the wash was discarded. The RNA pellets were air dried for 5-10 minutes and then resuspended in 50 µl RNase-free water. The solutions were then incubated in a heat block at 55-60°C for 10-15 minutes.

An RNeasy Plus Mini Kit (Qiagen, Venlo, Limburg, Netherlands) was used for the rest of the RNA isolation. After the solutions were incubated in a heat block, they were transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and 300 µl of RLT buffer containing β-mercaptoethanol (β-ME) was added to the column as well. Columns were centrifuged for 30 second at ≥8000 g and the flow-through was preserved. 350 µl of 70% ethanol was added to each flow-through

and mixed well. 700 µl of the samples were transferred to the RNeasy spin columns placed in 2 ml collection tubes. Then centrifuged for 15 seconds at ≥8000 g and the flow-through discarded. 700 µl of Buffer RW1 was added to the RNeasy Mini spin columns (in 2 ml collection tubes) and centrifuged for 15 seconds at ≥8000 g and the flow-through discarded. 500 µl Buffer RPE was added to the RNeasy spin columns. Then centrifuged for 15 seconds at ≥8000 g and the flow-through discarded. 500 µl Buffer RPE was added to the RNeasy spin columns. Then centrifuged for 2 minutes at ≥8000 g and the flow-through discarded. The RNeasy spin columns were placed in a new 1,5 ml collections tubes and 50 µl of RNase-free water was added directly to the spin column membranes. Spin columns were centrifuged for 1 minute at ≥8000 g to elute the RNA. The columns were then discarded and the collections tubes kept in -80°C until cDNA synthesis.

2.8.1.1 RNA quantification

The RNA was analysed for quantity by spectrophotometer NanoDrop ND-1000 (Thermo Scientific) according to manufacturer's instruction.

2.8.2 cDNA synthesis

After RNA had been isolated from all samples and quantified, cDNA was synthesized from the RNA using enzymatic reactions involving reverse transcriptase.

First, master mix components from table 10 were thawed on ice. Afterwards, the components were mixed together and the master mix was complete. The volumes given in table 10 are for a single sample so each volume was multiplied by the number of RNA samples, given each time this procedure was done.

When the samples and master mix were ready, 25 µl of master mix and 25 µl of RNA sample were added into RNAse free cDNA tubes (Nunc, Roskilde, Denmark) and mixed well, making each solution 50 µl. When all samples had been mixed with master mix, the tubes were centrifuged at 1244 x g for few seconds and then placed in the thermal cycler set to the four following steps. First, 10 min at 25°C, second, 120 min at 37°C, third, 5 sec at 85°C and finally, 4°C until samples were removed from the thermal cycler and placed in -20°C storage.

Table 10 Master mix components

Master mix was obtained by mixing all of these components (Applied Biosystems, Foster city, CA, USA).

	Volume (µl)/reaction
Nuclease free H_2O	8
10x RT buffer	5
10cx Random primers	5
RNAse inhibitor	2,5
25x dNTP	2
Multiscripe^{TM} Reverse Transcriptase	2,5
Total per reaction	**25 µl**

2.8.3 Real time qPCR

The gene expression of SOX9, COMP, Aggrecan and Collagen type I (Applied Biosystems) was analysed by real time quantitative PCR in Step One Real-Time PCR system (Applied Biosystems). Each cDNA sample was 1:10 diluted and 9 µl of the sample was mixed with 11 µl of an assay stock (10 µl Taqman master mix (Applied Biosystems) combined with 1 µl of the respective Taqman® Gene Expression assay (Applied Biosystems)). Samples were added to a 48 microwell plate in duplicates, the plate sealed and centrifuged at 1244 x g for few seconds. The plate was added to the PCR system and allowed to cycle for 40 cycles (Table 11). Two housekeeping genes, TBP and YWHAZ were also examined and used as reference genes. The reason why these housekeeping genes were used as reference genes was a result of an analysis performed on several housekeeping genes, which determined that these two genes are best suitable as reference genes for this type of stem cells. The data was analysed with REST-384 beta version 2 [August 2006] software within Microsoft excel, by comparing C_T values.

Table 11 RT-qPCR cycle protocol

Appropriate genes were analysed with RT-qPCR in Step One Real-Time PCR system. These two steps represent the procedure that samples undergo within the Step One Real-Time PCR system.

Step	Cycles	Temperature (°C)	Time
1	1	50°C	2 min
		95°C	10 min
2	40	95°C	15 sec
		60°C	1 min

2.9 Glycosaminoglycan assay

GAG assay is used to determine the production of extracellular matrix during chondrogenic differentiation. Chondrocytic pellets were harvested after 7, 14, 28 and 35 days and digested and then allowed to dissolve with 0,5 ml of papain extraction reagent (PER) in a heat block at 65°C for 2-4 hours. The ingredients of PER are listed in table 12. When the chondrocytic pellets had been dissolved, the samples were stored frozen at -80°C until the assay was performed.

Table 12 Ingredients of papain extraction reagent

Ingredients of papain extraction reagent	Amount
0,2 M sodium phosphate buffer pH 6,4	50 ml
0,1 M sodium acetate	400 mg
0,01 M Na_2EDTA	200 mg
0,005 M cysteine HCl	40 mg
Crystallized papain suspension	40 µl

A Blyscan kit (Biocolor Ltd., Carrickfergus, UK) contained all required ingredients for the rest of the assay. Standards were prepared by using deionised water and PER as blanks and aliquots that contained 1, 2, 3, 4 and 5 µg of the reference standard, making the volume of each standard 150 µl. These standards were used to obtain a calibration curve.

Micro-tubes were prepared and 1 ml of Blyscan dye reagent was added to all the tubes. In addition, 100 µl of each standard or sample was added as well to the appropriate tubes. The tubes were then placed on a mechanical shaker for 30 minutes. Afterwards, the tubes were centrifuged for 10 minutes at 14.300 x g. The unbound dye was drained carefully and 0,5 ml of dissociation reagent was added to each tube. Samples and standards were vortexed to release the bound dye into the solution and 200 µl of each tube was then transferred to individual wells in 96-well microplate and measured, in duplicates, at 656 nm in a microplate reader.

After measurement, calibration curve was prepared and the equation for the curve was then used to determine the GAG concentration in the samples.

2.10 Chondrocytic pellet staining

All processing and staining of chondrocytic pellets was performed by experienced biomedical scientists at the pathological laboratory at Landspítali Háskólasjúkrahús in Reykjavík. The chondrocytic pellets were stained with Haematoxylin and eosin (H&E), S100 immunostaining and Masson Trichrome.

2.11 Statistical analysis

CellQuestPro software 4.0.2 and GraphPad® Prism version 5.0 software were used to analyse results from FACS. Graphpad® and Microsoft Excel 2007 were used to analyse results from GAG assay and alkaline phosphatase activity assay. REST-384 beta software version 2 [August 2006] software within Microsoft excel 2007 and Graphpad® were used for gene expression analysis and the statistical significance examination for the gene expression analysis. Two-way and one-way ANOVA were used and student's t-test was used to confirm statistical significance for MSC chondrocytic pellets GAG assay and alkaline phosphatase activity assay.

3 Results

3.1 Phenotyping and osteogenic differentiation

In order to confirm the mesenchymal properties of the hES-MP cells and the MSC cells, we analysed the osteogenic differentiation and the phenotype of the cells. MSCs and hES-MP cells that had been expanded for two passages in either 10% FBS or 10% HPLO supplemented basal media, were harvested and cell surface marker expression was measured using flow cytometry. This was done in order to compare the phenotypes of hES-MP and MSCs (based on their cell surface marker expression), and to detect potential phenotype differences after expansion in 10% FBS or 10% HPLO. Cell surface markers CD73, CD45, CD105, CD29, CD90 and HLA-DR were analysed and their expression was considered positive (+) if they had an expression >95% and negative (-) if their expression was <2%. No difference in the expression of these surface markers between the two cell types was detected nor was there any difference between cells cultured in HPLO or FBS supplemented media. These results are displayed in table 13, located in Appendix A.

MSCs and hES-MP cells that had been expanded in either 10% FBS or 10% HPLO supplemented media for two passages were differentiated in osteogenic media to analyse their osteogenic potential. An ALP activity assay was performed to estimate their osteogenic differentiation. Both cell types, hES-MP and MSCs showed a potential for osteogenic differentiation as indicated by an increase in alkaline phosphatase activity over the three week differentiation period. These results are displayed in figure 30, located in Appendix B. A more detailed analysis of the osteogenic differentiation and immunomodulation properties of these cells has been performed by other members of our research group (data not shown).

3.2 Chondrogenic differentiation

MSCs and hES-MP cells were differentiated to a chondrogenic lineage using a micromass culture that forms cartilage-like pellet structures, using three types of an “in-house” formula basal differentiation media containing either 10% FBS, 10% HPLO or without additional supplements (N.S.). Cartilage pellets were harvested at days 7, 14, 28 and 35 and analysed for chondrogenic differentiation using immunohistochemistry, GAG assay and RT-qPCR. In order to better display the effects of the different media supplements we chose to present the data based on dates of differentiation both for hES-MP and MSCs in the following sections. Histological stainings of MSC D3 are shown in Appendix C.

3.2.1 Chondrogenic differentiation - Day 7

Histological staining of hES-MP pellets after 7 days of differentiation are shown in figure 6. The H&E staining displayed an elongated cellular morphology at the edges of all pellets. The Masson Trichrome staining showed that all pellets had begun forming collagen fibers (stained blue) and the S100 staining showed that S100 proteins were scattered in every pellet.

GAG concentrations in hES-MP pellets are displayed in figure 7, where the GAG concentration in the N.S. pellets (0,4002 ± 0,019 µg, N=2) was higher than the GAG concentration in both 10% HPLO (0,1804 ± 0,015 µg, N=2) and 10% FBS (0,3010 ± 0,006 µg, N=2) pellets. The 10% FBS pellets also had a higher GAG concentration than 10% HPLO pellets.

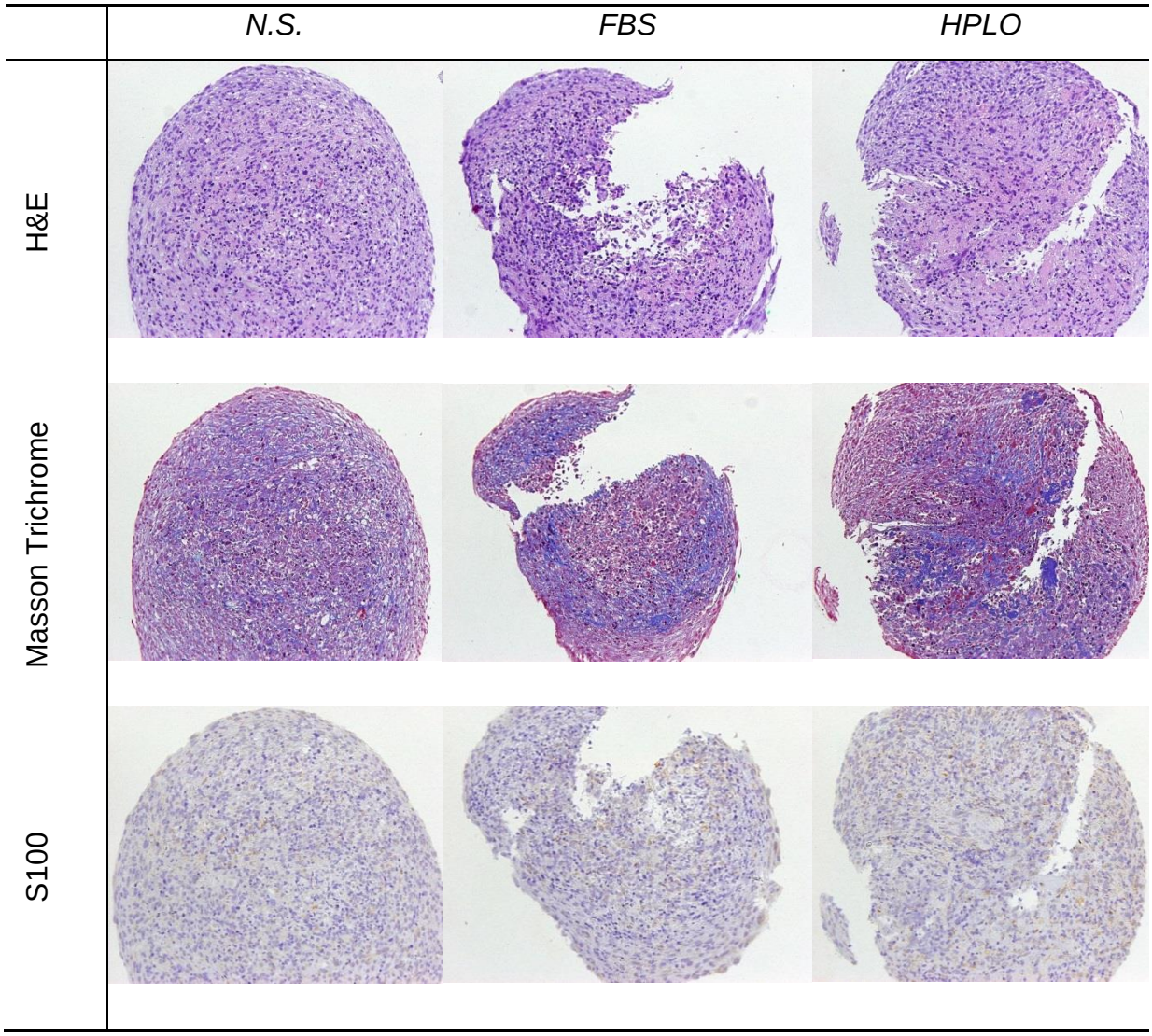

Figure 6 hES-MP chondrocytic pellets stained after 7 days of differentiation

The hES-MP cells were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

Gene expression of SOX9, and Collagen I is displayed in figure 8. The expression of COMP was not detected in hES-MP pellets differentiated without a supplementary media and Aggrecan was not detectable in all hES-MP pellets. The fold change of gene expression refers to an increase or a decrease of gene expression relative to that of pellets differentiated without a supplementary media

(N.S.), which is given the fold change of 1. Thus, the numbers in gene expression analysis represent the fold change when compared to the gene expression of N.S. pellets. The gene expression of SOX9 was lower in hES-MP pellets differentiated with 10% FBS (0,10, N=2) and 10% HPLO (0,24, N=2) when compared to N.S. pellets, and the gene expression of Collagen I was higher in hES-MP pellets differentiated with 10% HPLO (2,40, N=2).

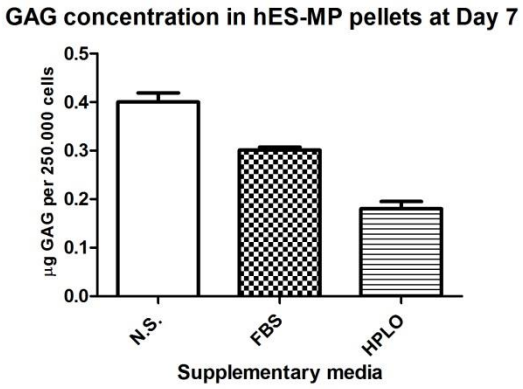

Figure 7 Glycosaminoglycan concentration in hES-MP pellets at day 7

The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of hES-MP pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in N.S. pellets was higher than the GAG concentration in both FBS and HPLO pellets. HPLO pellets also had a lower GAG concentration than FBS pellets. N.S. = No supplement.

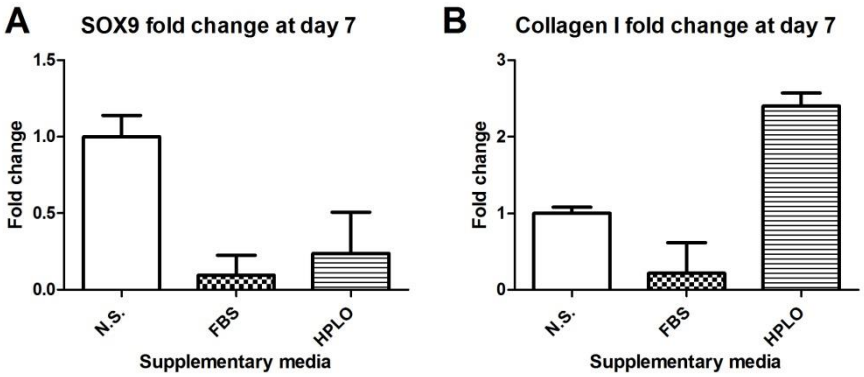

Figure 8 Comparison of gene expression between differentiation media at day 7

Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in hES-MP pellets (y-axis). A) SOX9 expression in both FBS and HPLO pellets was lower than SOX9 expression in N.S. pellets B) Collagen I expression in HPLO pellets was higher than in N.S. pellets. N.S. = No supplement.

The H&E staining for MSC pellets harvested at day 7 indicates that all pellets displayed an elongated cellular morphology at the edge of the pellets (Figure 9). HPLO pellets showed a greater amount of extracellular matrix (stained pink) than pellets from other media. The Masson Trichrome staining indicated that collagen fibers had started to form in all pellets. The HPLO pellets did however display more collagen fibers than other pellets, which corresponds to the greater amount of extracellular matrix, which is apparent in the H&E staining. The S100 staining demonstrated that the FBS pellets had a higher amount of S100 proteins than pellets differentiated with other media.

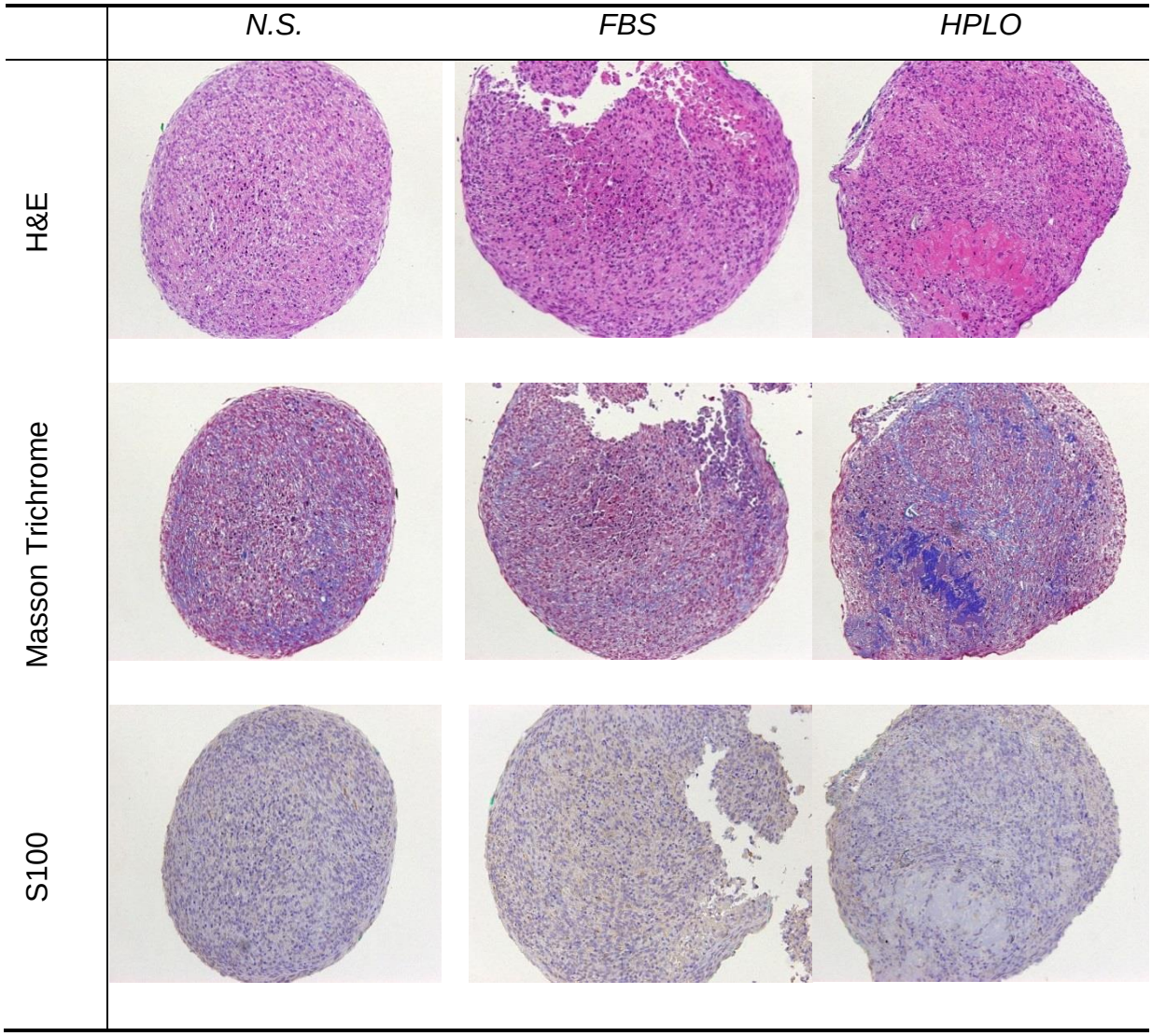

Figure 9 MSC D6 chondrocytic pellets stained after 7 days of differentiation

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

The GAG concentrations in these MSC pellets are displayed in figure 10. The HPLO pellets (0,45 ± 0,0063 µg, N=4) had a significantly higher concentration of GAG than the FBS pellets (0,31 ± 0,0054 µg, p<0,001, N=4) and N.S. pellets (0,27 ± 0,016 µg, p<0,001, N=4).

The gene expression of SOX9, Collagen I, Aggrecan and COMP is presented in figure 11. The SOX9 gene expression was significantly lower in FBS pellets (0,64, p<0,001, N=4) than in N.S. pellets. The expression was also lower in HPLO pellets (0,88, p>0,05, N=4) but without a significant difference. No significant difference was detected between differentiated pellets when the Collagen I gene expression was examined. The gene expression of Aggrecan and COMP was more than 10x lower in the HPLO pellets (0,08, N=4) in both examinations (p<0,001) with a significant difference from the gene expression in N.S. pellets.

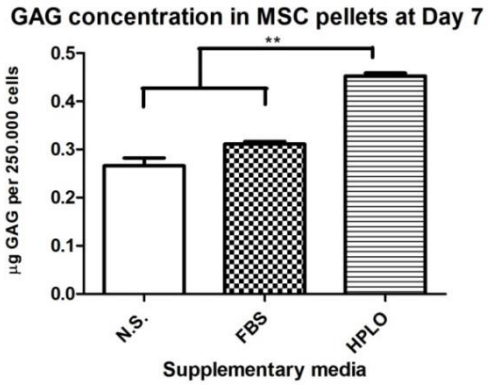

Figure 10 Glycosaminoglycan concentration in MSC pellets at day 7

*The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of MSC pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in HPLO pellets was significantly higher than the GAG concentration in both FBS and N.S. pellets (p<0,001). **=p<0,001. N.S. = No supplement.*

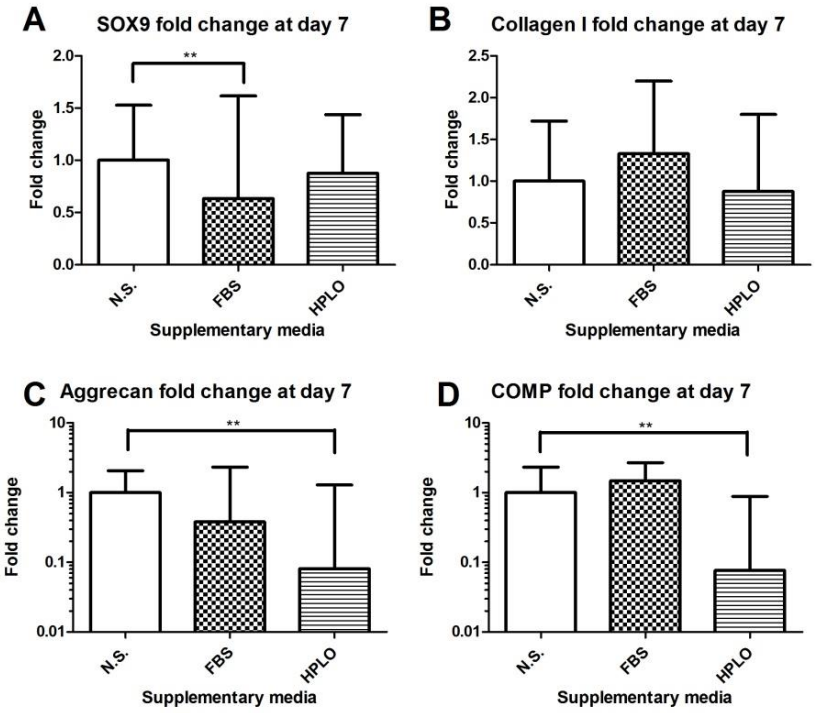

Figure 11 Comparison of gene expression between differentiation media at day 7

*Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in MSC pellets (y-axis). The y-axis is log scaled at log^{10} in graphs C and D. A) SOX9 expression in both MSC FBS and HPLO pellets was lower than SOX9 expression in N.S. pellets, but only with a significant difference between the expression in FBS and N.S. pellets (p<0,001). B) Collagen I expression was similar between media with no significant difference (p>0,05). C) Aggrecan expression was significantly lower in HPLO pellets (p<0,001). D) COMP expression was significantly lower in HPLO pellets (p<0,001). **=p<0,001. N.S. = No supplement.*

3.2.2 Chondrogenic differentiation - Day 14

hES-MP pellets differentiated with 10% HPLO showed an increased amount of extracellular matrix when the H&E staining was compared to the H&E staining at day 7 (Figure 12). The amount of nuclei (stained purple) also had decreased between time points and the elongated cellular morphology was more prominent. The N.S. pellets did not change between days 7 and 14, except the pellets appeared to have an increased amount of extracellular matrix. The elongated cellular morphology expanded within the FBS pellet. The collagen fibers had increased greatly from day 7 in all pellets and the S100 proteins seemed to have decreased in all pellets from day 7 to day 14.

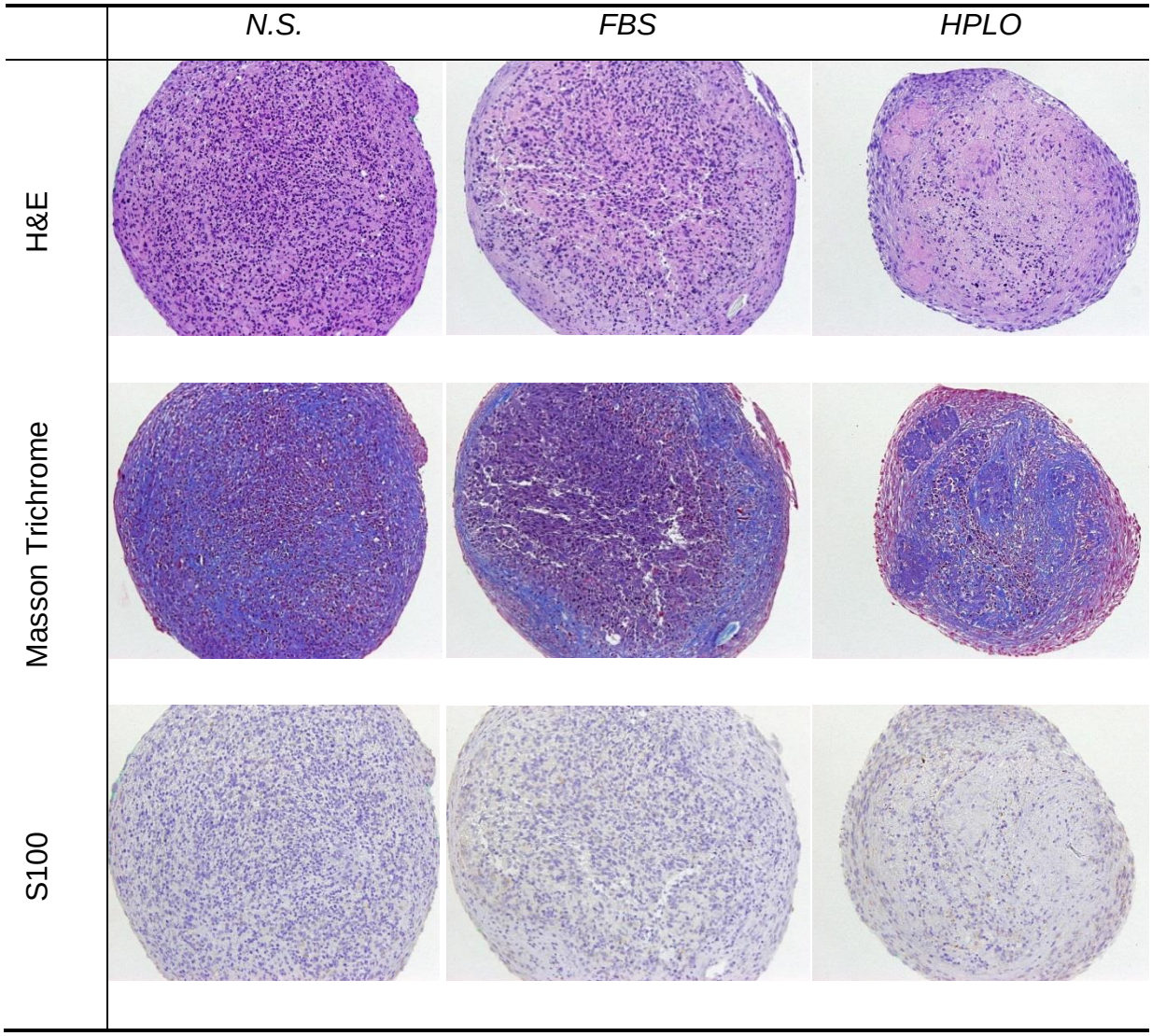

Figure 12 hES-MP chondrocytic pellets stained after 14 days of differentiation

The hES-MP cells were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

The GAG concentration (Figure 13) was similar in all types of pellets where the GAG concentration in N.S. pellets was 0,25 ± 0,067 (N=2), 0,21 ± 0,019 (N=2) in HPLO pellets and 0,20 ± 0,023 (N=2) in FBS pellets.

The gene expression (Figure 14) of SOX9 at day 14 was lower in FBS pellets, with the fold change of 0,7 when compared to N.S. pellets (N=2), and the SOX9 expression was higher in HPLO pellets (1,5, N=2). The gene expression of Collagen I was higher in both FBS pellets (4,2, N=2) and HPLO pellets (5,3, N=2).

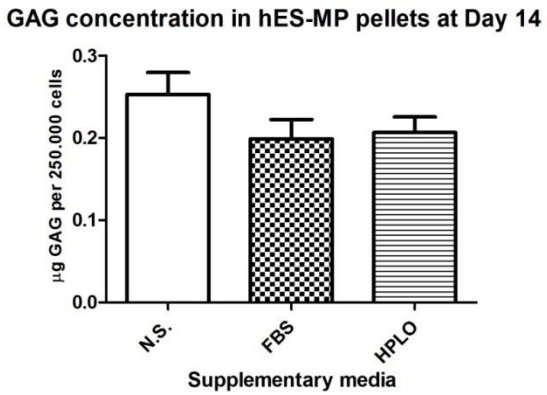

Figure 13 Glycosaminoglycan concentration in hES-MP pellets at day 14

The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of hES-MP pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration was similar in all types of pellets. N.S. = No supplement.

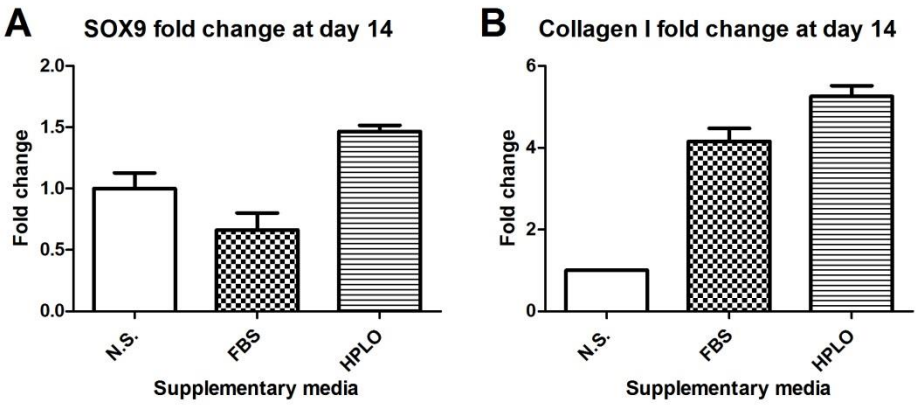

Figure 14 Comparison of gene expression between differentiation media at day 14

Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in hES-MP pellets (y-axis). A) The SOX9 expression in FBS pellets was lower than the expression in N.S. pellets. B) Collagen I expression in HPLO pellets was higher than in N.S. pellets. N.S. = No supplement.

The H&E staining of MSC pellets (Figure 15) harvested at day 14 showed that the elongated cellular morphology had greatly increased and moved farther inside the HPLO pellet, and the extracellular matrix has increased. The FBS pellets and the N.S. pellets showed an increased elongated cellular morphology but not as much as the HPLO pellets. The extracellular matrix seemed to have increased in both types of pellets. The Masson Trichrome staining showed that the collagen fibers had increased in all pellet types from day 7 (Figure 9) but with minor difference between the FBS pellets at day 7 and day 14. The amount of S100 proteins seemed similar between day 7 and day 14 in all types of pellets.

Similar to day 7 (Figure 10), the GAG concentration was higher in the HPLO pellets with however no significant difference between media (p>0,05) (Figure 16).

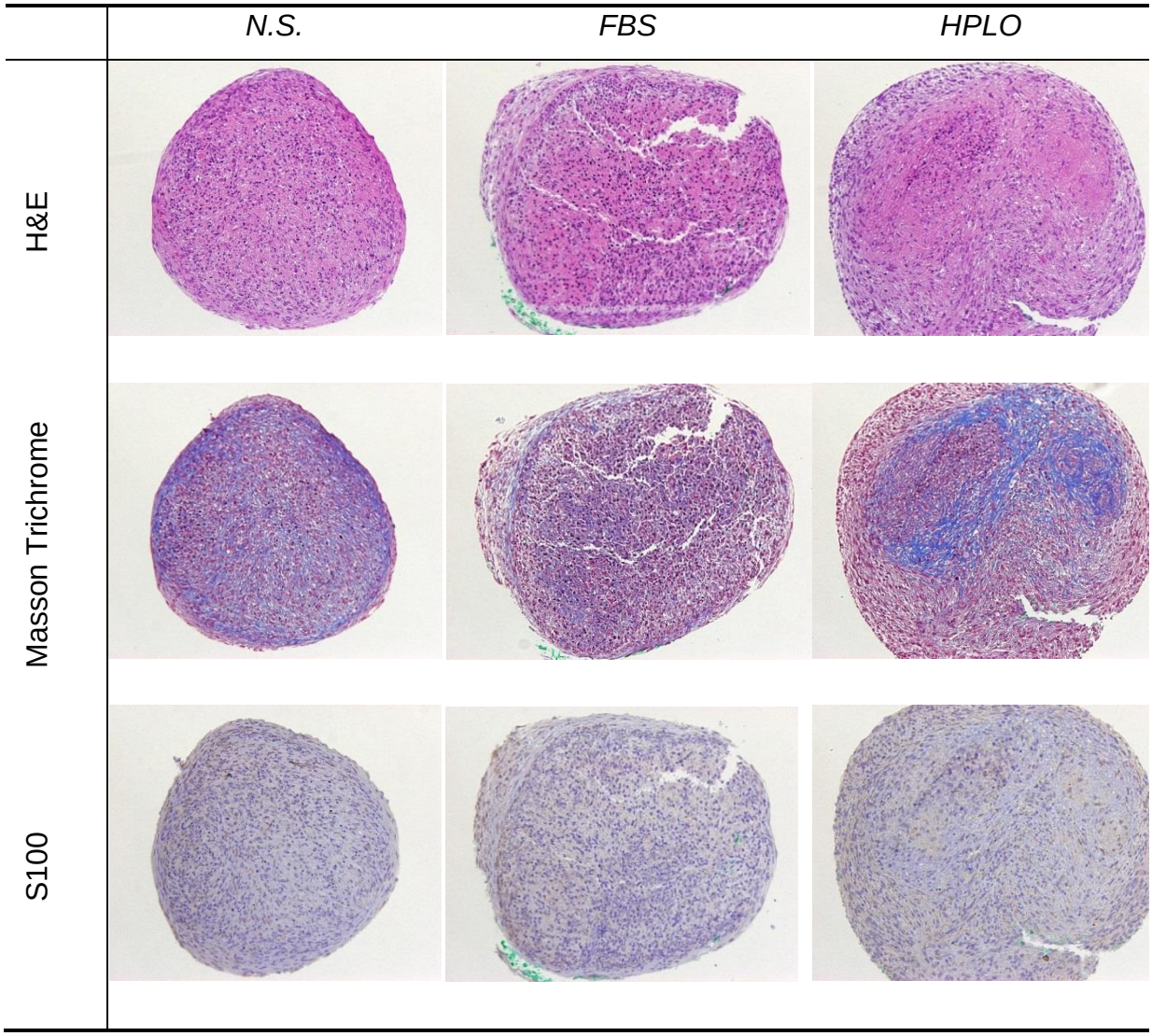

Figure 15 MSC D6 chondrocytic pellets stained after 14 days of differentiation

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

When the gene expression of SOX9, Collagen I, Aggrecan and COMP was examined in MSC pellets (Figure 17), it showed that there was no significant difference between any types of media except for the Aggrecan fold change. The FBS pellets (0,35, p<0,001, N=4) had a significantly lower expression than the N.S. pellets. The HPLO pellets had a decreased expression of all genes when compared to N.S. pellets, but with no significant difference (p>0,05).

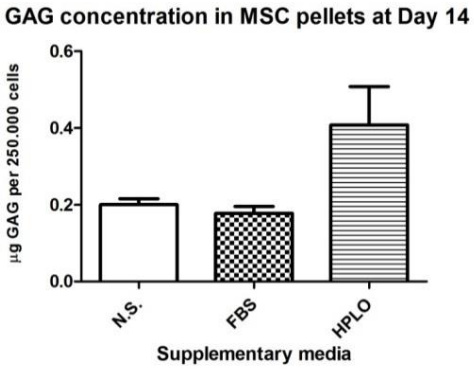

Figure 16 Glycosaminoglycan concentration in MSC pellets at day 14

The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of MSC pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in HPLO pellets was higher than the GAG concentration in both FBS and N.S. pellets, with however no significant difference (p>0,05). N.S. = No supplement.

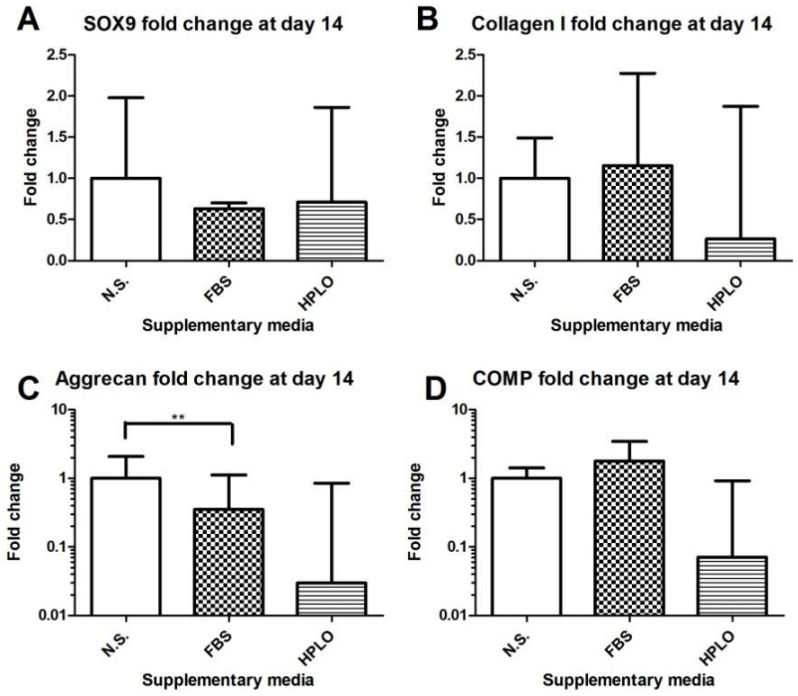

Figure 17 Comparison of gene expression between differentiation media at day 14

*Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in MSC pellets (y-axis). The y-axis is log scaled at log^{10} in graphs C and D. A) Gene expression was similar with no significant difference between media (p>0,05). B) Collagen I expression was without a significant difference between media (p>0,05). C) Aggrecan expression was significantly lower in FBS pellets (p<0,001). D) COMP expression was without a significant difference between media (p<0,05). **=p<0,001. N.S. = No supplement.*

3.2.3 Chondrogenic differentiation at day 28

The staining of hES-MP pellets after 28 days of differentiation can be seen in figure 18. The nuclei kept decreasing in the HPLO pellets while the extracellular matrix increased. The elongated cellular morphology kept increasing in both HPLO and FBS pellets but the N.S. pellets kept the same morphology from day 7 (Figure 7). The Masson Trichrome staining showed a noteworthy increase of collagen fibers in the HPLO pellet, making it almost completely covered with collagen fibers. The collagen fibers in the FBS pellets were mostly located at the edge of the pellet and they seemed to have decreased from day 14 (Figure 12) to day 28 in the N.S. pellets. The S100 staining showed that the amount of S100 proteins had decreased in HPLO and FBS pellets from day 14. The amount seemed to be similar between day 14 and 28 in the N.S. pellets.

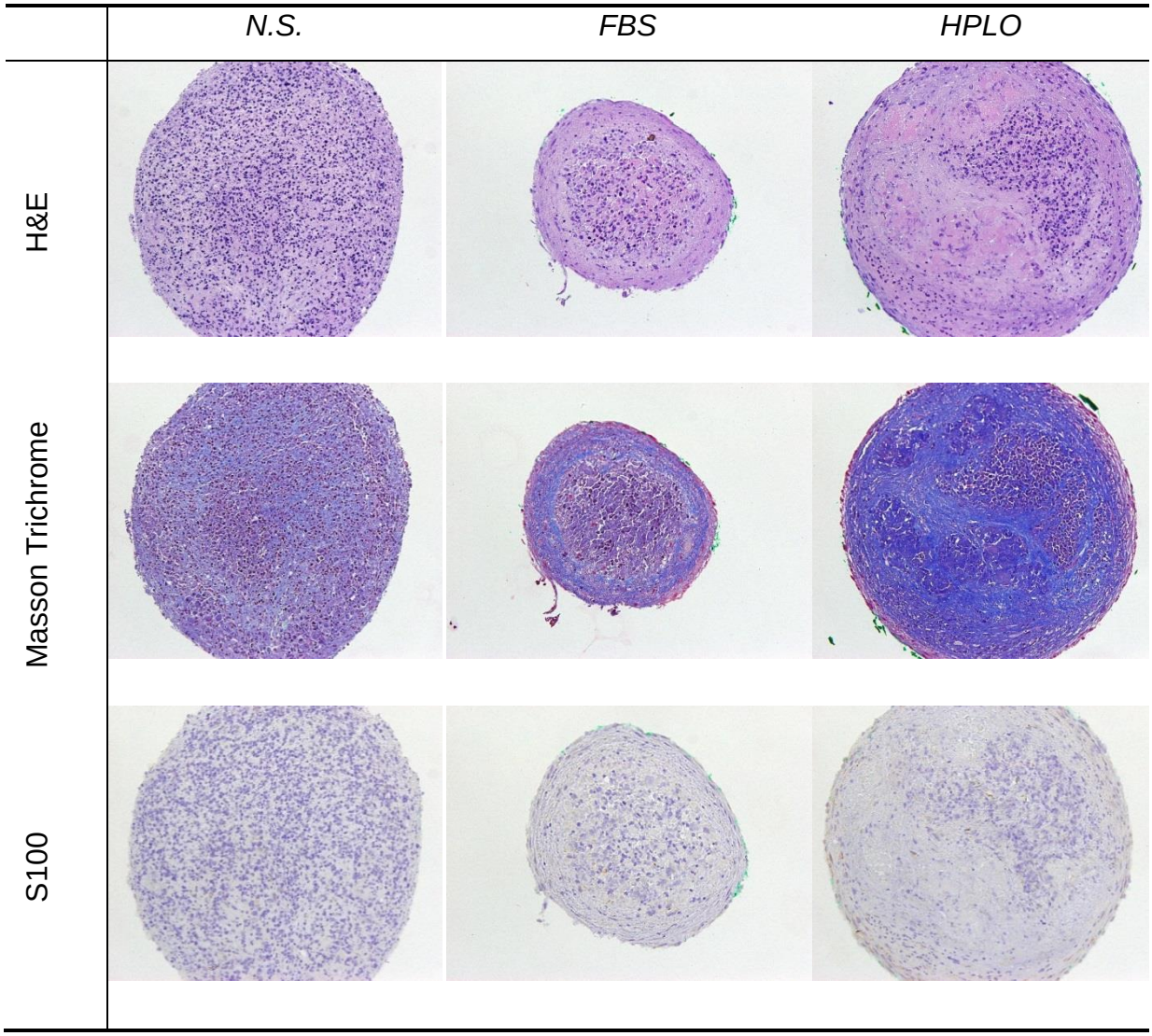

Figure 18 hES-MP chondrocytic pellets stained after 28 days of differentiation

The hES-MP cells were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

The GAG concentration in hES-MP pellets is shown in figure 19. The GAG concentration in HPLO pellets (0,12 ± 0,015 µg, N=2) was higher than in FBS pellets (0,05 ± 0,006 µg, N=2) and N.S. pellets (0,02 ± 0,005 µg, N=2).

The gene expression analysis of SOX9 showed that the FBS (0,072, N=2) and HPLO (0,202, N=2) pellets had a lower SOX9 expression than N.S. pellets (N=2). This is opposite to the Collagen I expression, where the FBS (6,360, N=2) and HPLO (5,053, N=2) pellets had a higher expression than N.S. pellets (Figure 20).

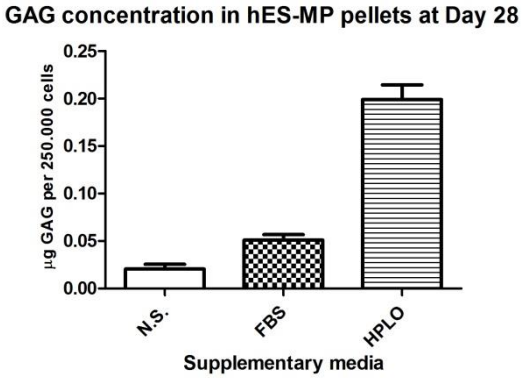

Figure 19 Glycosaminoglycan concentration in hES-MP pellets at day 28

The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of hES-MP pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in HPLO pellets was higher than the GAG concentration in both FBS and N.S. pellets. N.S. = No supplement.

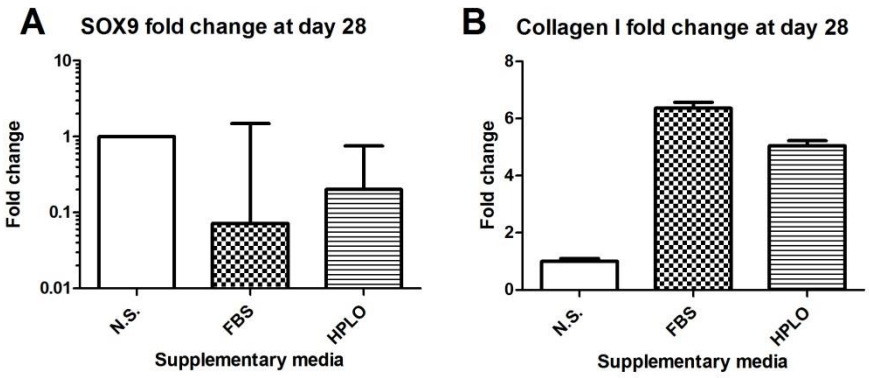

Figure 20 Comparison of gene expression between differentiation media at day 28

Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in hES-MP pellets (y-axis). The y-axis is log scaled at log^{10} in graph A. A) SOX9 expression in both FBS and HPLO pellets was lower than SOX9 expression in N.S. pellets. B) The Collagen I expression in HPLO and FBS pellets was higher than in N.S. pellets. N.S. = No supplement.

The staining of MSC pellets after 28 days of differentiation can be seen in figure 21. The H&E staining showed that the extracellular matrix had increased greatly and the nuclei had decreased in HPLO pellets. The morphology of N.S. and FBS pellets was similar to pellets harvested at day 14 (Figure 15). The Masson Trichrome staining showed that the collagen fibers had increased greatly in the HPLO pellet. The FBS and N.S. pellets showed an increased amount of collagen fibers but not as much as in the HPLO pellet. The S100 staining showed a decrease of S100 proteins in HPLO and FBS pellets but their amount seemed to have increased from day 14 in the N.S. pellets.

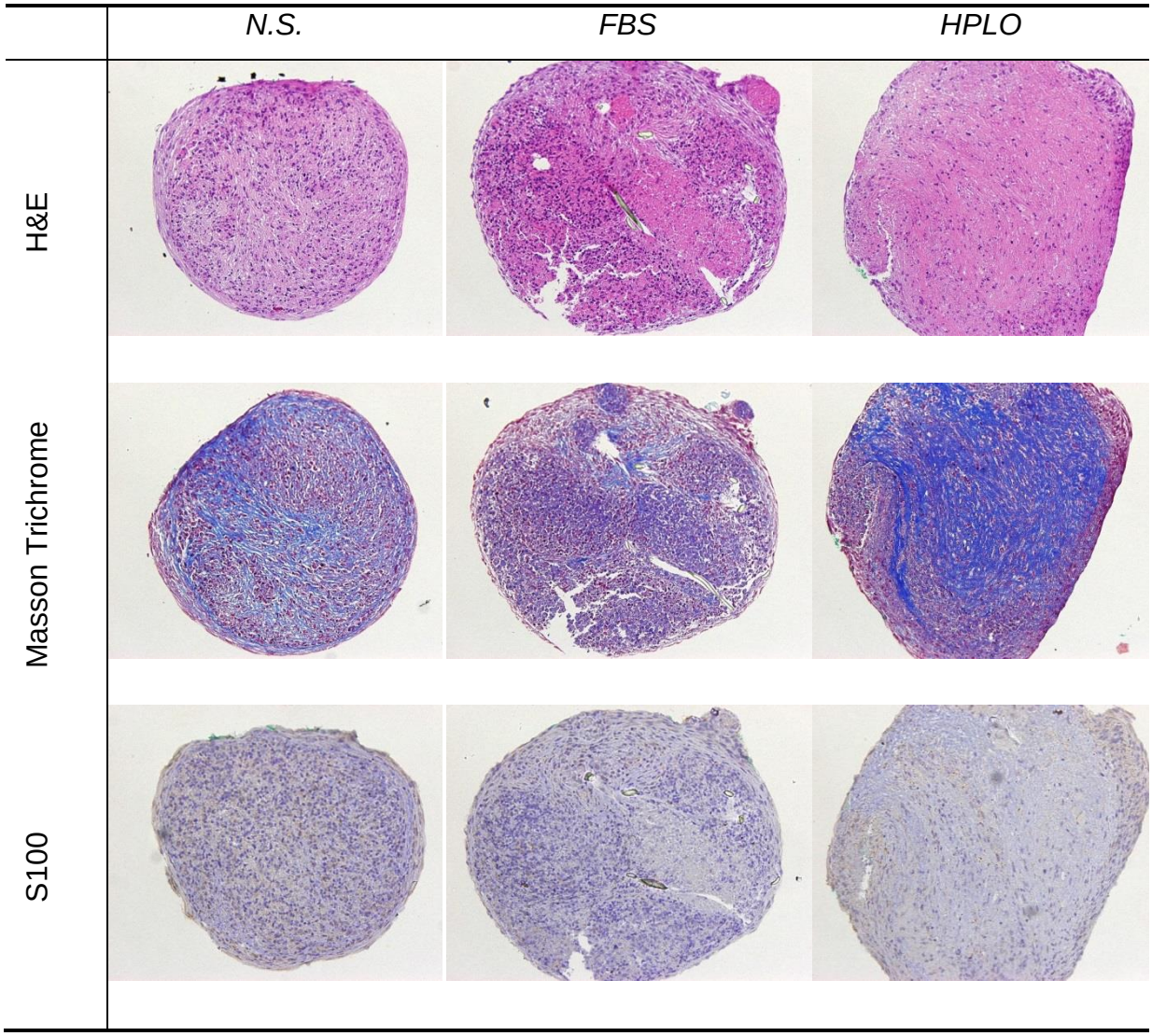

Figure 21 MSC D6 chondrocytic pellets stained after 28 days of differentiation

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

The GAG concentration in MSC pellets at day 28 is displayed in figure 23. The HPLO pellets (0,48 ± 0,0008 µg, N=4) had a greater GAG concentration than pellets from other media, but only had a significantly higher GAG concentration than FBS pellets (0,14 ± 0,08 µg, p<0,05, N=4).

The gene expression of SOX9, Collagen I, Aggrecan and COMP are shown in figure 22. No significant difference was between media when the SOX9 expression was analysed (p>0,05). Collagen I expression in HPLO (0,24, N=4) pellets was significantly lower than in N.S. pellets (p<0,001). The HPLO pellets (0,11, N=4) also had a significantly lower expression of Aggrecan compared to N.S. pellets (p<0,001). The COMP expression was significantly lower in both FBS (0,451, N=4) pellets and HPLO pellets (0,055, N=4) when compared to N.S. pellets (p<0,001).

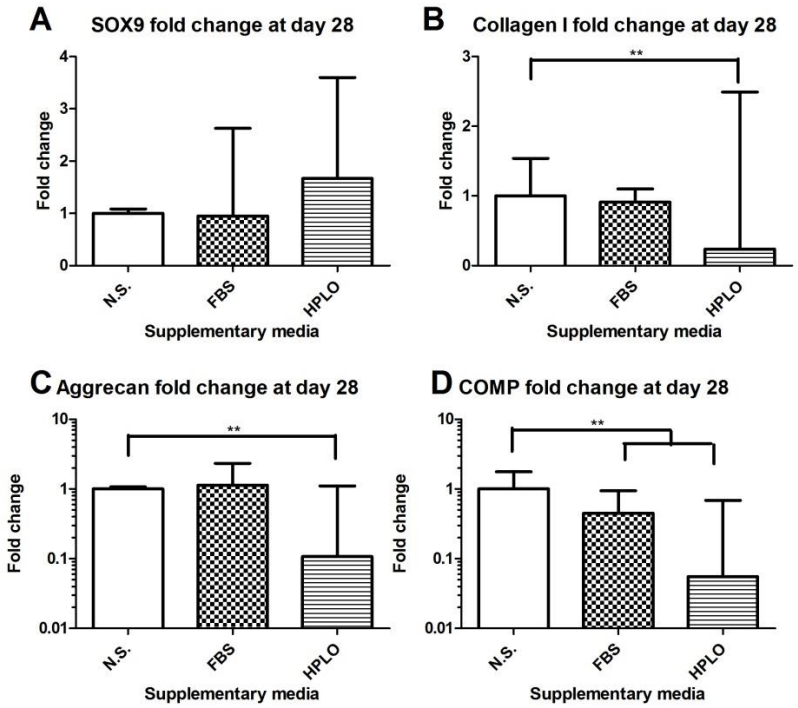

Figure 22 Comparison of gene expression between differentiation media at day 28

*Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in MSC pellets (y-axis). The y-axis is log scaled at log^{10} in graphs C and D. A) No significant difference was between media when SOX9 expression was examined (p<0,05). B) Collagen I expression in HPLO pellets was significant lower than the expression in N.S. pellets (p>0,001). C) Aggrecan expression was significantly lower in HPLO pellets (p<0,001). D) COMP expression was significantly lower in both FBS and HPLO pellets (p<0,001). **=p<0,001. N.S. = No supplement.*

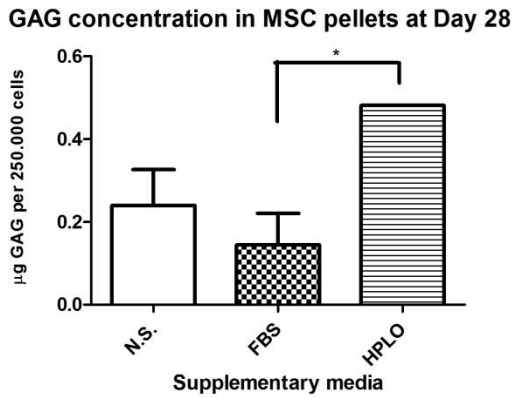

Figure 23 Glycosaminoglycan concentration in MSC pellets at day 28

*The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of MSC pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in HPLO pellets was significantly higher than the GAG concentration in FBS pellets (p<0,05). *=p<0,05. N.S. = No supplement.*

3.2.4 Chondrogenic differentiation at day 35

The staining of hES-MP pellets harvested after 35 days of differentiation can be seen in figure 24. The morphology of N.S. and FBS pellets did not alter from day 28. However, the HPLO pellets kept losing nuclei and gain extracellular matrix. The Masson Trichrome staining showed that the HPLO pellets were completely covered with collagen fibers while the collagen fibers in FBS pellets increased slightly. A change in collagen formation between time points was not detectable in N.S. pellets. S100 staining showed that the S100 proteins seemed to be decreasing in all pellets from day 28.

The GAG concentration in hES-MP pellets differentiated without a supplementary media (0,082 ± 0,0004 µg, N=2) was lower than the GAG concentration in both FBS (0,16 ± 0,012 µg, N=2) and HPLO pellets media (0,11 ± 0,007 µg, N=2) (Figure 25).

The gene expression of SOX9 and Collagen I is displayed in figure 26. The SOX9 expression analysis indicated that both FBS (0,20, N=2) and HPLO (0,23, N=2) pellets had a lower expression than N.S. pellets. The Collagen I expression was however more than four times higher in both FBS (5,64, N=2) and HPLO (4,63, N=2) pellets when compared to the expression in N.S. pellets.

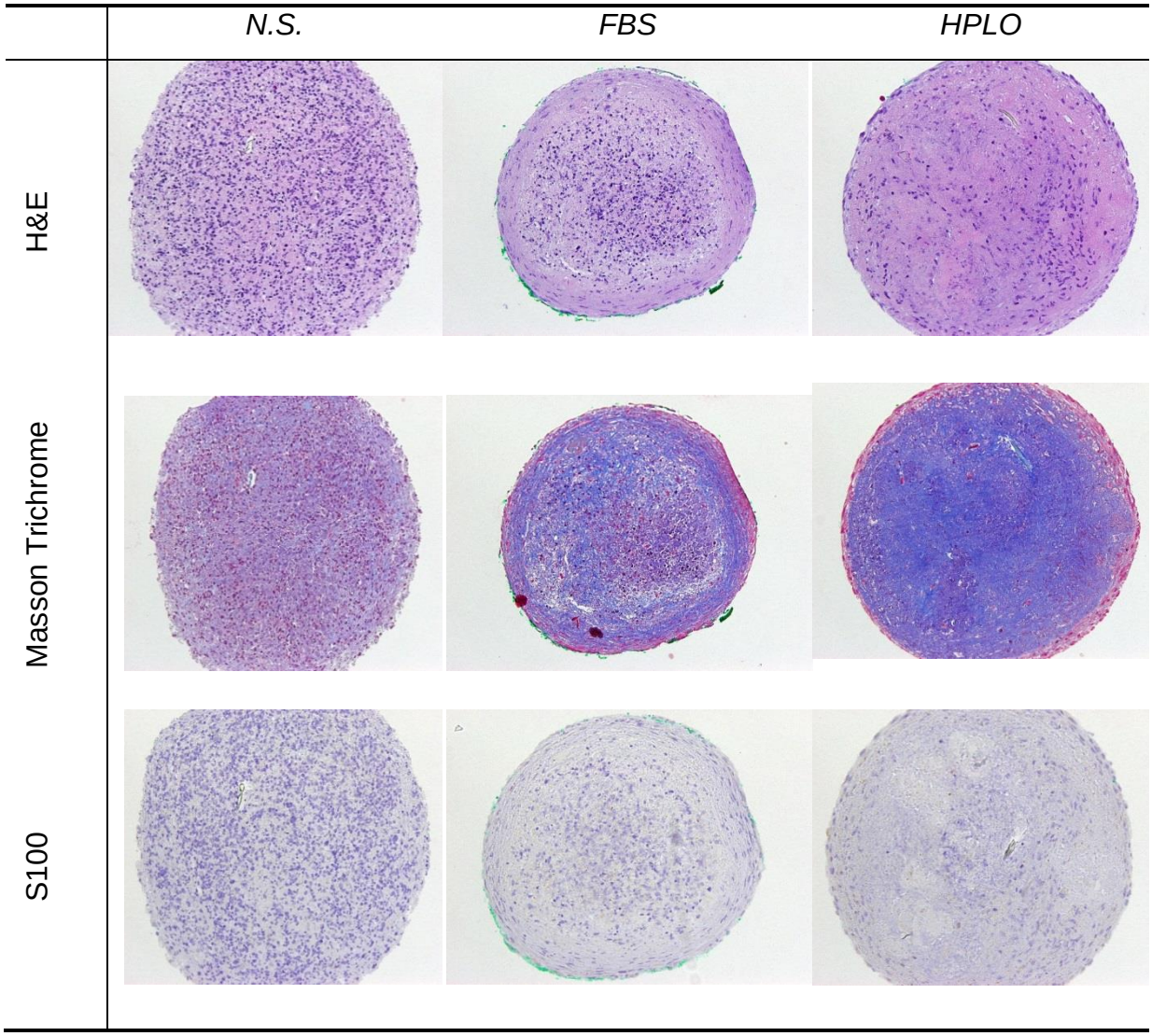

Figure 24 hES-MP chondrocytic pellets stained after 35 days of differentiation

The hES-MP cells were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

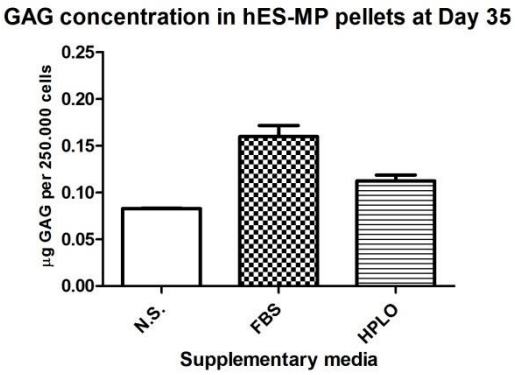

Figure 25 Glycosaminoglycan concentration in hES-MP pellets at day 35

The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of hES-MP pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in N.S. pellets was lower than the GAG concentration in both FBS and HPLO pellets. N.S. = No supplement.

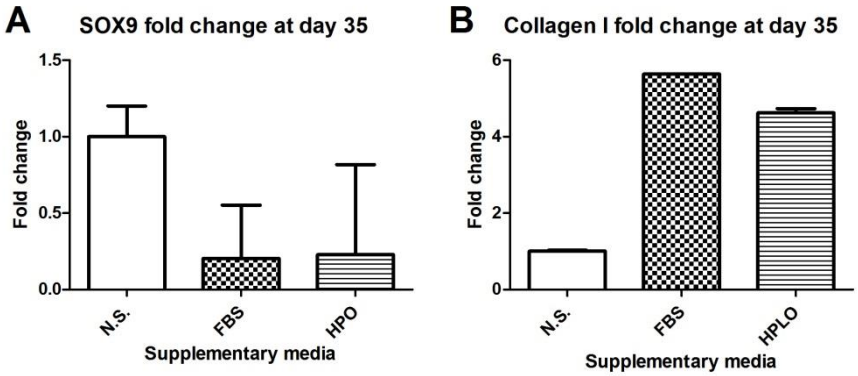

Figure 26 Comparison of gene expression between differentiation media at day 35

Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in hES-MP pellets (y-axis). A) SOX9 expression in FBS pellets was lower than SOX9 expression in N.S. pellets. B) The Collagen I expression in HPLO and FBS pellets was higher than in N.S. pellets. N.S. = No supplement.

The staining of MSC pellets harvested after 35 days of differentiation can be seen in figure 27. The elongated cellular morphology had increased in N.S. and FBS pellets. The HPLO pellets lost more nuclei and the extracellular matrix kept increasing. The Masson Trichrome staining displayed an increase of collage fibers in the HPLO pellets but no clear changes were visible in the N.S. and FBS pellets. The S100 staining showed that S100 proteins were scattered throughout the pellets in all media.

The GAG concentration in MSC pellets at day 35 is shown in figure 28. The HPLO pellets (0,49 ± 0,054 µg, N=4) had a significantly higher GAG concentration than N.S. pellets (0,14 ± 0,052 µg,

p<0,05, N=4). No significant difference was between HPLO and FBS pellets (0,13 ± 0,11 µg, p>0,05, N=4)

The SOX9 gene expression in MSC pellets differentiated with 10% HPLO showed some difference between media but without a significant difference (p>0,05) (Figure 29). The FBS pellets (0,82, N=4) had a significantly lower expression of Collagen I than N.S. pellets (p<0,001). The HPLO pellets (0,23, N=4) seemed to have a decreased expression of Collagen I but without a significant difference. The Aggrecan expression in HPLO pellets (0,11, N=4) was significantly lower than N.S. pellets (p<0,001). Lastly, the COMP expression was significantly lower in both FBS (0,33, N=4) and HPLO (0,08, N=4) pellets when compared N.S. pellets (p<0,001).

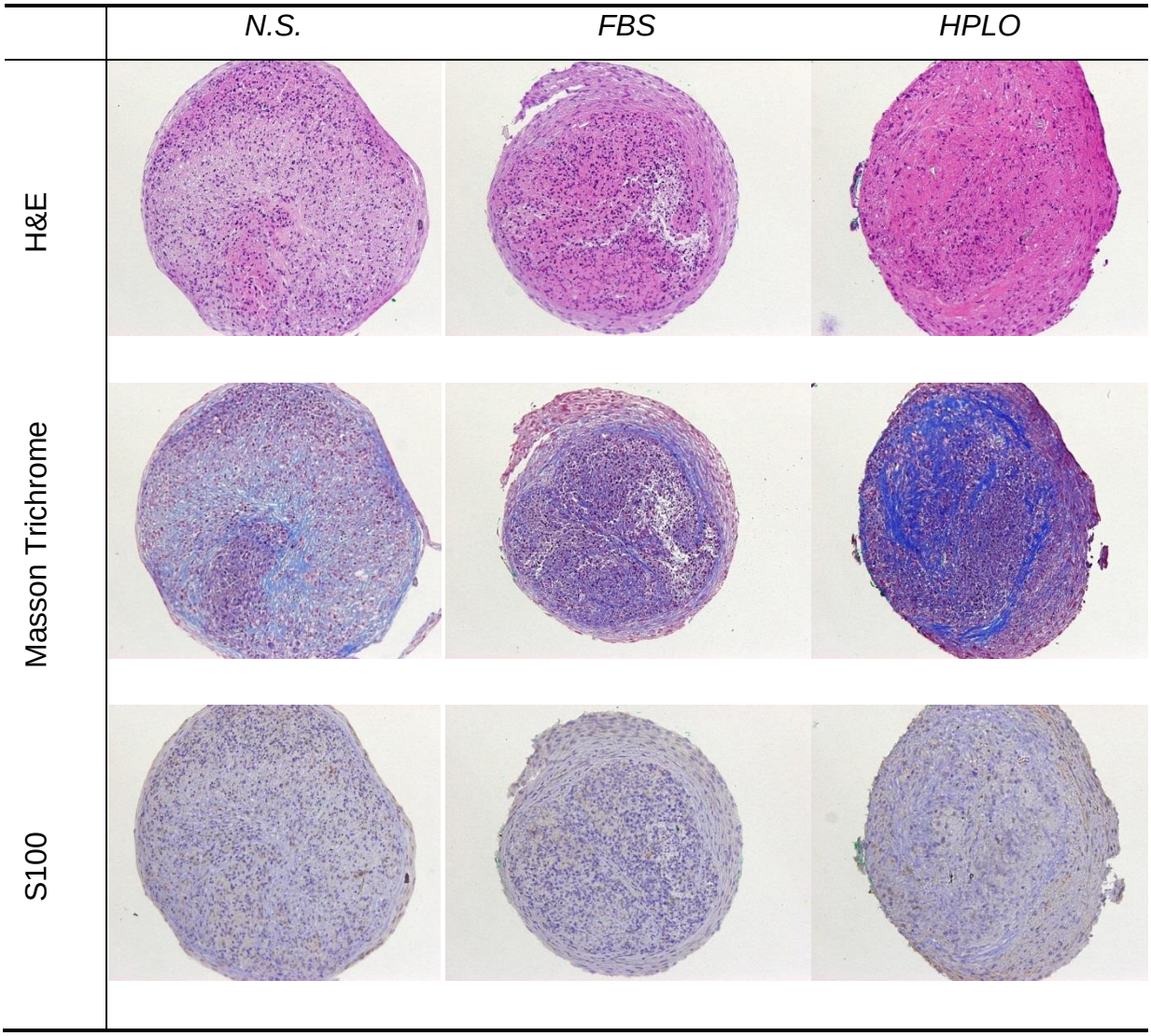

Figure 27 MSC D6 chondrocytic pellets stained after 35 days of differentiation

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the staining methods are displayed to the left of the image. All images were taken at 100x magnification. Nuclei are stained purple and extracellular matrix stained pink with H&E staining, Collagen fibers stained blue with Masson Trichrome staining and S100 proteins are stained light brown with S100 staining.

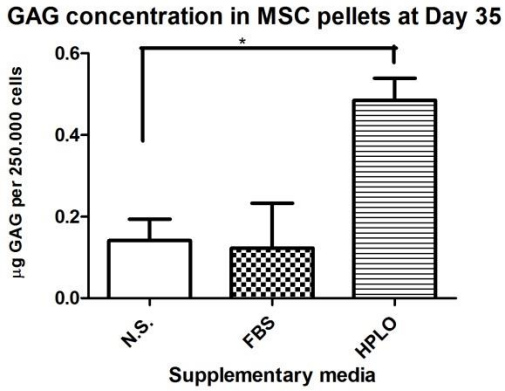

Figure 28 Glycosaminoglycan concentration in MSC pellets at day 35

*The GAG concentrations in µg, per one pellet or 250.000 cells, (y-axis) of MSC pellets in different types of media (x-axis) are displayed with SEM as error bars. The GAG concentration in HPLO pellets was significantly higher than the GAG concentration in N.S. pellets (p<0,001). *=p<0,05. N.S. = No supplement.*

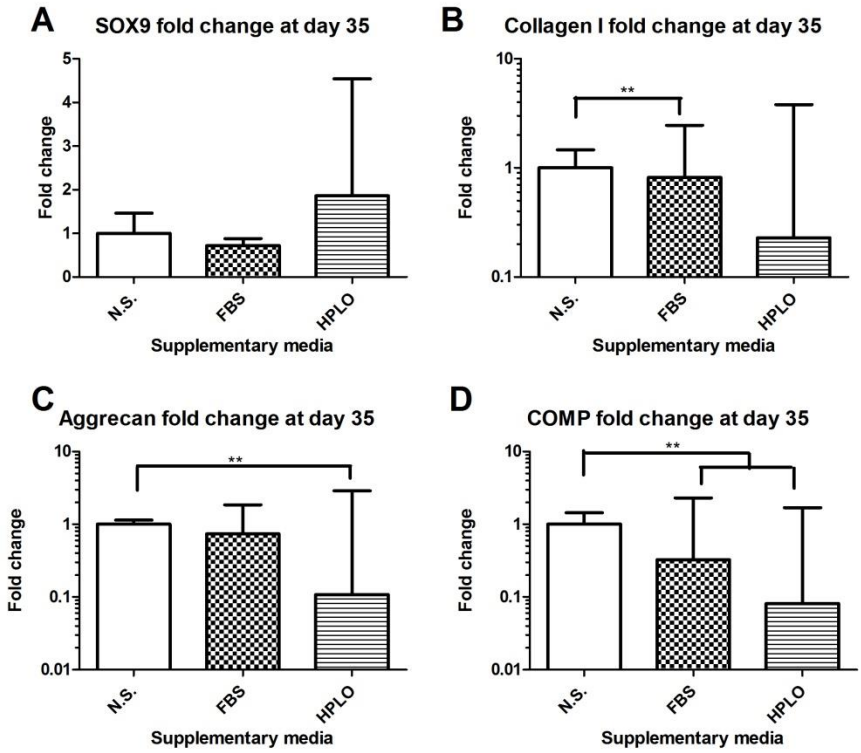

Figure 29 Comparison of gene expression between differentiation media at day 35

*Fold change in gene expression, presented with SEM as error bars, in different types of media (x-axis). The gene expression is presented as a fold change, assigning 1 to pellets differentiated without a supplementary media (N.S.) in MSC pellets (y-axis). The y-axis is log scaled at log^{10} in graphs B, C and D. A) No significant difference was between the SOX9 expression in pellets from all media (p<0,05). B) Collagen I expression in FBS pellets was significantly lower than the expression in N.S. pellets (p>0,001). C) Aggrecan expression was significantly lower in HPLO pellets (p<0,001). D) COMP expression was significantly lower in both FBS and HPLO pellets (p<0,001). **=p<0,001. N.S. = No supplement.*

4 Discussion

MSCs and hES-MP cells were differentiated to a chondrogenic lineage with three different in-house formula based differentiation media, 10% FBS supplemented media, 10% HPLO supplemented media, and a media without additional supplement (N.S.). The media without additional supplement is a basal media used for chondrogenic differentiation. It has all the necessary supplements needed for differentiation, but it does not contain any additional supplements such as FBS or HPLO. Their extracellular components, morphology and gene expression was analysed and compared between both media and cell types after 7, 14, 28 and 35 days of differentiation.

MSC and hES-MP cells have the ability to differentiate into chondrocytic pellets with all three types of differentiation media. The GAG concentration analysis and the histological staining demonstrated that hES-MP and MSC pellets create greater amount of extracellular matrix than pellets in other media. Aggrecan and COMP expression in MSC pellets seemed to be lower in HPLO derived pellets and the Collagen I expression in hES-MP HPLO and FBS pellets was higher than in N.S. pellets. The SOX9 expression was also lower in hES-MP HPLO and FBS pellets than in N.S. pellets.

4.1 Establishing mesodermal lineage in hES-MP cells

The phenotype of hES-MP cells and their osteogenic differentiation potential were analysed to establish their MSC-like abilities. MSC and hES-MP cells were expanded in either 10% HPLO or 10% FBS before being harvested for analysis.

The phenotype of MSC and hES-MP cells was examined with flow cytometry to determine if hES-MP cells had similar phenotype as MSCs, and whether it changed after expansion in 10% HPLO supplemented media. The expression of CD90, CD73, CD29, CD105, CD45 and HLA-DR surface markers were examined. As Table 13 displayed, both MSCs and hES-MP cells expressed the surface markers CD73, CD90, CD105 and CD29, and did not express CD45 and HLA-DR. These results are correlative to the ISCT guideline for identifying MSCs (Dominici et al., 2006; Salem, 2009; Williams et al., 2011). Table 13 additionally shows that the expression of these surface markers did not change between media in both MSCs and hES-MP cells. These results indicate that hES-MP cells have the same phenotype as MSCs when expanded in both 10% FBS and 10% HPLO.

The osteogenic differentiation potential of MSC and hES-MP cells was examined by determining the ALP activity. Figure 30 shows the results of the ALP activity assay after 0, 7, 14 and 21 days of osteogenic differentiation in MSCs and hES-MP. The ALP activity was similar in all samples at day 0 and day 7. However, at day 14, MSCs and hES-MP cells grown in 10% HPLO had a significantly higher ALP activity than both cell types grown in 10% FBS. On day 21, the ALP activity increased similar in MSCs and hES-MP cells grown in 10% FBS, and MSCs grown in 10% HPLO have a significantly higher ALP activity than other samples. Unlike other samples, the ALP activity stayed similar to the activity of day 14 in hES-MP cells grown in 10% HPLO. This lack of increase in ALP activity at day 21 in hES-MP HPLO cells is rather unusual. There is a possibility of pipetting inaccuracy

or a failure in harvesting the cells. However, these results indicate that both cell types, grown in 10% FBS or 10% HPLO supplementary media are differentiating to an osteogenic lineage.

These results further indicate that the ALP activity increased more rapidly in MSCs and hES-MP cells grown in 10% HPLO than MSCs and hES-MP cells grown in 10% FBS. These results are similar to results from Jonsdottir-Buch et.al. (Jonsdottir-Buch et al., 2013). Their results showed that MSCs have a greater increase in ALP activity after expansion in 10% HPLO than 10% FBS, from day 7 to 14. One possible explanation to this ALP activity difference between media is the presence of TNFα (Tumour necrosis factor-α) in platelet lysate (Fekete et al., 2012). TNFα is pro-inflammatory cytokine, which promotes MSX2 expression through the NF-κB pathway (Lee et al., 2010). MSX2 encodes a homeobox transcription factor that helps regulate osteoblast proliferation and differentiation (Lee et al., 2010). MSX2 encourages multipotent mesenchymal progenitor cells to osteogenic differentiation and suppresses their adipogenic differentiation potential (Cheng et al., 2003). Lee et al. showed that MSX2 induces expression of ALP in mineralization in vascular smooth muscle cells (Lee et al., 2010). There is a possibility that TNFα is causing this difference, but TNFα is also present in FBS. Whether there is a higher concentration of TNFα in platelet lysate than in FBS is unknown and more analysis is needed to determine what is affecting this increased ALP activity.

The results from both cell surface marker expression and the ALP activity assay indicate that hES-MP cells are differentiated to a mesodermal lineage, comparable to MSCs.

4.2 Chondrogenic lineage in MSCs and hES-MP cells

A GAG assay was performed to evaluate the GAG amount present in the chondrocytic pellets. This assay has the ability to estimate the production of extracellular matrix during differentiation at various time points. The results showed that the GAG concentration in MSC and hES-MP pellets, differentiated with 10% HPLO, was relatively stable throughout the differentiation period when compared to the pellets from the same cell type in other media. When taken together, there was an unusual decrease of GAG concentration in the hES-MP pellets in FBS or N.S. media, which increases again at the end of the differentiation period. In addition, the GAG concentration in MSC pellets in FBS or N.S. media decreased digressively throughout the differentiation period. Platelet-derived growth factor (PDGF) is a factor present in platelet lysate and could possibly be a factor in the regulation of GAG concentration in pellets differentiated with HPLO. PDGF is a polypeptide mitogen for mesenchymal cells and interacts with proteoglycans, especially via GAG chains (Zafiropoulos et al., 2008). The binding of PDGF to cell associated GAGs can regulate its mitogenic function on normal cells (Zafiropoulos et al., 2008). Bear in mind that PDGF is also present in FBS, but the concentration may be higher in platelet lysate and that could possibly explain the difference. Nevertheless, more analysis is needed to confirm that. Additionally, Elias et al. showed that recombinant TNF, when associated with Gamma Interferon (IFN-γ), are important factors in the regulation of fibroblast GAGs resided in the human lung (Elias et al., 1988). These are examples about what factors could be

regulating the GAG concentration throughout the differentiation period in the HPLO pellets. However, these are only speculations and further testing is needed for confirmation.

What is noticeable is the fact that MSC HPLO pellets had a higher GAG concentration than other pellet types and approximately 2 times more GAG concentration than the hES-MP HPLO pellets over the differentiation period. TGF-β1 is known to stimulate GAGs *in vitro* (Bassols & Massague, 1988) and platelet lysate is rich with TGF-β1 (Fekete et al., 2012). That could possibly explain why MSC pellets differentiated with 10% HPLO have such high concentration of GAGs compared to other media. Why the GAG concentration was lower in hES-MP HPLO pellets than in the MSC HPLO pellets is not clear. It could be on the account of their embryonic origin, but Gasimli et al. showed that the GAG structure of hESCs changed when differentiated to mesoderm or endoderm lineages (Gasimli et al., 2014). Still, as said before, these are speculations and further analysis is necessary.

Chondrocytic pellets from both cell types were sectioned, and stained in order to examine their morphology and extracellular components with H&E staining, Masson Trichrome staining and S100 staining. The results from the H&E staining demonstrate that the morphology of both MSC and hES-MP pellets differentiated with 10% HPLO have the highest resemblance to physiologically normal cartilage, demonstrated by the decreased number of nuclei and high concentration of ECM. They also gained more extracellular matrix than MSC and hES-MP pellets differentiated with 10% FBS or without a supplementary media. MSC and hES-MP pellets differentiated with 10% FBS or 10% HPLO attained a greater elongated cellular morphology, which is an indication of differentiation.

The Masson Trichrome staining confirmed this difference of ECM amount in pellets between media, showing that MSC pellets and hES-MP pellets differentiated with 10% HPLO contained greater amount of collagen fibers than pellets grown in other media. The staining was stronger in hES-MP pellets than in MSC pellets at day 28 and 35, which could mean that the amount of collagen fibers is greater in the hES-MP pellets. IGF-1 is present in cartilage and Yaeger et al. demonstrated that this factor has the ability to work with TGF-β2 and induce expression of type II Collagen in adult human articular chondrocytes (Yaeger et al., 1997). Thus, when chondrocytic pellets reach a good cartilage differentiation, there is a possibility that TGF-β2 could be interacting with IGF-1, which could result in this increase of collagen II fibers in pellets differentiated with HPLO. However, Masson Trichrome staining does not stain for any specific types of collagen, making it hard to analyse what elements present in HPLO are causing this unexpected increase of collagen fibers.

The S100 staining did not display the anticipated results comparable to the results from H&E staining and Masson Trichrome staining. The concentration of S100 was low in both cell types, making it hard to analyse the results. Figures 34 and 35 in Appendix D demonstrate the hES-MP and MSC D6 pellets after S100 staining in 600x magnification. These figures show that the S100 protein amount decreased minimally in both cell types throughout the differentiation period. This could be related to the loss of nuclei during the differentiation process. S100 proteins are calcium binding proteins that are expressed in vertebrates and are located in chondrocytes (Yammani, 2012). These findings are therefore parallel with normal cartilage differentiation, even though the quantity of S100 proteins is low in both cell types. When looking at the S100 protein amount between cell types, it seems that the MSC

pellets contained greater amount of S100 protein, thus making it possible to believe that MSC pellets carry more chondrocytes than hES-MP pellets.

When the results from the histological analysis are summarized, it is clear that the MSCs and hES-MP cells differentiated with 10% HPLO generate better chondrocytic pellets than MSCs and hES-MP cells differentiated with 10% FBS or without additional supplements. However, it seems that there is a difference between the cell types regarding the amount of S100 proteins and collagen fibers, when looking closely. More experiments are needed to confirm and then determine what could possibly be causing these differences.

Lastly, the gene expression of SOX9, Collagen I, Aggrecan and COMP was examined with real-time qPCR to evaluate the chondrogenic differentiation.

The gene expression of SOX9 in hES-MP pellets showed that the N.S. pellets had a higher expression of SOX9 than FBS and HPLO pellets at every time-point, except for day 14 where HPLO expressed higher expression of SOX9 than N.S. pellets. The gene expression of SOX9 in MSC pellets showed that HPLO and FBS pellets had a lower expression than N.S. pellets but there was no significant difference between media, except for at day 7, where the SOX9 expression in FBS pellets was significantly lower. As stated before, SOX9 is a transcription factor that is necessary for cartilage formation and the expression of cartilage-specific matrix proteins, such as Collagen type II and Aggrecan (Dancer et al., 2010). TNF-α and interleukin-1 (IL-1) are cytokines that exist both in platelet lysate and FBS. Murakami et al. showed that those two factors have the ability to cause a reduction of SOX9 levels in chondrocytes (Murakami et al., 2000). Interleukin-6 (IL-6), also present in platelet lysate, and its soluble receptor (sIL-6R) have also been shown to down-regulate SOX9 at both protein and mRNA levels (Legendre et al., 2003). These factors could possibly be down-regulating SOX9 expression in hES-MP pellets, after differentiation with HPLO. The hES-MP FBS pellets also had a down-regulated expression of SOX9 when compared to N.S. pellets. Thus, we may assume that adding additional supplements, whether it is HPLO or FBS, may not have a positive effect on SOX9 expression.

The gene expression of Collagen I in MSC HPLO pellets was lower than in N.S. pellets, but there was only a significant difference at day 28 and 35. When the Collagen I expression was examined in hES-MP pellets, it was clear that hES-MP HPLO pellets had a greater expression than N.S. pellets. The hES-MP FBS pellets also had a greater expression of Collagen I than N.S. pellets, except for on day 7. Why hES-MP pellets have a higher expression of Collagen I than the N.S. pellets, and why the MSC pellets do not is undetermined. The gene expression of Collagen I in hES-MP cells has not been analysed before, to the best of our knowledge, making it hard to determine what elements in hES-MP cells could be up-regulating Collagen I expression. One of the possible reasons why hES-MP HPLO and express higher expression of Collagen I, and MSC HPLO lower expression of Collagen I may be due to TGF-β1. Kim et al. examined mesangial cells and the connection between TGF-β1, Collagen I and autophagy. Their results showed that TGF-β1 both induces the synthesis of Collagen I, and encourages autophagy and thus stimulate deterioration of Collagen I (Kim et al., 2012). This dual function of TGF-β1 may be influencing the expressions. This example is however just a theory about what could be effecting the Collagen I expression in HPLO pellets and more analysis is needed.

The gene expression of Aggrecan was only detectable in MSC pellets, and not in hES-MP pellets. The gene expression of Aggrecan in FBS pellets was rather similar to the gene expression in N.S. pellets throughout the differentiation period. However, the Aggrecan expression in HPLO pellets was significantly lower than the expression in N.S. pellets at every time point except at day 14. As stated before, Aggrecan is an important proteoglycan in cartilage and its high content in hyaline cartilage is one of its definition (Roughley, 2006). A study by Legendre et al. showed that IL-6, a pleiotropic factor located in platelet lysate as previously stated, activated signal transducers and activators of transcription (STAT) signaling pathway collaborate in down-regulating the cartilage-specific matrix genes Collagen type II and Aggrecan (Legendre et al., 2003). However, the concentration of IL-6 in platelet lysate is not great and further analysis is required to figure out what is causing this Aggrecan decrease in MSC pellets differentiated with HPLO. Why the gene expression of Aggrecan was not detectable in hES-MP pellets is unknown. A study executed by Li et al. demonstrated that hES-MP cells from the same cell line as we use (hES-MP002,5 cells) express Aggrecan with an immunostaining (Li et al., 2013). Nevertheless, no published study has confirmed Aggrecan expression in hES-MP pellets with RT-qPCR analysis.

Similar to the gene expression of Aggrecan, the gene expression of COMP in MSC FBS pellets was comparable to the gene expression in N.S. pellets and the gene expression in MSC HPLO pellets was significantly lower than in N.S. pellets at every time point except day 14. Several members of the matrix metalloproteinase (MMPs) such as collagenase-3 (MMP-13) and gelatinase-B (MMP-9) have been reported to digest purified COMP *in vitro* (Luan et al., 2008). MMP-9 is a factor that human platelets release and it inhibits platelet aggregation forced by thrombin (Fernandez-Patron et al., 1999). A connection between the low expression of COMP in MSC HPLO and MMP-9 is a possibility but more analysis is needed.

To summarise, MSCs and hES-MP cells differentiated to a chondrogenic lineage with 10% HPLO media demonstrate a better cartilage formation than cell types in other media, when the histological stainings and the GAG concentration are analysed. The expression of Aggrecan and COMP was lower in MSC HPLO pellets when compared to the expression in N.S. pellets. The Collagen I expression was higher in hES-MP HPLO pellets than in hES-MP N.S. pellets, but there was not a significant difference between MSC pellets, except at day 28. The SOX9 expression was lower in hES-MP HPLO and FBS pellets than in hES-MP N.S. pellets.

4.3 Advantages and disadvantages

One advantage regarding this research is that the chondrogenic differentiation period was longer than in many other researches and more time points were analysed. That gives a better insight to what effects these different types of differentiation media are having during chondrogenesis. Another advantage is that trained personnel at the pathological lab performed the histological stainings.

The disadvantages in this thesis are several. For one, no statistical significant difference was possible to obtain in the analysis of hES-MP cells after chondrogenic differentiation. Each sample was only measured in duplicates, making it impossible to calculate a p value. These results would have been more reliable if the hES-MP cells had been differentiated more often. In addition, due to donor variation in MSCs, it would have been a great improvement to differentiate three donors instead of just two, but the lack of the third donor explains the big SEM bars shown in all graphs. However, this was not possible due to a lack of time. Another disadvantage is regarding the RNA isolation in chondrogenic pellets. The RNA isolation was very problematic due to their 3D pellet culture. The cells are compressed together in this form and to isolate the RNA from these pellets, they have to be minced. This has to be carefully done and pellets must be fully grinded before the isolation procedure begins. In addition, there is always a risk of not enough RNA being isolated due to their variable size. The Masson Trichrome staining did show an increase in collagen fibers but there was no way of knowing what type of collagen could be found in these chondrocytic pellets. hES-MP cells are a new cell type and not many articles have been published about their chondrogenic differentiation potential, making it hard to compare the results given here to other results. This may be the first detailed research on hES-MP cells and their chondrogenic differentiation potential.

4.4 Future directions

According to the results presented in this thesis, it is possible to use lysate from pathogen inactivated platelets in chondrogenic differentiation media. MSCs and hES-MP cells differentiated with HPLO do obtain greater amount of extracellular matrix but the gene expression of cartilage matrix specific proteins must be further analysed. In addition, the reasons to why the gene expression is so atypical need to be examined further. As stated above, the isolation of RNA from these chondrocytic pellets is troublesome and this may be affecting the gene expression analysis. An improved method for the extraction of RNA from pellets would be helpful for this type of project. The addition of Collagen II staining to the Masson Trichrome staining would also add value to the analysis.

In addition, further analysis on hES-MP cells and their potential to replace MSCs for chondrogenic purposes is imperative. Our results indicate that the hES-MP cells are less stable than MSCs with regards to the gene expression and further research on these cells is therefore critical before their *in vivo* use.

5 Conclusion

What this thesis has established is that hES-MP cells have in fact differentiated to a mesodermal lineage and that outdated pathogen inactivated platelet lysate (HPLO) has a positive effect on their osteogenic potential. This thesis also demonstrates that MSCs and hES-MP cells have the ability differentiate to a chondrogenic lineage when cultured in a chondrogenic differentiation media, supplemented with either 10% FBS or 10% HPLO. MSCs and hES-MP cells differentiated with 10% HPLO produce a great amount of extracellular matrix, but the gene expression is atypical and further analysis needs to be performed before any conclusions can be drawn regarding their use for the chondrogenic differentiation of these cell types.

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Appendix A

Table 13 demonstrates the result from FACS analysis on MSCs and hES-MP cells, grown in either 10% FBS or 10% HPLO.

MSCs, expanded in both 10% FBS and 10% HPLO, were positive for CD73, CD105, CD29 and CD90 and negative for CD45 and HLA-DR. hES-MP cells, expanded in both 10% FBS and 10% HPLO, expressed the same surface markers as MSCs (CD73, CD105, CD29, and CD90) and were also negative for the same surface markers (CD45 and HLA-DR).

Table 13 FACS analysis on MSCs and hES-MP cells

MSCs and hES-MP cells, in both types of media, were positive for CD73, CD105, CD29 and CD90, and they were negative for CD45 and HLA-DR.

	MSC HPLO	MSC FBS	hES-MP HPLO	hES-MP FBS
CD73	+	+	+	+
CD45	-	-	-	-
CD105	+	+	+	+
CD29	+	+	+	+
HLA-DR	-	-	-	-
CD90	+	+	+	+

Appendix B

Figure 30 demonstrate the results from the ALP activity assay on MSC and hES-MP cells that had been expanded in either 10% FBS or 10% HPLO, and then differentiated to a osteogenic lineage.

The ALP activity of hES-MP cells, grown in HPLO, was similar from day 0 (0,4274 ± 0,006123, N=3) to day 7 (0,4588 ± 0,01045, N=3) with no statistical significant difference (p>0,05). It increased between day 7 and 14 (0,7164 ± 0,01253, p<0,0001, N=3) and held steady to day 21 (0,7093 ± 0,0007185, p>0,05, N=2). The ALP activity of hES-MP cells, grown in FBS, on day 0 (0,3695 ± 0,009054) is statistically different (p<0,05) from day 7 (0,4210 ± 0,01127, N=3). There was no significant difference between day 7 and day 14 (0,4625 ± 0,01392, p>0,05, N=3), but ALP activity increased significantly between day 14 and day 21 (1,046 ± 0,09653, p<0,01, N=3). The ALP activity of MSCs, grown in HPLO, did not statistically differ between day 0 (0,4092 ± 0,009927) and day 7 (0,4425 ± 0,006762, p>0,05, N=3). There was however a significant difference (p<0,001) between day 7 and day 14 (0,7678 ± 0,03416, N=3), and day 14 and day 21 (1,612 ± 0,1422, p<0,01, N=3). The ALP activity of MSCs, grown in FBS, did statistically differ between day 0 (0,3604 ± 0,008957) and day 7 (0,4083 ± 0,005391, p<0,05, N=3), but there was no significant difference between day 7 and day 14 (0,4197 ± 0,008030, p>0,05, N=3). Finally, there was a significant increase of ALP activity between day 14 and day 21 (1,231 ± 0,09484, p>0,01, N=3).

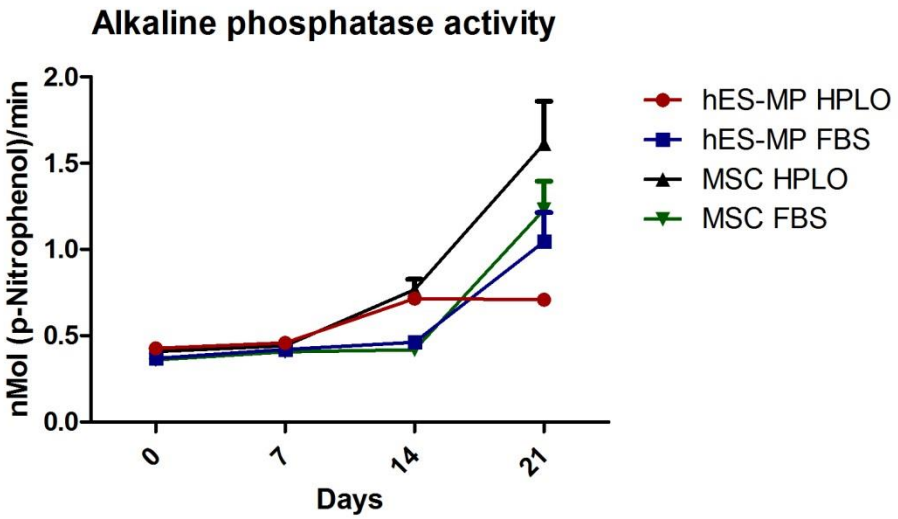

Figure 30 ALP activity in MSCs and hES-MP cells

The ALP activity (y-axis) from MSCs and hES-MP cells after 0, 7, 14 and 21 days (x-axis) in osteogenic culture is presented with SEM (standard error of the mean) as error bars. There was no statistical significant difference between cell types or growth media on day 0 or day 7 (p>0,05).On day 14, there was a significant difference between hES-MP HPLO and FBS (p<0,05), and MSC HPLO and FBS (p<0,001). On day 21, there was a significant difference between hES-MP HPLO and hES-MP FBS (p<0,01), hES-MP HPLO and MSC HPLO (p<0,0001), hES-MP HPLO and MSC FBS (p<0,0001), hES-MP FBS and MSC HPLO (p<0,0001) and MSC HPLO and MSC FBS (p<0,001). The ALP activity from day 0 to day 21 increased significantly in hES-MP HPLO (p<0,001), in hES-MP FBS (p<0,01), in MSC HPLO (p<0,01) and in MSC FBS (p<0,001).

The ALP activity was similar for day 0 ($p>0,05$) and day 7 ($p>0,05$) in both cell types, grown in either HPLO or FBS. MSCs and hES-MP cells, grown in HPLO, had a similar amount of ALP activity on day 14 with no significant difference between cell types ($p>0,05$). There is however a significant difference between hES-MP cells and MSCs on day 21 ($p<0,0001$). hES-MP cells and MSCs grown in FBS also have a similar amount of ALP with no significant difference ($p>0,05$) for all time points. When hES-MP cells, grown in either HPLO or FBS, are compared, there is a significant difference between day 14 ($p<0,05$) and day 21 ($p<0,01$). MSCs, grown in either HPLO or FBS, also have a significant difference between day 14 and day 21 ($p<0,001$).

Appendix C

The following figures display sliced MSC D3 pellets after histological staining.

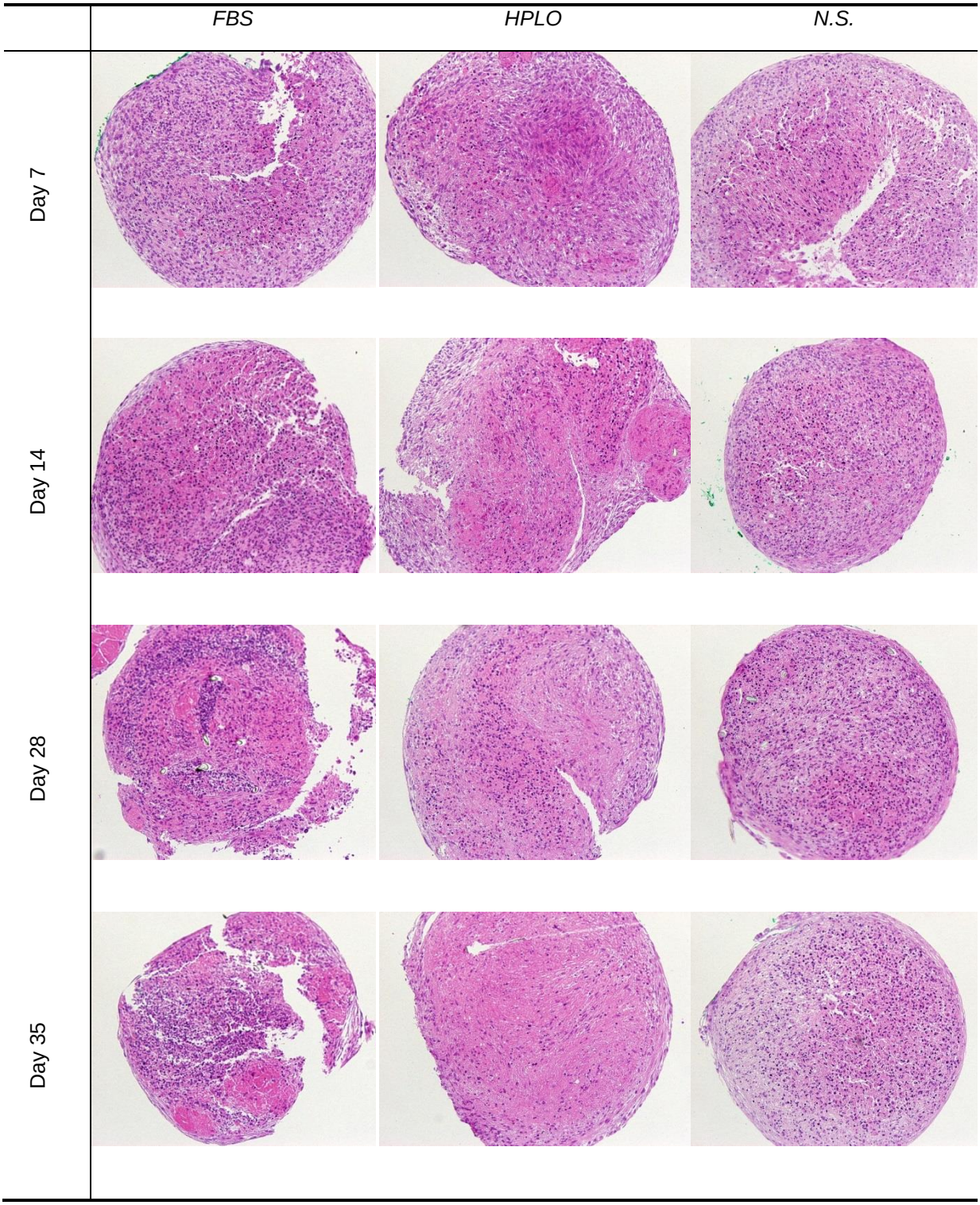

Figure 31 MSC D3 chondrocytic pellet staining with Hematoxylin and Eosin

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the days displayed to the left of the image. All images were taken at 100x magnification. Nucleic are stained purple and extracellular matrix stained pink.

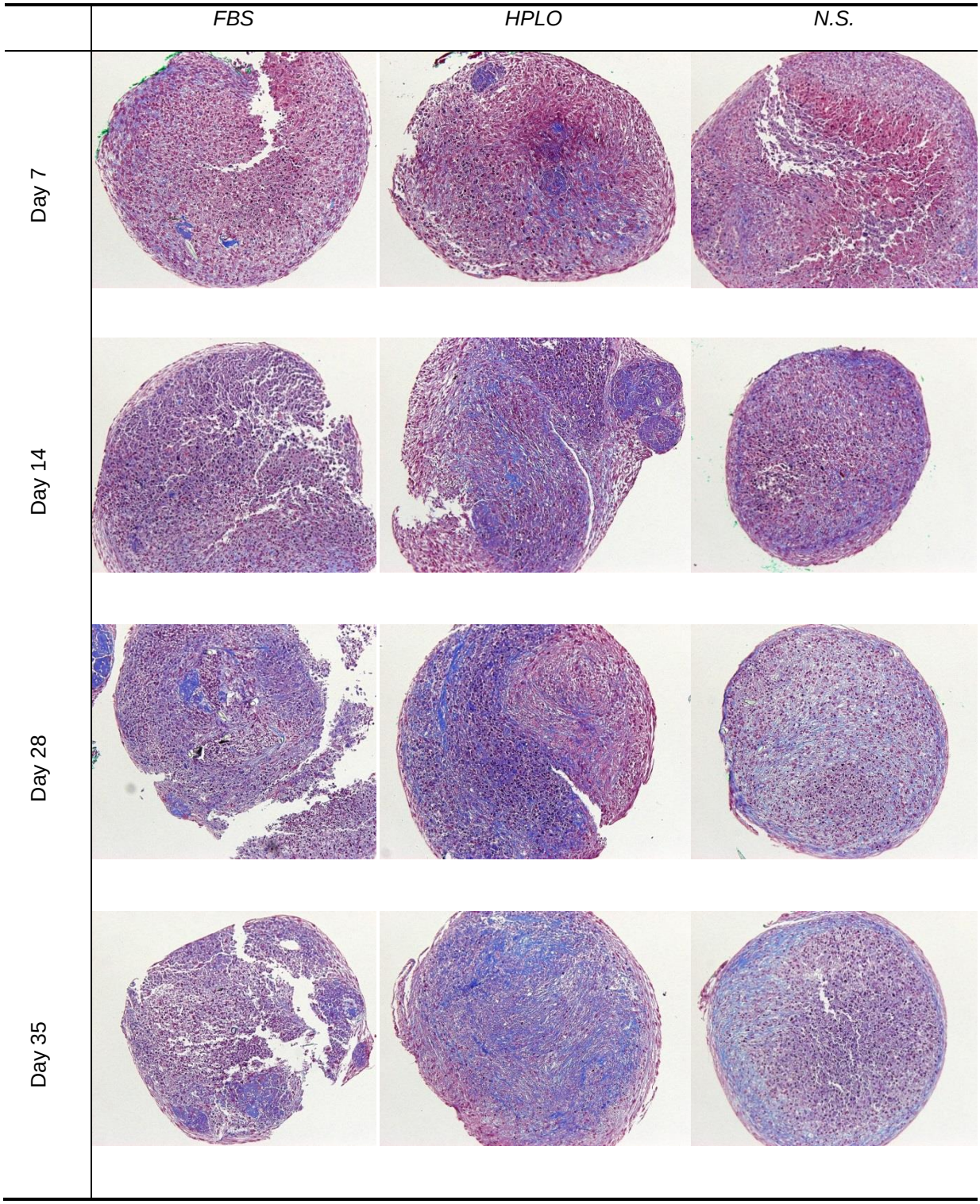

Figure 32 MSC D3 chondrocytic pellet staining with Masson Trichrome

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the days displayed to the left of the image. All images were taken at 100x magnification. Collagen fibers are stained blue.

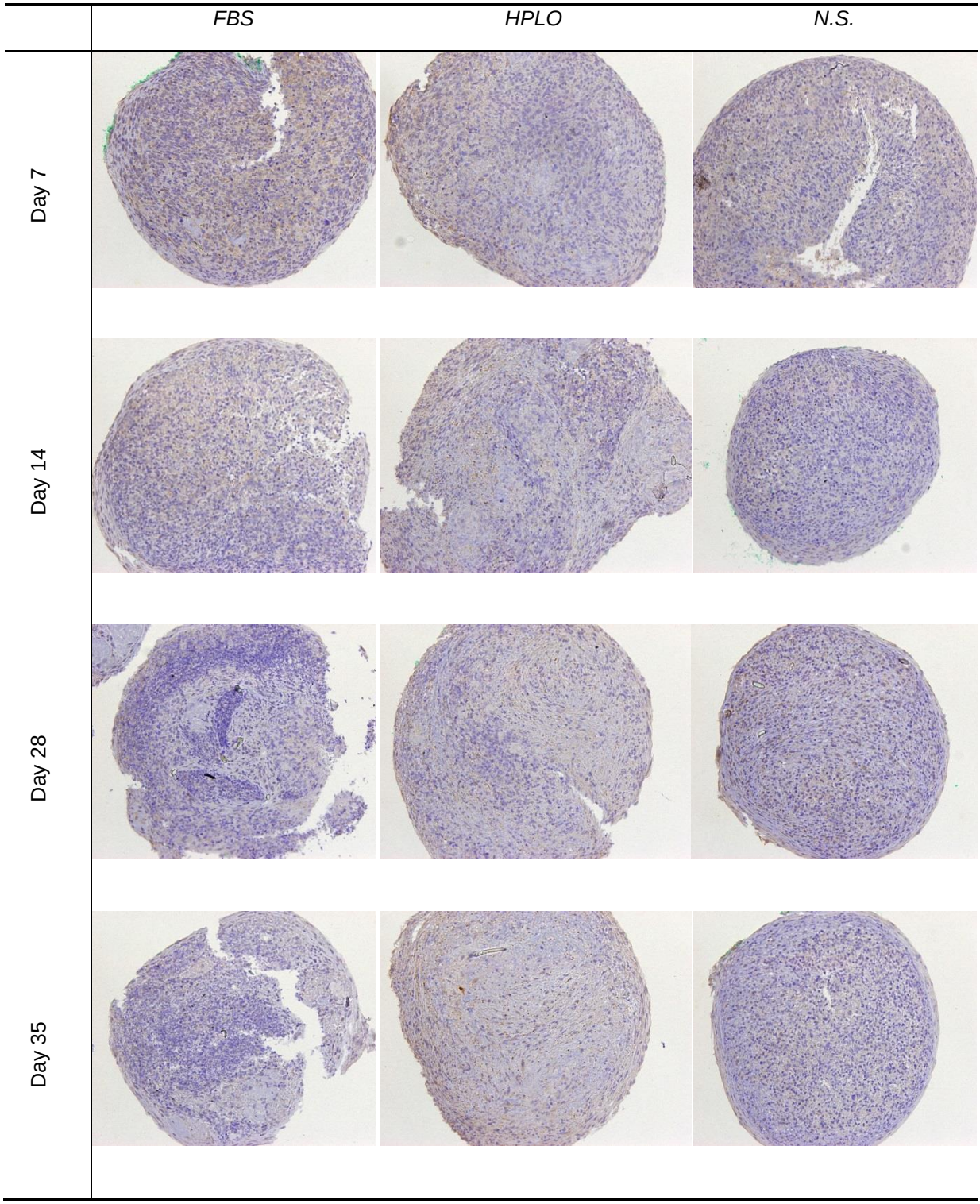

Figure 33 MSC D3 chondrocytic pellet staining with S100

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the days displayed to the left of the image. All images were taken at 100x magnification. S100 proteins are stained light brown.

Appendix D

Following figures show sliced MSC D6 and hES-MP pellets after S100 staining in 600x magnification.

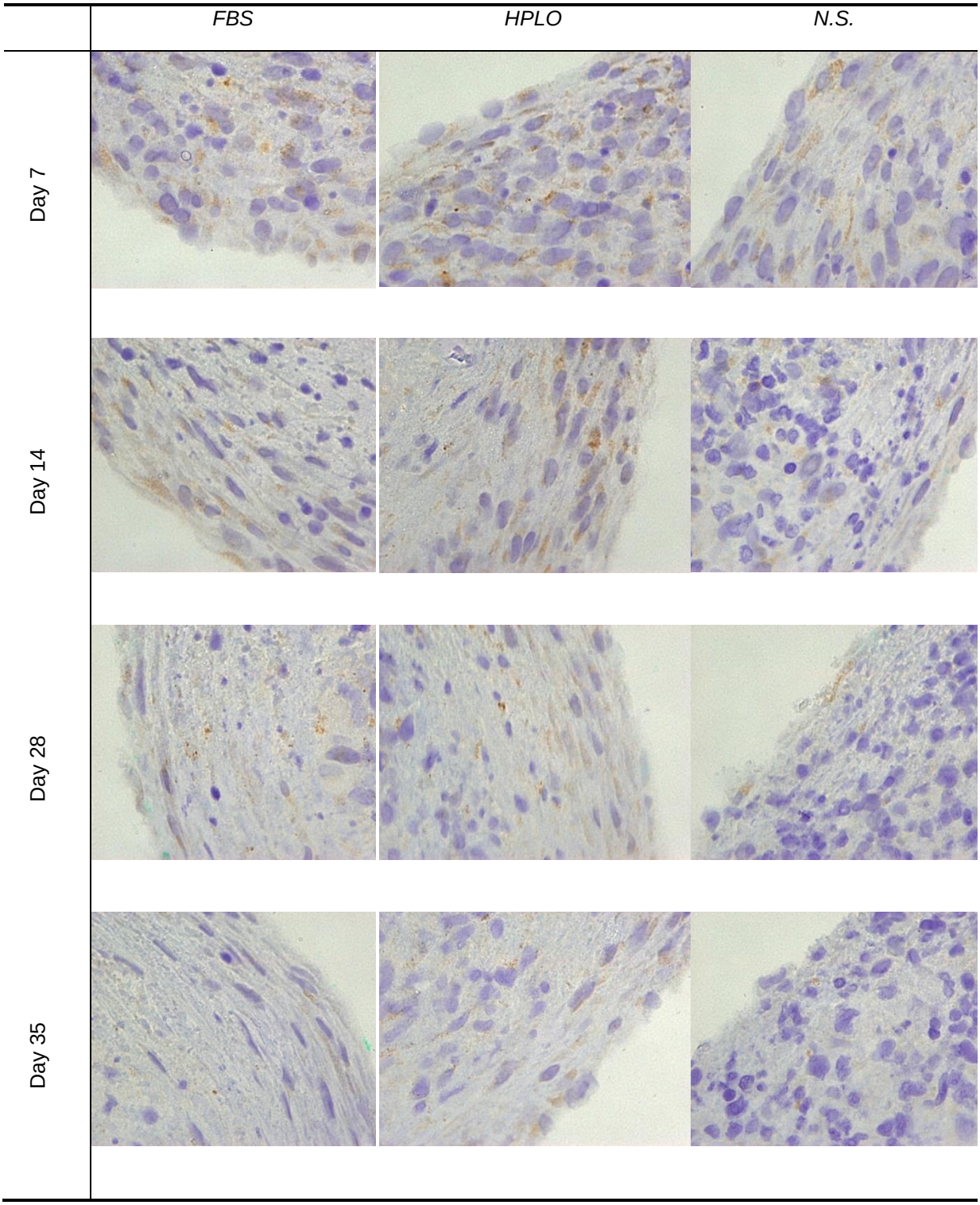

Figure 34 hES-MP chondrocytic pellet staining with S100 in 600x magnification

The hES-MP cells were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the days displayed to the left of the image. All images were taken at 600x magnification. S100 proteins are stained light brown.

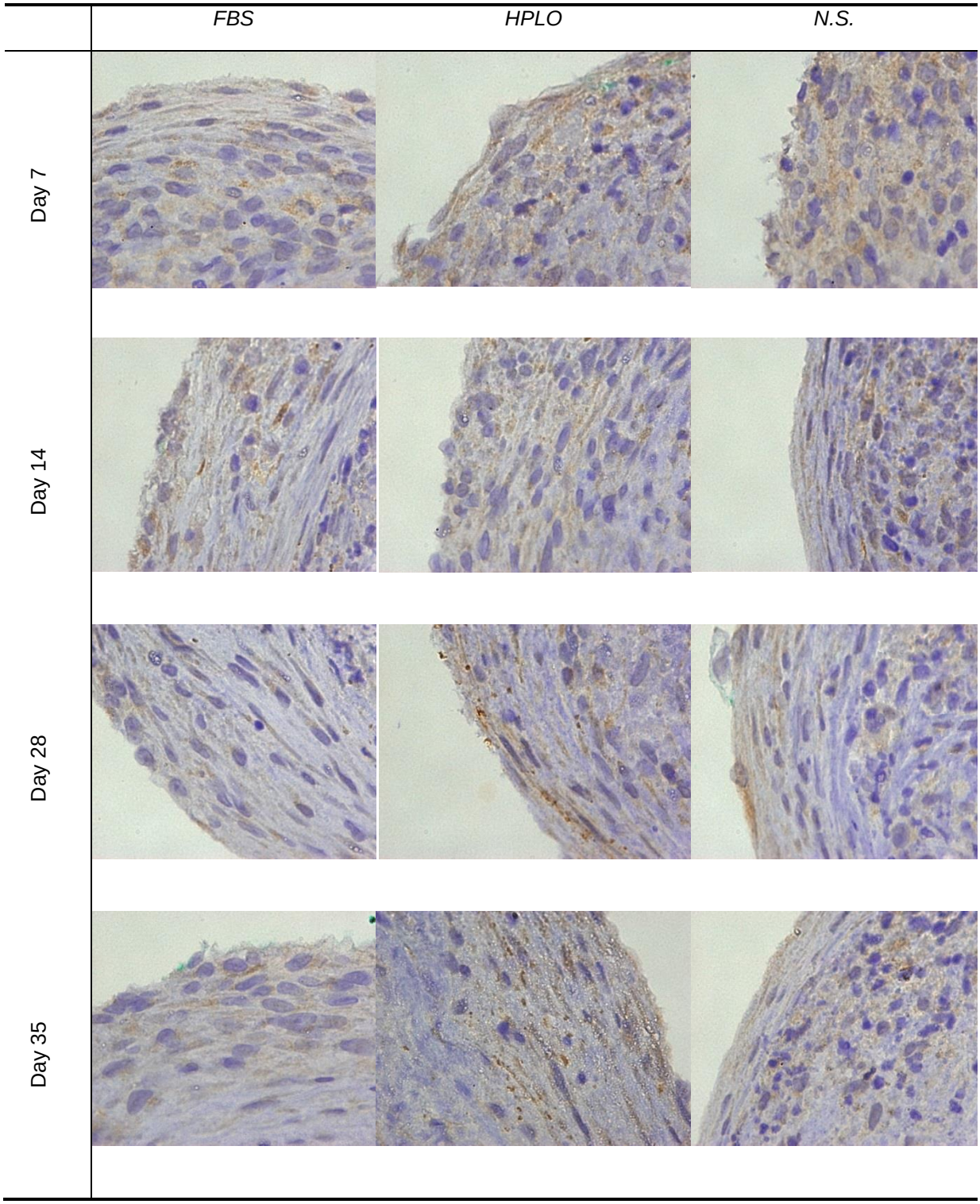

Figure 35 MSC D6 chondrocytic pellet staining with S100 in 600x magnification

The MSCs were differentiated with three media types (10% FBS, 10% HPLO and without a supplemented media (N.S.)) displayed at the top and harvested on the days displayed to the left of the image. All images were taken at 600x magnification. S100 proteins are stained light brown.