Comparison of hES-MP cell immunophenotype after expansion in fetal bovine serum or in platelet lysates, manufactured from expired platelet concentrate

Kristbjörg Gunnarsdóttir

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University of Iceland
Faculty of Medicine
Department of Biomedical science
School of Health Sciences

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Samanburður á svipgerð hES-MP frumna eftir ræktun í blóðflögulýsati og kálfasermi

Kristbjörg Gunnarsdóttir

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Umsjónarkennari: Martha Ásdís Hjálmarsdóttir
Leiðbeinendur: Dr. Ólafur Eysteinn Sigurjónsson og Sandra Mjöll Jónsdóttir Buch

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Ágrip

Mesenchymal stofnfrumur (MSC) hafa verið mikið rannsakaðar síðustu ár en þær eru fjölhæfar frumur sem hægt er að sérhæfa yfir í bein-, brjósk- og fitufrumur við vissar aðstæður. Hægt er að einangra þær úr ýmsum vefjum fullorðinna einstaklinga, til dæmis úr beinmerg. Hinsvegar hefur gengið erfiðlega að rannsaka MSC frumum vegna meðal annars ræktunaraðferða, erfiðleika við að skilgreina frumurnar og einnig geta einangrunaraðferðir verið sársaukafullar, eins og beinmergsástunga. Nýlega tókst að sérhæfa fósturstofnfrumur yfir í mesenchymal forverafrumur (hES-MP) og gætu þessar frumur verið lykllinn að lausn þeirra vandamála sem einkenna rannsóknir á MSC. Sýnt hefur verið fram á að hES-MP frumur hafi svipaða eiginleika og MSC hvað varðar útlit, svipgerðartjáningu og fleira. Miklar vonir eru því bundnar við þessar stofnfrumur og eru rannsóknir á þeim tiltölulega nýjar af nálinni. Kálfasermi er það ræktunaræti sem er mest notað við ræktun stofnfrumna. Það er hins vegar ekki fýsilegur kostur sem æti fyrir læknisfræðilegar meðferðir vegna dýrborinna smita og siðferðislegra vandamála, svo dæmi séu tekin. Útrunnin blóðflögulýsöt úr blóðflögum manna gætu mögulega gegnt hlutverki kálfasermis og hefur notkun þess sem ræktunaræti reynst vel á MSC frumur, ekki er þó búið að rannsaka áhrif þess á hES-MP frumur.

hES-MP frumur voru ræktaðar í ræktunaræti sem innihélt kálfasermi og ræktunaræti sem innihélt útrunnin blóðflögulýsöt. Með svipgerðargreiningu var athugað hvaða áhrif blóðflögulýsatið hafði á svipgerð frumnanna og einnig hvort svipgerð frumnanna líktist frekar fósturstofnfrumum eða MSC frumum.

Svipgerðagreining hES-MP frumna var svipuð milli ræktunaræta en þó var munur á milli nokkurra yfirborðssameinda. Þrátt fyrir þennan mun benda niðurstöðurnar til þess að svipgerð hES-MP frumnanna líkist svipgerð MSC frumnanna.

Mögulega er hægt að nota útrunnin blóðflögulýsöt sem ræktunaræti fyrir hES-MP frumur en nauðsynlegt er að gera sérhæfðari próf til að rannsaka hvað veldur þessari breyttu tjáningu á yfirborðsmarkerum í HPLO.

Abstract

Mesenchymal stem cells (MSC) have been researched extensively for the last few years. These cells are versatile and can differentiate into osteocytes, chondrocytes and adipocytes. They can be isolated from various human adult tissues such as bone marrow and fat. However, studying MSC can be troublesome due to difficulties with expansion, characterization and isolation methods of the cells but isolation methods, such as bone marrow aspiration, can be unpleasant for the patient. Recently, mesenchymal progenitors (hES-MP) were derived from human embryonic stem cells. These cells may be the key in eliminating the problems that occur when examining MSC. It has been shown that the morphology and immunophenotype, among other characteristics, of hES-MP cells and MSC are similar. These cells open up for new possibilities in the future of stem cell research. Fetal bovine serum (FBS) is the most used supplementary media for stem cell expansion. Nevertheless, due to complications, such as ethical issues and contamination from animal molecules that involve the application of fetal bovine serum use, a substitute is needed. Outdated human platelet lysates derived from platelets are a possible substitute for fetal bovine serum and they have already been analysed with MSC cells with good results. However, hES-MP cells have not been analysed in outdated human platelet lysate (HPLO).

hES-MP cells were expanded in two different supplemented medias, more precisely FBS and HPLO. Immunophenotype in hES-MP cells were analysed in both media and compared. The immunophenotype of hES-MP cells was analysed in correlation with the immunophenotype of MSC and embryonic stem cells (ESC) to see whether they are more alike MSC or ESC.

Immunophenotype analysis of the two media was similar but there was a slight difference in the fluorescence intensity in a few of the surface markers. Even so, the results indicate that hES-MP cells immunophenotype is similar to the immunophenotype of MSC. Thus, they are derived to a mesoderm lineage.

It is possible to use HPLO as a supplementary media for hES-MP cells but studies with more decisive results are necessary to determine what is affecting the expression of surface markers in HPLO.

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Table of Contents

Figures

Tables

Abbreviations

ADP	Adenosine diphosphate
ALL	Acute lymphatic leukaemia
ALP	Alkaline phosphatase
APN	Aminopeptidase N
APS	Ammonium persulfate
ASC	Adult stem cells
bFGF	basic fibroblast growth factor
BM	Bone marrow
BSA	Bovine serum albumin
CD	Cluster of Differentiation
CFU-Fs	Colony-forming unit fibroblasts
D1	Donor one
DMEM	Dulbecco's minimal essential medium
ECC	Embryonic carcinoma cells
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
ESC	Embryonic stem cells
F12	Ham's F12 supplement
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
hESC	Human embryonic stem cells
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
hPLT	Human platelets
hrbFGF	Human recombinant basic fibroblast growth factor
HSC	Haematopoietic stem cells
ICM	Inner cell mass
IL-6	Interleukin 6
iPSC	induced pluripotent stem cells

ISCT	The International Society for Cellular Therapy
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
mESC	Mouse embryonic stem cells
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cells
OCS	Open Canalicular system
P1	Passage one
PBS	Phosphate buffered saline
PC	Platelet concentrates
PDGF	Platelet-derived growth factors
PMSF	Phenylmethanesulfonyl fluoride
PRP	Platelet rich plasma
RIPA	Radio immuno precipitation assay
SCF	Stem cell factor
SDS	Sodium Dodecyl sulphate
SOCS	Suppressor of cytokine signaling
SP	Substance P
TBS	Tris buffer saline
TGFβ	Transforming growth factor
TNFα	Tumour necrosis factor-α
TβRI	Type I TGF beta receptor
UCBS	Umbilical cord blood serum
UTF1	Undifferentiated embryonic cell transcription factor-1

1. Introduction

1.1 Stem cell overview

1.1.1 History

The German biologist, Ernst Haeckel, first published the term “stem cell” in scientific literature in 1868. He described stem cells as the progenitor of all unicellular organisms, which he postulated that all multicellular organisms were derived from (Appasani et al., 2011). This theory was constructed to explain the ability of tissues, such as blood and skin, to self-renew over the course of a lifetime even though they are constructed from short-lived cells. Many years later, stem cells were identified as cellular entities. This identification was made possible due to methods developed for prospective isolation of stem cell candidates and more accurate analysis on their potency after transplantation (Bianco et al., 2008). Current knowledge of stem cell biology dates to the 1960s when stromal cells and multipotent haematopoietic stem cells were identified in the bone marrow. Numerous studies from then on have reported about isolations of stem cells from early embryos from adult tissues such as bone marrow and adipose tissue (de Peppo et al., 2012).

1.1.2 Definition and characterization

Stem cells can be defined as the building material of our anatomy, working as the main functional units of embryonic formation throughout development and regeneration of adult tissue following damage (de Peppo & Marolt, 2012). Stem cells are unspecialized cells that are capable of multilineage differentiation and self-renewal, which indicates their ability to generate a daughter cell with equivalent potential (Luca, 2013; Weissman, 2000). They have two methods for dividing themselves. Firstly, there is a symmetrical division where they divide into two identical daughter cells. Secondly, asymmetrical division where they divide into one identical daughter cell and one differentiated cell (Figure 1) (Mountford, 2008). Stem cell lineages are originated from the ectoderm, mesoderm and endoderm germ layers. It is possible to create all cells in the human body from these germ layers (Luca, 2013).

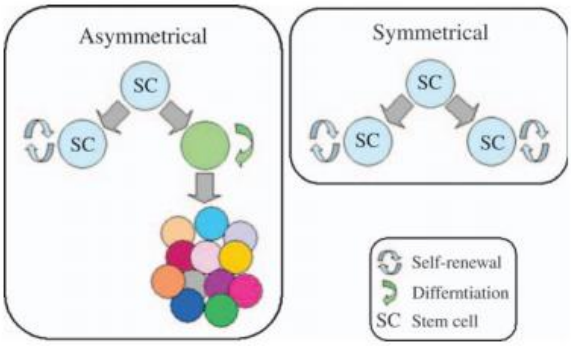

Figure 1 Self-renewing stem cell divisions

Stem cells divide in two different ways. When they divide to two identical daughter cells, it is called a symmetrical division. When they divide into one identical daughter cell and to one differentiated cell it’s referred to an asymmetrical division (Mountford, 2008).

Potency is referred to as the differentiation potential of stem cells into particular cell types and their ability to give rise to mature cell types. Stem cell differentiation potential is categorized into five groups of gradual potency (Hima Bindu et al., 2011; Kolios et al., 2012):

1. Totipotent stem cells are cell such as Zygotes (a fusion between an egg and a

sperm cell) and cells produced from the first few divisions of a fertilized egg are the only cells that can be called totipotent. Zygotes have the ability to differentiate into embryonic and extraembryonic cell types.

2. *Pluripotent* stem cells can differentiate into all cells that are derived from any of the three germ layers. Their main aspects are their self-renewal and their ability to differentiate into all cell types of the adult organism. Embryonic Stem Cells and induced pluripotent stem cells (iPSC) have pluripotency.
3. *Multipotent* stem cells can only differentiate into cells that belong to the same germ layer. For example, Mesenchymal stem cells are multipotent stem cells that can differentiate to cells from the mesodermal layer such as osteoblasts.
4. *Oligopotent* stem cells can differentiate into two or more cell type lineages that belong to their residing tissue or organ. Haematopoietic stem cells are an example of oligopotent stem cells and they can differentiate into both myeloid and lymphoid lineages.
5. *Unipotent* stem cells can only differentiate into one cell type, which is their own. The only thing that distinguishes them from non-stem cells is their self-renewal ability. Muscle stem cells are examples of unipotent stem cells.

Stem cells can be roughly classified as embryonic, umbilical cord and adult stem cells based on their derivation development (de Peppo & Marolt, 2012).

1.1.2.1 Embryonic stem cells

Human embryonic stem cells (hESC) are pluripotent cells that are derived from the inner cell mass of the blastocysts. They have the ability to give rise to all three embryonic germ layers; ectoderm, endoderm and mesoderm (L. Li et al., 2005). They are capable of indefinite self-renewal due to their replicative senescence (Mountford, 2008). For example, these cells have been expanded continuously for two years without undergoing senescence and preserving their karyotype over time (Mountford, 2008). This shows how much genomic stability hESC have (Mountford, 2008). These cells will be described more precisely in chapter 1.2.

1.1.2.2 Umbilical cord stem cells

Right after birth, the majority of haematopoietic stem cells are found in the placenta and umbilical cord. But within hours of delivery, they migrate to the bone marrow where they provide a permanent source of stem cells and blood-forming progenitors, such as platelets and erythrocytes, for a lifetime (Rogers et al., 2004). Umbilical cord blood has more haematopoietic stem cells per volume than in bone marrow and it seems to be more tolerant of human leukocyte antigen (HLA) mismatches. Many umbilical cord blood transplantations have been done to treat patients with malignant and non-malignant diseases but most of these have been from sibling donors with partial or complete HLA matching (Ballen et al., 2001; Rogers & Casper, 2004). Collection of umbilical cord blood units is non-invasive for the donor, contrary to collection from the bone marrow, so they can be easily collected. Also, this procedure averts ethical and technical issues that are linked to embryonic stem cell harvest from embryos (Malgieri et al., 2010; Wu et al., 2009).

1.1.2.3 Somatic stem cells

Somatic stem cells, also referred to as adult stem cells (ASC), are defined as undifferentiated cells that can be found among other differentiated cells in organs and tissues (Health, 2012). The main characteristic of somatic stem cells is their ability to maintain and repair the tissue they are located in but somatic stem cells have been identified in organs and tissues like brain, skeletal muscle, heart, liver and bone marrow for example (Health, 2012). Their differentiation and self-renewal are promoted by symmetrical and asymmetrical divisions as previously described (Figure 1) and have been classified as either multipotent, oligopotent or unipotent (Kolios & Moodley, 2012; Lazzeri et al., 2012). Two kinds of somatic stem cells were located in the bone marrow in the 1950s. Firstly, there are haematopoietic stem cells that produces all types of blood cells in the body and secondly, mesenchymal stem cells that produces chondrocytes, osteocytes and adipocytes (Health, 2012). Mesenchymal stem cells will be described more precisely in chapter 1.3.

1.1.3 Stem cell niches

The concept of a niche was proposed by Schofield in 1978 when he described the microenvironment that supports stem cells (Schofield, 1978). Some stem cell types lie in their quiescent state but are awakened in particular stages of the life cycle and when injuries occur. These elements are controlled within restricted tissue microenvironments known as niches (Morrison et al., 2008). The stem cell niche has been referred to as an anatomical compartment that includes both cellular and acellular components. These components accommodate both local and systemic signals to regulate the biology of stem cells (Mohyeldin et al., 2010). These niches are important to preserve stem cells potency during homeostasis and it is only when they evacuate from their niches, that they differentiate (Kiefer, 2011). The niche has a specific role; to maintain stem cells and to keep their microenvironment stable. It builds a specific reserve for stem cells with signaling molecules and cell adhesion. By secretion of cytokines and growth factors, differentiation is avoided and self-renewal is promoted (Kiefer, 2011).

It has been difficult to examine and locate the relationship between stem cells and their niche in mammalian organs due to its enormity and complexity (Kiefer, 2011). However, it has proven useful to examine other genetic model systems like *Drosophila melanogaster* and *Caernorhabditis elegans* due to their defined stem cell niche structure *(L. Li & Xie, 2005; Walker et al., 2009)*.

1.1.4 Stem cell surface markers

Each cell type in the body has specialized proteins present on their surface. These proteins have the ability to bind or interact selectively with other signaling molecules and receptors in their environment and are used as surface markers for cellular identification (Health, 2009). In normal situations, cellular communication is facilitated by the binding of receptors to their ligands, triggering intracellular signaling that may appear in altered cellular behaviour and responses (Prentice, 2003). All types of stem cells express different surface markers and since they have not differentiated to a specific function, identification of these cells relies heavily on these surface markers (Prentice, 2003).

1.1.5 Use of stem cells in regenerative medicine

> "The development of cell lines that may produce almost every tissue of the human body is an unprecedented scientific breakthrough. It is not too unrealistic to say that stem cell research has the potential to revolutionize the practice of medicine and improve the quality and length of life (Appasani & Appasani, 2011)."

Harold Varmos, the former director of National Institutes of Health and a 1989 Nobel recipient in physiology or medicine, spoke these words before the U.S. Senate Appropriations Subcommittee in December 1998 (Appasani & Appasani, 2011) . Ever since the discovery of stem cells, there has been a great deal of expectations due to their abilities regarding self-renewal and proliferation. As stated earlier, stem cells are characterized by their ability to self-renew and their ability to differentiate along multiple lineage pathways but stem cells for regenerative medicinal application have to meet the following criteria (Gimble, 2003):

1. They need to be available in abundant quantities (millions to billions of cells)
2. They can be harvested with a minimally invasive procedure
3. They can be differentiated along multiple cell lineage pathways in a regulatable manner
4. They should be transplanted safely and effectively to either an autologous or allogeneic host
5. They should be manufactured in accordance with current Good Manufacturing Practice guidelines.

Even though there is huge potential for using stem cells as a treatment for a broad range of genetic disorders and irregular degenerative disorders such as Alzheimer's disease and Parkinson's disease, many obstacles challenge stem cell therapy. The foremost of obstacles is the inability to assess fate of cells and know the differentiation outcome before transplantation (Appasani & Appasani, 2011). Before there is a chance to use stem cells for therapy it is necessary to find new technology to be able to assess cell viability before transplantation (Appasani & Appasani, 2011).

There will be a more definitive and a deeper discussion regarding the use of stem cells in regenerative medicine, with emphasis on embryonic stem cells (chapter 1.2.4) and mesenchymal stem cells (chapter 1.3.3).

1.2 Embryonic stem cells

1.2.1 Historical overview

It was not until the early 1980s when Gail Martins of the University of California at San Francisco in the United States, and Martin Evans and Matthew Kaufman of the University of Cambridge in England isolated stem cells from mouse embryos independently. These cells were called embryonic stem cells (Appasani & Appasani, 2011).

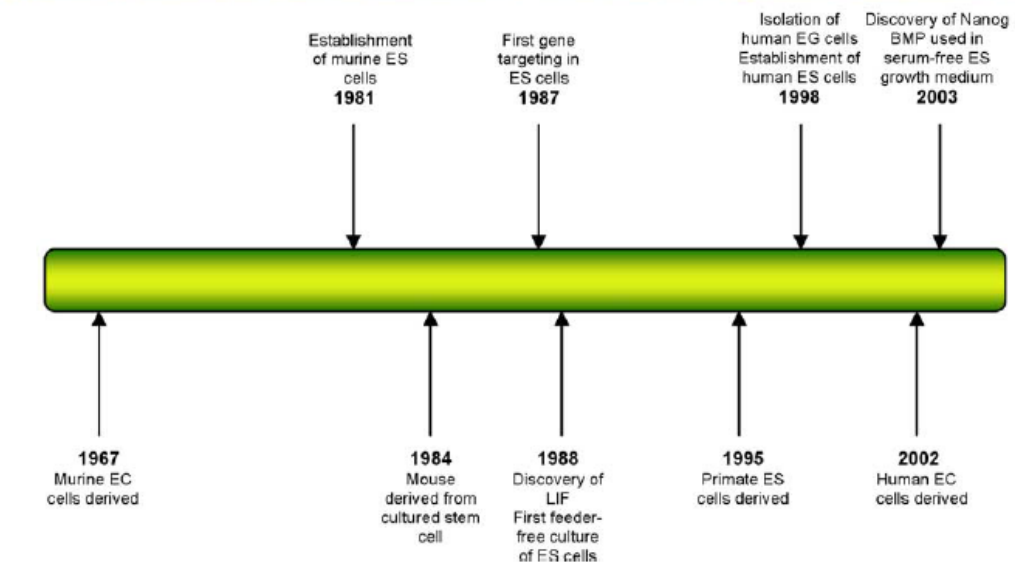

Figure 2 Timeline showing crucial discoveries in embryonic stem cell biology

This timeline reviews briefly the short history of embryonic stem cells until 2003(Friel et al., 2005) .

Thomson and colleagues were the first to derive hESC lines from the inner cell mass (ICM) of human blastocysts generated *in vitro* (Thomson et al., 1998). The inner cell mass is destined to give rise to all tissues of the body and ES cells are derived from these cells (Friel et al., 2005). Since then, many decisive discoveries have put their mark on embryonic stem cell biology (Figure 2).

1.2.2 Definition

hESC are usually grown in dense colonies on feeder layers of murine embryonic fibroblasts or human cells, like cells from fetal muscle and fetal skin that generates extracellular matrix for cell adhesion and modify the culture medium with secretion of paracrine factors (de Peppo & Marolt, 2012; Richards et al., 2002). As stated above in chapter 1.1.2.1, hESC withhold their normal karyotype during a long time of proliferation due to their unlimited self-renewal potential. The main cause of replicative senescence is telomere shortening due to deterioration on the ends of individual chromosomes, which forms a cellular replicative clock. This explains why somatic stem cells do not have unlimited proliferation. hESC have high expression of telomerase activity so they have the ability to replace their telomeres and reset their replicative clock which allows them to divide continuously (Mountford, 2008).

One of their main characteristic is that they can differentiate into cells from all three germ layers, i.e. they can differentiate into all types of cells in the body (Mountford, 2008). Mechanisms in ESC control their pluripotent state. The factor that promotes self-renewal and inhibits differentiation in mouse embryonic stem cells (mESC) is provided by the feeder cells. It was identified in the late 1980s and was called leukaemia inhibitory factor (LIF), which is a member of the IL-6 cytokine family (Friel et al., 2005). LIF activates the Janus-associated tyrosine kinase, a signal transducer and activator of the Jak/Stat3 transcription pathway by binding a heterodimer of the LIF receptor (LIFR) and gp130, which is an IL-6 signal transducer. This triggers the stimulation of mESC renewal and suppresses differentiation (Hyslop et al., 2005). In contrast to mESC, hESC do not express gp130 and LIFR enough to control the high expression of suppressor of cytokine signaling (SOCS) genes, which leads to the inhibition of LIF-mediated signaling. Therefore, the LIF/Stat3 signaling pathway in hESC is inadequate to inhibit differentiation (Hyslop et al., 2005).

Reports have shown that TGFβ, Activin, Nodal signaling, WNT signaling and SMAD2/3 activation are essential for the maintenance of the undifferentiated state in hESC (James et al., 2005; Vallier et al., 2005).

1.2.3 ESC markers and self-renewal

There have been reports of a wide range of cell surface markers and generic molecular markers in recent years that can be used to identify and locate undifferentiated ESC. However, some ESC markers overlap with those of tumour stem cells so it can be problematic to use these markers for ESC isolation and identification (Zhao et al., 2012). ESC markers can be categorized into five different groups (Table 1).

Table 1 Categories and examples of ESC markers

ESC markers can be divided to five categories based on their location. Each category contains numerous markers and only examples are given in this table.

Categories	Examples
Surface markers	SSEA-3, SEE-4, CD90, CD324, CD326, CD29
Transcription factors	Oct-4, Sox2, Nanog, ZFX, Stat3, Sall4
Pathway related markers	SMAD1/5/8, SMAD2/3, SMAD4, β-catenin
Enzymatic markers	ALP
Other markers	Lectins, Peptides

Many transcription factors are essential for promoting self-renewal. Nanog, Oct-4 and Sox-2 are few of those transcription factors and they cooperatively conserve pluripotency in embryonic stem cells (Babaie et al., 2007). Oct-4, also referred to as Oct-3/4, is a member of the POU domain transcription factor family and was first recognized due to its expression in undifferentiated stem cells, which is diminished during differentiation (Niwa, 2004). The function of Oct-4 includes operating with Sox-2, a Sry-related transcription factor. These factors activate transcription of target genes such as UTF-1 (Undifferentiated embryonic cell transcription factor-1) (Niwa, 2004). UTF-1 is a chromatin component and is essential for ESC and embryonic carcinoma cell (ECC) differentiation (Kooistra et al., 2010). This cooperation between Oct-4 and Sox-2 also regulates Sox-2 expression, which indicates that a positive feedback mechanism may potentially be a part of ESC self-renewal maintenance (Niwa, 2004). Nanog is a homeodomain factor and works as a marker for all pluripotent cell lines. Nanog is essential for maintaining undifferentiation in ESC, similar to Oct-4 (Cavaleri et al., 2004).

1.2.4 Use of embryonic stem cells in regenerative medicine

Scientists believe that stem cells from human embryos could hold the key to understanding and even controlling the human development. However, there has been an ethical debate between researchers and those who are pro-life due to the derivation of ESC from early embryos, which is necessary for *in vitro* research (Robertson, 2010). Pro-life advocates believe this treatment of embryos is identical to abortion, i.e. murder or a human sacrifice for science, while the other side sees the embryo not as a human being since the embryo is too undeveloped to have feelings or human rights (Robertson, 2010). In 2006, Takahashi K. et al. published an article about induced pluripotent stem cells. By transducing embryonic or epidermal fibroblasts with four transgenes Oct4, c-Myc, SOX2 and Klf4, they obtained cells with typical aspects of stem cells (Takahashi et al., 2006). It is possible to overcome these ethical issues if pluripotent stem cells can be derived from somatic cells (Okita et al., 2007).

Even though there is a lot of hope surrounding the use of embryonic stem cells to cure diseases due to their differentiation potential, they have a number of limitations. Their unrestricted growth potential is a potential limitation due to possible teratoma formation. Teratoma formation could occur if differentiation cues are avoided after transplantation (D.J. Prockop et al., 2010). Scientist have been trying to ensure ESC differentiation prior to transplantation by using growth factors, hormones and cytokines for example (D.J. Prockop et al., 2010). One of the most challenging limitations is the possibility of immunological rejection from the transplant recipient. When ESC are engrafted into an adult tissue, it is considered an allogeneic transplantation. Even though ESC are usually linked to allogeneic transplantation and identified as such, they also possess the ability to engraft into hosts that are not identical, with minimal immunosuppression (D.J. Prockop et al., 2010).

When data from clinicaltrials.gov is examined, it is evident that there are currently nine open ongoing clinical trials involving ESC with eight open clinical trials focusing on human ESC. However, even though ESC can differentiate into all cells of the body, there are many obstacles that the scientific community has to overcome before they can be used in regenerative medicine.

1.2.5 hES-MP cells

1.2.5.1 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).

1.2.5.2 Characterization of hES-MP and how they resemble MSCs

hES-MP cells have a typical fibroblast like morphology that resembles MSC, i.e. they form an elongated spindle-shaped cell morphology with branching pseudopodia and they do have a significantly different morphology from those of undifferentiated hES cells (Karlsson et al., 2009). It has been shown by Camilla Karlsson et al. that hES-MP cells do not express markers that are typically found in undifferentiated hESC like Oct-4, Nanog, SSEA-3, and SSEA-4. Instead they express the early mesodermal markers Desmin and Vimentin, which is the same as MSC express, and they noticed that the hES-MP lines could expand for 16-20 passages before a decrease in proliferation was detected (Karlsson et al., 2009). They also showed that hES-MP cells had not the potential to de-differentiate and could not attain pluripotency again. Thus, their stability is acceptable (Karlsson et al., 2009). Guiseppe Maria de Peppo et al. looked at gene expression in hES-MP cell lines, including SA002.5. They also found that hES-MP cells repress genes such as Nanog, Sox-2 in hES-MP derivation, which provides evidence for a lineage commitment detected in hES-MP cells compared with hESC. Also, there is evidence for a decreased chance of teratoma formation in contrast to hESC due to their induced expression of NRF2F and p53 associated genes LTPP2 and TFAP2A (Giuseppe Maria de Peppo 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 (Giuseppe Maria de Peppo et al., 2010). This is essential for a possible future use of hES-MP cells usage in regenerative medicine.

1.3 Mesenchymal stem cells

1.3.1 Historical Overview

The advancement of stem cell research arose when it was clear that all blood cells are derived from the same stem cells (Nombela-Arrieta et al., 2011). These stem cells are called haematopoietic stem cells (HSC) and are a rare subset of the bone marrow but they can be isolated and assayed *in vitro* and *in vivo (*Nombela-*Arrieta et al., 2011).* After the discovery of HSC, Alexander Friedenstein and his

colleagues discovered the mesenchymal stem cell. It was about 40 years ago when they conducted a research and found out that pieces of bone marrow transplanted under the renal capsule of mice formed a heterotropic osseous tissue that was self-renewing, self-maintaining and capable of supporting host cell haematopoiesis (Darwin J. Prockop et al., 2008). Friedenstein additionally showed that osseous-forming activity of the bone marrow was contained within the fibroblastoid cell fraction isolated by preferential attachment to tissue culture plastic (Darwin J. Prockop et al., 2008). These results suggested that a non-haematopoietic bone marrow multi-potent precursor cells with adipose and skeletal potential existed (Nombela-Arrieta et al., 2011). Friedenstein described these cells as multi-potential stromal precursor cells that were spindle-shaped and clonogenic in monolayer cultures. MSC were defined as colony-forming unit fibroblasts (CFU-Fs) (Salem, 2009; Uccelli et al., 2008). CFU-F-derived stromal cells can serve as feeder layers for the expansion of haematopoietic stem cells and they can differentiate into adipocytes, chondrocytes, osteoblasts and myoblasts 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, and meanwhile preserving their ability to differentiate to osteoblasts, adipocytes and chondrocytes *in vitro* (Nombela-Arrieta et al., 2011; Pittenger et al., 1999). Proliferation *in vitro* and multilineage capacities were interpreted as significant for *in vivo* multipotency and self-renewal. The term mesenchymal stem cell was accepted for these newly identified precursor cells (Nombela-Arrieta et al., 2011).

1.3.2 Definition and Characteristics

Even though MSC have been intensively investigated throughout the years, the definition of MSC is solely constructed from *in vitro* experiments (Augello, Kurth, et al., 2010). Their role and location within their tissue of origin *in vivo* are not known due to the lack of specific markers that can distinctively identify them (Augello, Kurth, et al., 2010). There have been speculations whether MSC abilities and phenotype differ between *in vivo* and *in vitro* due to the removal from their natural environment. However, these cells are known to undergo phenotypic rearrangements during *ex vivo* manipulations that would result in acquiring new and losing expression of some existing markers (Augello, Kurth, et al., 2010; Jones et al., 2002). MSC demonstrate a heterogeneous morphology. Several terms have been used to describe their appearance. These terms include fibroblastoid cells, giant fat cells and blanket cells, spindle shaped, flattened cells, and very small round cells (Pevsner-fischer et al., 2011). Seeding density can have an altering effect on morphology of these cells and their shape can change dramatically (Pevsner-fischer et al., 2011; Wong, 2011).

The International Society for Cellular Therapy (ISCT) has provided minimum criteria for defining multi-potent MSC in standard cultivation (Dominici et al., 2006; Salem, 2009). This criterion is as follows:

1) They are plastic-adherent under standard culture conditions.
2) They are positive for expression of CD73, CD105 and CD90, and don't express the haematopoietic 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 catalyst.

These characteristics apply to all cultivated MSC but there could be some differences between tissues of origins. For example, adipose-tissue-derived MSC express the membrane antigens CD34 and CD54 (De Ugarte et al., 2003).

1.3.2.1 Characterization with surface markers

MSC compose a heterogeneous population of cells in terms of their morphology and expression of surface markers. They express various surface markers (Table 2) but there are no distinctive surface markers that can selectively identify MSC so it can be difficult to assess their actual numbers or identify their specific locations (Caplan & Bruder, 2001; Docheva et al., 2008). Various methods have been used to up-regulate signals from membrane proteins due to the difficulty of identifying them by mass spectrometry, especially those membrane proteins that can be used as cell surface markers (Niehage et al., 2011). However, a comprehensive understanding of the cell surface proteome is restricted to the complexity of the MSC proteome when undergoing differentiation toward distinct cell lineages (Niehage et al., 2011).

Table 2 CD antigens that can been used to characterize MSC

These CD antigens can be used to define and characterize MSC. MSC express CD13, CD29, CD44, CD73 and CD105 but not CD10, CD45 and CD184) (Murphy et al., 2008).

CD antigen	Cellular expression	Molecular weight(kDA)	Function
CD10	B- and T-cell precursors, bone marrow stromal cells	100	Zinc metalloproteinase, marker for pre-B acute lymphatic leukaemia (ALL)
CD13	Myelomonocytic cells	53-55	Zinc metalloproteinase
CD29	Leukocytes	130	Integrin β-1 subunit, associates with CD49a in VLA-1 integrin
CD44	Leukocytes	80-95	Binds hyaluronic acid, mediates adhesion of leukocytes
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
CD73	B-cell subsets, T-cell subsets	69	Ecto-5'-nucleotidase, dephosphorylates nucleotides to allow nucleoside uptake
CD105	Endothelial cells, activated monocytes and macrophages, bone marrow cell subsets	90	Binds TGF-β
CD184	Preferentially expressed on the more immature CD34+ haematopoietic stem cells	46-52	Binding to SDF-1 (LESTR/Fusin); acts as a cofactor for fusion and entry of T-cell line; trophic strains of HIV-1

Expressions of CD (Cluster of Differentiation) markers in MSC have been thoroughly examined due to the lack of distinctive markers for MSC characterization. Their phenotypic characterization varies between research papers. Mafi et al. examined 29 studies that had been focusing MSC expression of cell surface markers (Mafi et al., 2011). Their conclusion was that markers like CD105, CD13, CD44, CD73 and CD29 for example were among the most common positive surface markers on mesenchymal cells in these studies and surface markers like CD10, CD45 and HLA-DR were most commonly negative (Mafi et al., 2011). HLA-DR is a human leukocyte antigen and belongs to the MHC class II molecules. These MHC (major histocompatibility complex) class II molecules bind peptides from proteins that have been degraded in endosomes (Murphy et al., 2008). Martins et al. examined MSC surface markers and by their analysis, CD184 is also a positive surface marker on mesenchymal stem cells (Martins et al., 2009).

Brachyury is used as a mesodermal marker and functions as a transcription factor. Brachyury is a part of the T-box gene family but these genes encode transcription factors that share similar characteristic sequence within the DNA-binding domain. This protein function involves binding DNA in a sequence-specific manner in the nucleus (Showell et al., 2004).

Even though MSC consistently express these markers either positive or negative, they are not necessarily representative of MSC *in vivo*. They can be expressed in other cells as well so it is preferable to follow all of the minimum criteria that ISCT published.

1.3.2.2 The MSC microenvironment

The bone marrow (BM) is a dominant groundwork for HSC. These cells renew red blood cells, monocytes, granulocytes and platelets. They inhabit within the BM due to cells, other than HSC, that support the microenvironment and help HSC to develop and differentiate. MSC are one of the cell types that support HSC microenvironment. This microenvironment is also called the “haematopoietic niche” (Williams et al., 2011). Inside this HSC microenvironment, HSC are believed to dwell in enclosed niches that are built by surrounding cells, extracellular matrix proteins and soluble factors that promote HSC maintenance (Nombela-Arrieta et al., 2011). MSC in BM are thought to provide haematopoietic progenitors modulatory signals because a derived culture from adherent fraction of BM stroma promotes HSC ability for proliferation and survival *ex vivo*. Therefore, MSC main function is considered to be configuration in the HSC niche by organizing vascular networks or through direct interaction (Nombela-Arrieta et al., 2011).

MSC can be found in almost all tissues in perivascular niches that have close association with blood vessels (Mohyeldin et al., 2010). They have been compared to pericytes, cells that are located near blood vessels. Pericytes have similar characteristics as MSC in terms of morphology and expression of surface markers. They can also differentiate into adipocytes, osteoblasts and chondrocytes (Nombela-Arrieta et al., 2011). Bear in mind that pericytes and MSC are analogous, not equivalent. Pericytes are only found near capillaries and MSC-like precursors can be found near the walls of vascular types, like arteries and veins (Nombela-Arrieta et al., 2011).

1.3.3 MSC differentiation

MSC are non-haematopoietic stem cells that possess a multilineage potential and give rise to cells such as skeletal muscle cells, connective tissues (tendons and muscles for example) and cells that belong to the vascular system (Figure 3) (Salem, 2009). MSC are most often derived from the bone marrow, but they have been derived from other tissues such as adipose tissue and periosteum (Augello & De Bari, 2010).

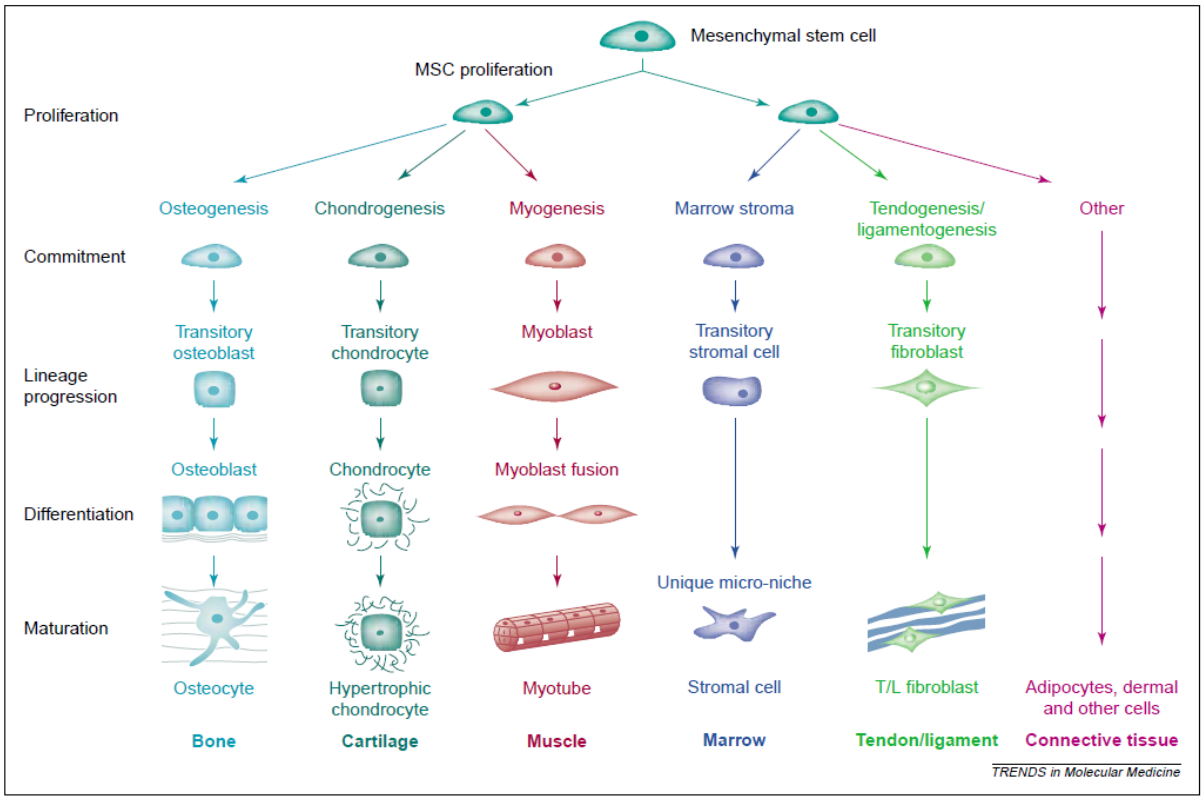

Figure 3 The MSC multilineage potential

The process from a mesenchymal stem cell to a differentiated tissue showed schematically. This figure does not show the detailed process of differentiation (Caplan et al., 2001).

As stated before, MSC can be characterized by their ability to differentiate into osteogenic, chondrogenic and adipogenic lineage (Augello & De Bari, 2010). Differentiation is activated by incubating MSC with certain chemicals and morphogens (Kassem et al., 2008). MSC differentiation to osteoblasts *in vitro* activates in the presence of β-glycerol-phosphate, ascorbic acid and dexamethasone (Chamberlain et al., 2007). When incubated with these supplements, they up-regulate Alkaline phosphatase (ALP) and obtain osteoblastic morphology (Barry et al., 2004). Adipogenic differentiation is activated by expanding MSC with dexamethasone, isobutyl methyl xanthine, indomethacin and insulin (Chamberlain et al., 2007). The cells start to produce large vacuoles filled with lipids (Barry & Murphy, 2004). Chondrogenic differentiation is promoted by centrifuging MSC to make a pellet and then the pellets are expanded with TGF-β. The cells start to produce type II collagen and a great amount of glycosaminoglycans forms within the extracellular matrix (Chamberlain

et al., 2007). MSC can also differentiate into Myocytes when promoted with 5-aza-cytidine and amphotericin B (Chamberlain et al., 2007).

There have been studies about MSC plasticity and their ability to differentiate into non-mesodermal cells, for example hepatocytes and neuronal cells. However, there is no concrete evidence to support that notion (Kassem et al., 2008).

1.3.4 Use of mesenchymal stem cells in regenerative medicine

MSC have gained popularity among stem cell researchers in the recent years, not only for their ability to self-renew and their multilineage differentiation potential, but also because it is easier to deal with them in contrast to ESC for example (Wong, 2011). MSC are almost all MHC I positive and MHC II negative. Even though they express low levels of MHC I and can activate T cells, the absence of co-stimulatory molecules (CD40, CD80 and CD84) leaves the T cells inactive. Thus, MSC are immune privileged cells so they dodge the immune surveillance for a long time (Wong, 2011). Currently, there are 146 ongoing open studies involving MSC according to Clinicaltrials.gov, with 51 clinical trials focusing on human MSC. Hence, there is a great amount of interest in using these cells for regenerative medicine.

Despite these interesting properties, MSC withhold important limitations from a tissue engineering perspective. Firstly, donors or patients have to undergo invasive procedures, like bone marrow aspiration. MSC are then isolated and enriched through partly developed procedures, which usually results in high degree of heterogeneity (G. M. de Peppo et al., 2010; Ho et al., 2008). Secondly, analysis of MSC has shown that they lose their replicative capability after extended *ex vivo* expansion but that limits cell production for tissue engineering (G. M. de Peppo et al., 2010). However, they can be replicated into numerous cells *ex vivo* for an abbreviated expansion time, which is an advantage for stem cell therapies. Extended *ex vivo* cultivation of MSC has also been affiliated with karyotypic instability and uncontrolled malignant transformation (G. M. de Peppo et al., 2010).

1.4 Platelet lysates

1.4.1 Platelet biology and structure

Platelets are fragments that are derived from megakaryocytes, which are produced in the bone marrow. The production of platelets is encouraged by the growth factor thrombopoietin (Denise M Harmening et al., 2005; Kickler, 2006). Megakaryocytes do migrate into the bloodstream so platelet formation has been proposed to happen in other tissues than the bone marrow, such as blood and lungs (Italiano Jr et al., 2007). Nucleus is not a part of the platelet organelles but the mitochondria contain DNA. The bloodstream has an abundance of platelets where the normal concentration in the bloodstream is 150.000 to 350.000 platelets per µl, making them the second most numerous cells in the blood stream (Denise M Harmening & Moroff, 2005; Harrison, 2005; Kickler, 2006).

Their shapes has been described as discoid with an average diameter of 2 to 4 μm and have a mean volume of 7-11 femtoliters (fL). (Denise M Harmening & Moroff, 2005; Harrison, 2005). Due to their size and shape, platelets have the ability to place themselves at the edge of vessels. This is an ideal position for platelets so they can analyze the vasculature (Harrison, 2005). The platelet structure is divided to three different zones. Firstly, 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 (Denise M. 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 that need the hemostatic response (White, 2007). Secondly, there is the Sol-gel 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 the platelet (Denise M. Harmening et al., 2009). Thirdly, there is the organelle zone, where metabolic activities are conducted.

Three major morphologically distinct types of secretory organelles belong to platelets; dense bodies (δ granules), α granules and lysosomes (Denise M. Harmening et al., 2009; White, 2007). α granules are the most common of the platelet organelles with 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 3) (Reed, 2007).

Table 3 Categorization of α-granules content

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

Adhesion molecules	P-Selectin, von Willebrand factor, thrombospondin, fibrinogen, integrin αIIbβ3, integrin αvβ3, fibrinoectin
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
Immunolgic 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

Dense bodies are more or less acidic with a pH of 6,1. They contain small molecules like ADP and serotonin and also lysosomal membrane proteins like CD63 (LAMP-3) (Reed, 2007). Lysosome contain lysosomal membrane proteins like dense bodies plus acid hydrolases and cathepsins (Reed, 2007).

1.4.2 Platelet function during Haemostasis

The main role of platelets is in the haemostatic process where they stop bleeding after vascular injury and tissue trauma (Davi et al., 2007). Haemostasis is a pivotal process that keeps a complete balance between bleeding and clotting in the human body. This process is sensitive for any imbalance between the communications of five factors. Those five factors are blood vessels, platelets, fibrinolysis, serine protease inhibitors and coagulation proteins (Denise M. Harmening et al., 2009). Haemostasis is classified into primary and secondary haemostasis. Primary haemostasis involves a vascular injury response that forms a platelet plug at the side of damage. This platelet plug adheres to the endothelial wall and limits the bleeding. Secondary haemostasis 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. Haemostasis is completed when platelet-derived growth factor (PDGF) is released and promotes repair to the vasculature (Denise M. Harmening et al., 2009).

1.4.3 Supplementary serum

The diverse role of supplementary serum in expansion media is to provide transport proteins, lipid, hormonal factors and to promote differentiated functions for example (Rauch et al., 2011). Basal media for cell expansion is generally supplemented with animal serum but fetal bovine serum (FBS) is the optimal choice for MSC expansion (Vis et al., 2010). FBS is an excellent supplement due to its low gamma-globulin content, but gamma-globulin are known to have reductive effects on proliferation and growth (Rauch et al., 2011). However, problems have been encountered due to animal serum like FBS in cell expansion. Problems such as unexpected cell growth characteristics and the risk of possible contamination with prions, bacteria, fungi, viruses and endotoxins makes FBS an undesirable choice for MSC expansion (Rauch et al., 2011; Tekkatte et al., 2011). In addition, ethical issues around FBS harvest have had an impact but FBS is harvested from bovine fetuses from pregnant cows before they are slaughtered (Rauch et al., 2011). Therefore, human blood-derived additives have been investigated to see if they can replace FBS as a supplementary media. Alternatives such as human platelet lysate (HPL), umbilical cord blood serum (UCBS) and more have been discovered (Tekkatte et al., 2011). These human growth supplements prevent any risk of secondary effects in expansion that exists in FBS expansion. There is a possibility of contamination from these human growth supplements. However that risk can be kept at bay with strict protocols in blood banks (Tekkatte et al., 2011). Human platelet lysate is one of these alternatives that have been studied extensively as a substitute for FBS.

1.4.4 Platelet lysates as a media supplement

Human platelets (PLT), enriched in PLT lysate, contain PLT-derived growth factors (PDGF), epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and basic fibroblast growth factor (bFGF) and they are mitogenic for MSC, bone cells and chondrocytes (Schallmoser et al., 2007). This rich amount of growth factors are required for MSC expansion so this suggests that platelet lysate can in fact 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) but the process starts with storing PC in a freezer. Fresh HPL (HPLF) is produced by freezing PC immediately after sampling and outdated HPL (HPLO) is produced by keeping PC in room temperature for five to seven days before freezing. The preparation of platelet lysate can be performed by lysing PC with chemicals or mechanical disruption. In mechanical disruption, PC undergoes repeated freeze-thaw cycles, which involves freezing PC and then thawing it to approx. 37°C. This generates disruption of the platelets, which leads to a release of stored growth factors into the solution. HPL is filtered into a pooling double bag but this disruption causes high amounts of platelet molecules in the pooled HPL, which leads to unwanted aggregates. The pooled HPL is therefore centrifuged and a supernatant solution is transferred to storage vials (Schallmoser et al., 2013; Tekkatte et al., 2011). Studies have already demonstrated the possibility of MSC expansion with platelet lysates. Pérez-Ilzarbe et al. for example concluded that the immunophenotype, differentiation and growth potential of MSC did not vary between FBS and platelet lysate (Pérez-Ilzarbe et al., 2009). HPL is also said to accelerate the expansion rate of MSC and spontaneously induce expression of osteoblastic genes (Chevallier et al., 2010). Our group has also shown that lysates from expired platelet concentrates can be used as a supplemented media for cell expansion (Jónsdóttir-Buch et al., 2013).

This makes HPL an attractive choice as a substitution of FBS in cell expansion but the use of *in vitro* human cell expansions for cell therapies and tissue engineering have escalated in the last decade. The production cost for large amount of HPL is relatively low and no ethical issues are involved, unlike FBS preparation (Johansson et al., 2003; Rauch et al., 2011).

Objectives

The objective of this study was to determine what effects expansion of hES-MP cells with lysates produced from expired platelets (HPLO) has on their phenotype in contrast to using media supplemented with fetal bovine serum (FBS). These objectives were analysed by:

1. Performing a fluorescence-activated cell sorting (flow cytometry (FACS)) analysis on hES-MP cells that were expanded in either HPLO or FBS by analysing known surface markers.
2. Western blotting for the embryonic marker Nanog and the mesodermal marker Brachyury in both hES-MP and MSC cells that were expanded in either HPLO or FBS.

2 Materials and methods

2.1 Cell cultures

hES-MP cells were seeded and cultured in two different mediums (10% FBS and 10% HPLO). They were expanded to passages 4, 6 and 10 and used in Fluorescence-activated cell sorting (FACS) analysis. hES-MP cells were also expanded to passage 5, which was used for Western blot analysis. Mesenchymal stem cells from two different donors (D1 and D3) were seeded and expanded in two different mediums (10% FBS and 10% HPLO) to passage 4 to use in Western blot analysis.

2.1.1 Seeding of cells

2.1.1.1 General Seeding procedure

Basal medium was prepared by adding 0.1% penicillin/streptomycin (Gibco, Grand Island, NY, USA) to DMEM F12+glutamax growth medium (Gibco). Expansion mediums were prepared by adding either HPLO (The Bloodbank, Landspitali-University Hospital, Reykjavik, Iceland) or MSC-FBS (Gibco), to the basal medium, making it 10% concentrated. These expansion media were heated to 37°C in an incubator before use.

Cell expansion flasks and plates intended for hES-MP expansion were coated with 0,1% gelatin (SIGMA-ALDRICH® Taufkirchen, Germany) and the gelatin was allowed to completely cover the surface for at least 30 minutes at 4-8°C. Cell expansion flasks and plates intended for MSC expansion remained uncoated. Cryovials containing approximately 0,5 $x10^6$ cells were removed from -80°C storage and thawed carefully in 37°C water and then pipetted gently into 5 mL of warmed medium (either 10% FBS or 10% HPLO). The media, containing the cells, were centrifuged at 545 g for 5 minutes, supernatant poured off and the pellet was gently resuspended in 1 mL of warmed media. Expansion flasks or plates were prepared by replacing the 0.1% gelatin with 22 mL (75 cm^2 flasks), 6,25 mL (25 cm^2 flasks) or 2 mL (for each well in the 6-well plate) of either 10% FBS or 10% HPLO prewarmed media. The cell suspension was then added to the media. All cell cultures were cultured in a 5% CO_2 incubator at 95% humidity and 37°C. This is referred to as standard culture conditions hereafter.

2.1.1.2 Seeding of hES-MP cells for FACS

First, hES-MP cells (Cellartis Inc. Gothenburg, Sweden) from passage 2 and 4 (P2 and P4) were warmed from -80°C and they seeded in 75 cm^2 culture flasks, containing either 10% FBS or 10% HPLO medium. Approximately 6500 cells/cm^2 were seeded in every 75 cm^2 flask. Cells from P9 were provided by courtesy of Sandra Mjöll Jónsdóttir Buch who had an ongoing hES-MP culture. Approximately 400.000 cells were seeded in every 75 cm^2 culture flask, three flasks containing 10% FBS and other three containing 10% HPLO.

2.1.1.3 Seeding of hES-MP and MSC for Western Blotting

hES-MP cells from P4 were thawed from -80°C and seeded in 6,25 mL of either 10% FBS or 10% HPLO media in 25 cm^2 culture flasks. Approximately $0,5x10^6$ cells were seeded in both flasks. MSC, negative for HIV-I, hepatitis B and C viruses (Lonza, Walkersville, MD, USA), from P3 were provided by courtesy of Sandra Mjöll Jónsdóttir Buch, who had been culturing mesenchymal stem cells from D1 and D3. MSC were counted and 50.000 cells were seeded in 2 mL of either 10% FBS or 10% HPLO in a 6-well plate. Each type was seeded in duplicate.

2.1.2 Subculturing of cells

2.1.2.1 Subculturing procedure

Culture flasks were coated with 0,1% gelatin as previously described. Trypsin (Gibco) was warmed up to 37°C as well as 10% FBS and 10% HPLO media. Medium in the culture flasks, which were being subcultured, was poured into waste disposal container. Phosphate buffered saline (Gibco, PBS) was pipetted into the flasks to cover the cell cultured side of the flasks. After rocking the flasks carefully, PBS was disposed into the waste disposal unit. Trypsin was added into the flasks and then incubated at 37°C and 5% CO^2 for 3-5 minutes. Trypsin released cells from the cell cultured side of the flasks. Once cells were released from the flasks, warmed media was added onto the cell culture side of the flasks to neutralize the trypsin and release the remaining cells. Contents of the flasks were pipetted into plastic tubes and they centrifuged at 509 g for five minutes. Supernatant was poured into a waste disposal unit and the pellet was resuspended in warmed medium. Gelatin in the newly coated cell culture flasks was replaced by warmed media. The resuspended pellet was added to the flask as well. All these culture flasks were cultured in a 5% CO_2 incubator at 95% humidity and 37°C.

2.1.3 Subculturing of hES-MP and MSC

2.1.3.1 Subculturing for Flow cytometry

When the confluency for seeded cells reached 80-90%, the cells were subcultured. hES-MP cells from P3 and P5, cultured for FACS, were subcultured to three 75 cm^2 culture flasks for each media. To divide the cells equally to all three of the expansion flasks, 3 mL of media was used to resuspend the pellet. Cells from P10 were already expanded in three 75 cm^2 flasks so there was no need for subculturing. To summarize, all three passages and types were seeded in triplicate.

2.1.3.2 Subculturing for Western blotting

hES-MP cells were subcultured to a 6-well plate, two wells for each sample. 50.000 cells were counted and seeded into 2 mL of either 10% FBS or 10% HPLO media. One of the hES-MP well in FBS got infected so only one well could be used for western blotting. Because MSC cells were already seeded with 50.000 cells in each well, two wells for each media and for each donor, there was no need for subculturing.

2.2 Cell counting

To subculture both western blot and FACS expansion, and for FACS analysis, It was needed to calculate the right amount of cells for seeding and analysis. After resuspending the pellet with 1 mL of media, 20 µl of sample is mixed with 50 µl of Trypan blue (Gibco) for staining, after diluting it with 30 µl of phosphate buffered saline (PBS). After staining, each sample was counted two times with an improved Neubauer hemocytometer in an inverted microscope at 10x magnification (Figure 4). Cells were counted in the white squares and cell count per ml was then determined by using the following 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.

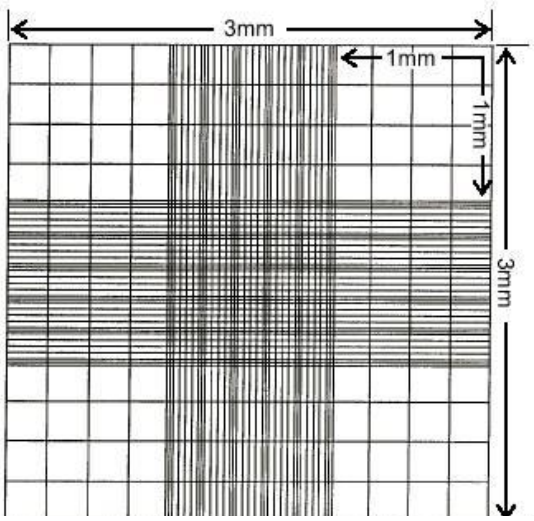

Figure 4 A improved Neubauer hemocytometer

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

2.3 Flow cytometry analysis

2.3.1 Harvesting of cells

When the cells had reached 80-90% confluency in their expansion flasks, they had to be harvested. After pouring medium into waste disposal container, PBS was pipetted into the flask, covering the cell cultured side of the flask, and then poured into a waste disposal container. Trypsin, heated to 37°C, was added into the flasks and then incubated for 3-5 minutes in 37°C and 5% CO_2, or until the cells did no longer adhere to the cell culture surface of the flask. Once cells were released from the surface, warmed media was pipetted onto the cell culture side of the flask to neutralize the trypsin and release the remaining cells. Contents of the flasks were pipetted into plastic tubes and centrifuged at 509 g for five minutes. Supernatant was poured off and pellet resuspended in warmed medium with a pipette. By staining the sample as described above, the cells were counted and cell count per mL was then determined. The required amount of cells for FACS analysis was about 100.000 cells so 100.000 cells from all three expansion flasks in HPLO (P4) were harvested. However, 150.000 cells were harvested from all other expansion flasks. Cells available in the HPLO (P4) flasks were fewer than in the other flasks.

2.3.2 FACS analysis on hES-MP cells

After acquiring the right amount of sample needed from each expansion, the samples had to be prepared for FACS analysis. The antibodies used in this analysis were as follows: CD10, CD105, CD13, CD29, CD44, CD45, CD184, CD73 and HLA-DR. Volume of 10 µl of each antibody was pipetted in different vials and the right amount of sample (cells per mL) added to the antibody, making it nine vials for each expansion flask. Vials were mixed on a shaker for a couple of seconds and stored at room temperature in the dark for at least 20 minutes.

Vials were centrifuged in 4°C for five minutes at 500 g and 500 µl of 0,5% paraformaldehyde in PBS was pipetted in every vial. After this process, the cells were ready to be analysed. Vials were mixed on a shaker for couple of seconds before being measured in FacsCalibur (BD Biosciences, San Jose, CA, USA). FACS function is briefly described in figure 5.

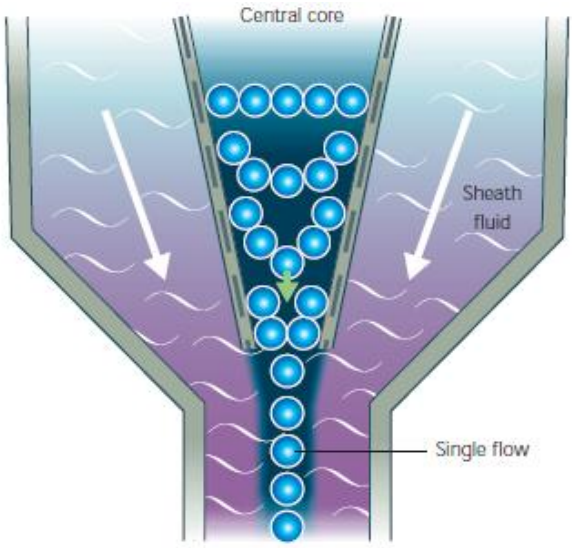

Figure 5 Animated description of FACS function

Samples are extracted with a probe and it flows through a channel that is enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves it develops a drag effect on the narrow central chamber. This alters the velocity of the central fluid and creates a single file of cells (Rahman, 2006).

2.4 Western blotting

2.4.1 Protein isolation

Protein isolation from the cells needed to be performed prior to Western blot analysis. It is crucial to keep the cells and every agent used in this procedure on ice to prevent protein deterioration. Each well was rinsed with 300 µl of ice cold PBS and then removed as much PBS as possible because high protein concentration depends on having low amount of PBS. 100 µl of cold RIPA lysis buffer was added, allowing it to cover the entire cell expansion surface and the cell expansion plate was then placed on ice for ten minutes. RIPA (Radio Immuno Precipitation Assay buffer) lysis buffer was made by adding 5 µl NaF (Sigma-Aldrich), 5 µl Sodium orthovanadate (Sigma-Aldrich), 5 µl PMSF (Sigma-Aldrich) and 1 µl protease inhibitor to 1ml RIPA base (0,86 H_2O, 2,4 mg Tris, pH tuned to 7,4 with HCL, 1% Triton (10 µl), 1% Sodium Deoxy cholate (10 mg), 8,76 mg NaCl and 1,3 mg EDTA). Cells were then scraped off with a pipette tip and transferred to an eppendorf vial on ice. Afterwards, vials were sonicated for three cycles at medium intensity for approximately two minutes and then placed on ice for ten minutes. Cells were centrifuged for 20 minutes at 4°C at 12000 g. This protein isolation protocol was obtained from the stem cell research unit (SCRU) in Læknagarður, University of Iceland.

Afterwards, the supernatant was transferred to a new eppendorf vial and stored at -80°C until all samples had been obtained and processed.

2.4.2 Western blot analysis on hES-MP and MSC

2.4.2.1 Making gel for electrophoresis

Complete gels for electrophoresis were made out of two different gels, the lower gel and the upper gel. First, 12,5% lower gel (12,5% symbolizes the dilution of Acrylamide in the solution) was added to the cassette and allowed to coagulate for 15 minutes with 200 µl of water on top to even out the surface and prevent interaction with oxygen. The water was then removed and upper gel added to the cassette. The description of how lower and upper gels were made can be seen in table 3. The gel was allowed to coagulate in the cassette and after coagulation, the cassette was removed and the prepared gel was put in plastic and stored at 4-8°C until the electrophoresis was implemented. These solutions are enough for two gels. However, four gels were made, so both solutions were made twice.

Table 4 Substance ingredients for Lower and Upper Gel

12,5% Lower gel was made by mixing H_2O, 40% Acrylamide (AppliChem GmbH, Darmstadt, Germany), Lower Tris Buffer (36.4g Tris base (Life Technologies™, Carlsbad, CA, USA) in 100 mL of dH_2O, pH to 8,8 and 0,8 g of SDS (AppliChem)), 10% APS (Ammonium persulfate) and Temed together. Temed causes polymerization. Upper gel was made by mixing H_2O, 40% Acrylamide, Upper Tris Buffer (6,05 g Tris base in 100 mL of dH_2O, pH to 6,8 and 0,4 g of SDS) 10% APS and Temed together.

		12.5% Lower gel	**Upper gel**
Ingredients	H_2O	4,15 mL	3,61 mL
	40% Acrylamide	3,15 mL	3,15 ml
	Lower Tris Buffer	2,6 mL	N/A
	Upper Tris Buffer	N/A	0,68 mL
	10% APS	100 µl	50 µl
	Temed	10 µl	5 µl

2.4.2.2 Electrophoretic separation of proteins and transferring them to a membrane

Before samples could be added to the gels, they had to be mixed with SDS loading buffer (AppliChem). Each sample was mixed with 1:1 ratio, so 40 µl of sample was mixed with 40 µl of SDS loading buffer. Samples and whole cell lysates were heated in 90°C for ten minutes and then centrifuged for couple of seconds. A549 Whole Cell Lysate: sc-2413 (Santa Cruz Biotechnology Inc., Dallas, TX, USA), derived from the A549 cell line and originating from a human lung. This cell lysate was used as a positive control for Brachyury while HeLa Whole Cell Lysate: sc-2200 (Santa Cruz), P27 and EV (provided by courtesy of Anne Richter) were used as a positive control for Nanog. HeLa cell lysate was derived from the HeLa cell line, originated from epithelial cells in human cervix while P27 and EV were human ESC transduced with two different control vectors (Empty vector and pLKO.1). 15 µl of each sample, 2 µl of Ladder #SM0671 (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 20 µl of lysates were added to each well as can be seen in Appendix A. Gels were then subjected to electrophoresis in constant 110 volt for 110 minutes in a running buffer. After 110 minutes, gels were released from the cassettes and trimmed before proteins were transferred to a nitrocellulose membrane. The setup for protein transfer as can be seen in figure 6, although two filter papers were used instead of just one. The cassette was put in a box with a cooling block. Transfer buffer was poured into the box until the cassette was fully submerged. The cassette was transferred for 60 minutes in constant 400 mA and the membrane dried overnight in the dark after transfer.

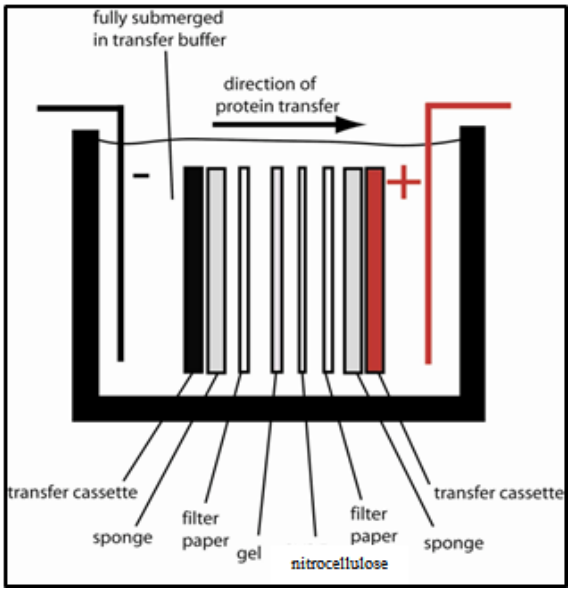

Figure 6 Setup of cassette for protein transferring

Proteins were transferred from gels to a nitrocellulose membrane after electrophoresis by submerging the cassette in transfer buffer and allowed to transfer for 60 minutes in constant 400 mA (Adapted from figure provided by Steinunn Guðmundsdóttir).

2.4.2.3 Primary and secondary antibodies

Gels for both Nanog and Brachyury were transferred to the same membrane so it was cut appropriately, making two membranes. One had the samples for the Brachyury antibodies and the second had the samples for the Nanog antibodies. Membranes were added in 1x TBS (Tris Buffer Saline) and then moved and submerged in a blocking buffer (2 mL 10xTBS, 18 mL dH_2O and 5% BSA (Sigma-Aldrich) (Nanog membrane)/5% skim milk powder (MS, Selfoss, Iceland) (Brachyury membrane)) for 30 minutes at room temperature on a mixer. After 30 minutes, the Nanog membrane was added to 5 mL of primary antibody buffer (5 mL 1xTBS, 0,1% Tween (Sigma-Aldrich) and 5%

BSA (Sigma-Aldrich)) that contained 2,5 µl of 1:2000 Nanog (D73G4) XP rabbit monoclonal antibody (Cell Signaling Technology Inc., Danvers, MA, USA). Brachyury membrane was added to 5 mL of primary antibody buffer (5 mL 1xTBS, 0,1% Tween and 5% skim milk powder (MS, Selfoss)) that contained 10 µl of 1:200 Brachyury (H-210): sc-20109 rabbit polyclonal antibody (Santa Cruz). In addition, 0,5 µl of 1:10.000 diluted anti-actin (EMD Millepore Corporation, Billerica, MA, USA) was added to each primary antibody buffers. Membranes were kept soaked in these primary antibody buffers overnight at 4°C on a mixer.

Membranes were washed four times in TBST (1x TBS and 0,1% Tween) for five minutes and then submerged in a second antibody buffer (10 mL TBST + 0,5 µl Rabbit 800 (LI-COR®) and 0,5 µl Mouse 700 (LI-COR®)). Rabbit 800 was used to detect Nanog and Brachyury and Mouse 700 was used to detect *β*-Actin. Membranes were left in secondary antibody buffer for 60 minutes, covered with aluminium foil for protection from light, and then washed again four times in a five minutes period in TBST. Then the membranes were ready for scanning.

2.4.2.4 Data imaging

Membranes were scanned with Odyssey® infrared imaging system and transferred into Image Studio Software (LI-COR® Biosciences, Lincoln, NE, USA) for imaging analysis.

2.5 Statistical analysis

GraphPad® Prism version 5.0 software and CellQuestPro software 4.0.2 were used to analyse results from FACS. One-way and two-way ANOVA were used and student's t-test used to confirm statistical significance. Image Studio Software and GraphPad® Prism version 5.0 software was used to analyse Western blot results and Gimp, an image-processing program, was used to put the final touches on images.

3 Results

3.1 Flow cytometry

hES-MP cells, expanded to passages 4, 6 and 10 were harvested and measured in FacsCalibur to analyse the difference between expansion in FBS and HPLO on hES-MP immunophenotype. The surface markers CD10, CD105, CD13, CD29, CD44, CD45, CD184, CD73 and HLA-DR were analysed.

There was a significant difference in expression of CD105, CD10 and CD13 between hES-MP cells grown in HPLO and FBS as can be seen in figure 7. Cells were positive for CD10 in HPLO and negative in FBS. They were positive for CD105 in FBS and P6 in HPLO but P4 and P6 were barely postive. Cells were positive for CD13 in both mediums but there was an accelerated expression in cells expanded in HPLO, whereas the expression in FBS was balanced.

Expression of CD29, CD45, CD184, CD73 and HLA-DR was not significant between media where CD29, CD44 and CD73 were postive in both media and CD45, CD184 and HLA-DR were negative in both media (Figure 8). The expression of surface markers were measured with geometric mean fluorescence intensity (gMFI). Geometric mean was used because the fluorescent scale was logscaled.

hES-MP cells in HPLO showed a statistically significant higher expression of CD10 than hES-MP cells in FBS in all passages ($p<0,001$). CD10 expression in P6 in HPLO was higher than in P4 but because it lowers again in P10 it is not thought to be important. hES-MP cells in FBS had a significantly higher expression of CD105 than hES-MP cells in HPLO, in contrast to the cellular expression of CD10. They showed a statistically significant increase in expression of CD105 in P4 and P10 (P4 $p<0,01$, P10 $p<0,001$) as compared to hES-MP cells in HPLO. However, there was not a significant difference in P6 ($p>0,05$). hES-MP cells in HPLO showed an escalated expression of CD13 that increased in a step-wise manner over time but hES-MP cells in FBS showed a steady expression of CD13 in all passages. No significant difference of expression was between hES-MP cells in HPLO and FBS in P4 ($p>0,05$) but P6 and P10 showed a significant difference in expression ($p<0,001$).

Expression of CD184 in FBS was higher than in HPLO in all passages with a significant expression in P4 and P6 ($p<0,001$) but no significance in P10 ($p>0,05$). There was a significant expression of CD29 in FBS in contrast to HPLO in P4 ($p<0,001$). There was a significantly higher expression of CD44 in FBS in P4 and P10 (P4 $p<0,001$, P10 $p<0,01$). Expression of CD45 in FBS was significantly higher ($p<0,001$). CD73 was positive in both mediums but no significant difference was between HPLO and FBS. Expression of HLA-DR in FBS was higher in all passages. However, there was only a statistical significance in P4 and P10 ($p<0,001$).

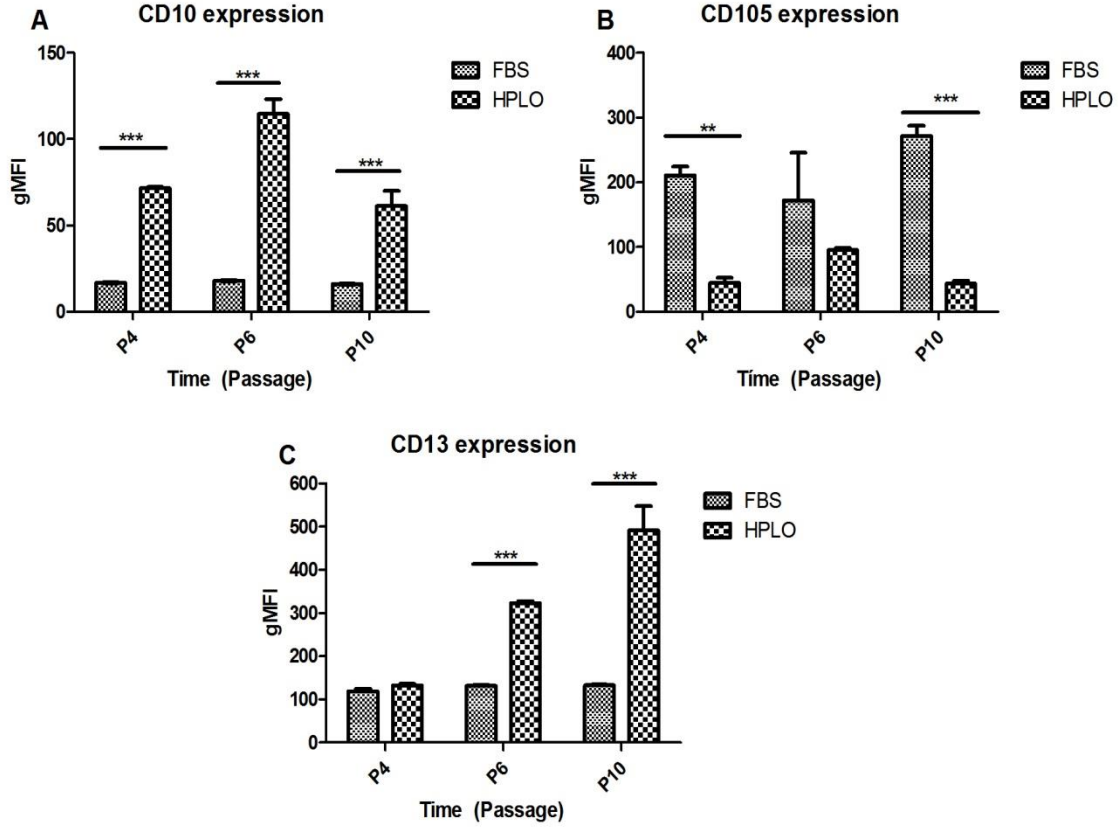

Figure 7 FACS analysis on CD10, CD105 and CD13

*Expression of CD10, CD105 and CD13 is presented with SEM (standard error of the mean) as error bars. A) hES-MP cells were positive for CD10 in HPLO but negative in FBS. Expression of CD10 in HPLO was statistical significant higher in all passages compared to expression of CD10 in FBS ($p<0,001$). A significant expression between P6 and P10 in FBS was present ($p<0,05$) as well as significant expression between P4 and P6 ($p<0,05$) and between P6 and P10 ($p<0,01$) in HPLO. B) hES-MP cells were positive for CD105 in FBS and P6 in HPLO. Cells in P4 and P10 in HPLO barely expressed CD105. CD105 was higher expressed in FBS than in HPLO in all passages but a significant expression was only detected in P4 and P10 (P4 $p<0,01$, P10 $p<0,001$). A significant expression between P4 and P10 in FBS was present ($p<0,05$) as well as a significant expression between P4 and P6 ($p<0,01$) and between P6 and P10 ($p<0,01$) in HPLO. C) hES-MP was positive for CD13 in both media. Cells in HPLO showed an escalated expression of CD13 overtime whereas expression of CD13 in FBS was balanced between passages. A significant expression was seen in P6 and P10 ($p<0,001$) but no significant difference between cells in HPLO and FBS in P4 ($p>0,05$).A significant expression of CD13 was present between all passages in HPLO ($p<0,05$ between P4 vs. P6 and P6 vs. P10, $p<0,001$ between P4 vs. P10). ** = $p<0,01$, *** = $p<0,001$. gMFI = geometric mean fluorescence intensity.*

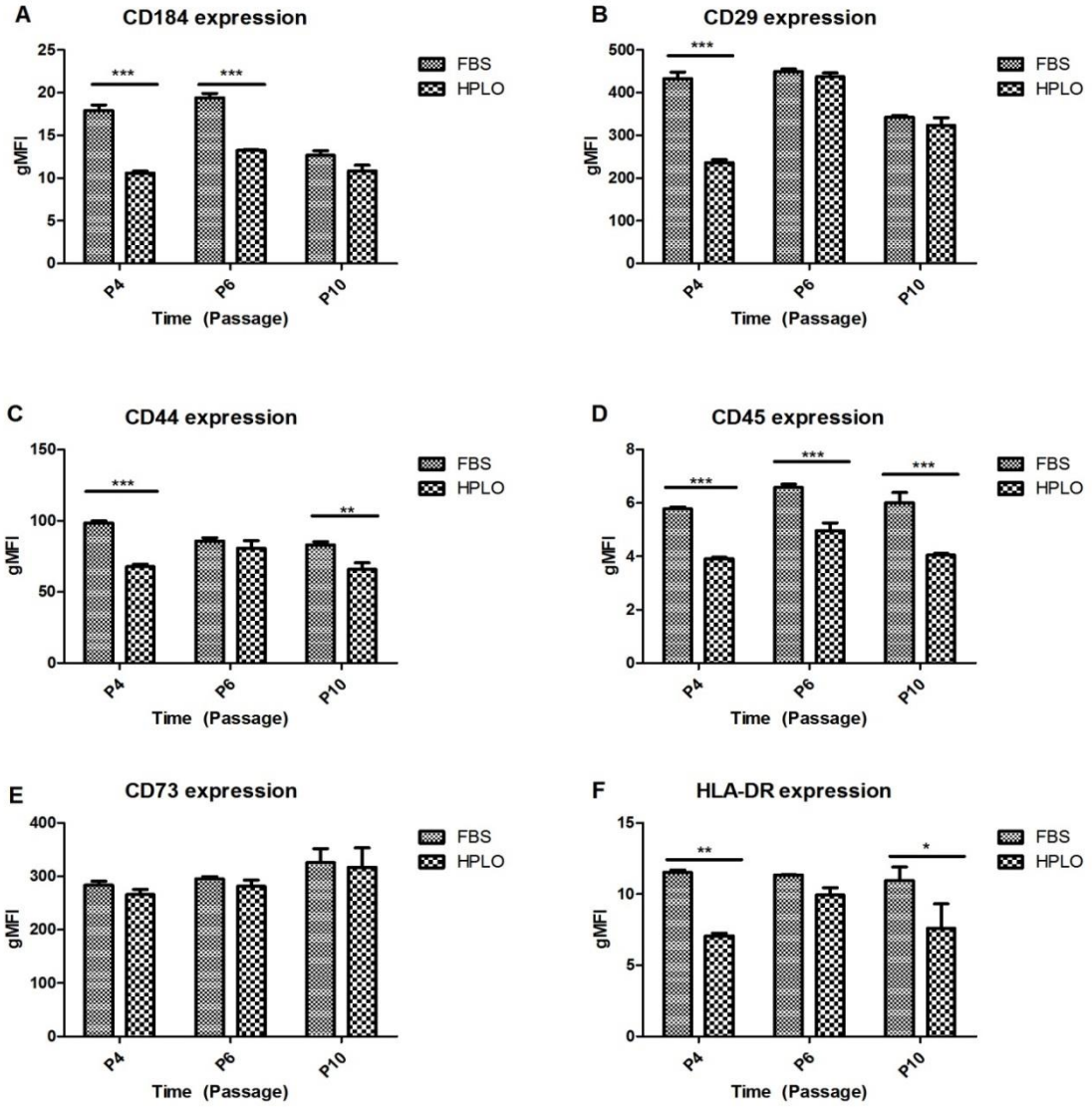

Figure 8 FACS analysis on CD184, CD29, CD44, CD45, CD73 and HLA-DR

*Expression of CD184, CD29, CD44, CD45, CD73 and HLA-DR is presented with SEM (standard error of the mean) as error bars. Cells were positive for CD29, CD44 and CD73 and negative for CD184, CD45 and HLA-DR regardless of what media was used. A) CD184 came out negative in both HPLO and FBS. Expression of CD184 was statistically significant higher in FBS than in HPLO with a significance in P4 and P6 (<0,001). A significant expression was between P4 and P10 (p<0,01) and P6 and P10 (p<0,001) in FBS. There was also a significant expression between passages in HPLO (p<0,05 between P4 and P6 firstly, and P6 and P10 secondly). B) CD29 came out positive in both mediums. Higher expression of CD29 was present in P4 (p<0,001). There was a significant expression between all passages in HPLO (P4 vs. P6 p<0,001, P4 vs. P10 and P6 vs. P10 p<0,01) and between P4 and P10 (p<0,01) and P6 and P10 (p<0,001) in FBS. C) CD44 was positive in both media where there was a higher expression of CD44 in FBS with a statistical significance in P4 (p<0,001) and P10 (p<0,01). P4 had a higher expression in FBS in contrast to P6 (p<0,05) and P10 (p<0,01). D) CD45 was negative in both media where FBS was expressed significantly higher than HPLO in all passages (p<0,001). P6 had a higher expression of CD45 in HPLO in both P4 and P10 (p<0,05). E) CD73 was positive in both media with no significant difference in expression (p>0,05) between media. F) HLA-DR was negative in both media. FBS was higher expressed in all passages with a statistical significance in P4 (p<0,01) and P10 (p<0,05). ** = p<0,01, *** = p<0,001. gMFI = geometric mean fluorescence intensity.*

3.2 Western blot

The embryonic marker Nanog and mesodermal marker Brachyury were used to analyse whether hES-MP cells are more like MSC or ESC. hES-MP cells (P5) were analysed and MSC (P4) from two different donors, expanded in HPLO and FBS, and cell lysates from human ESC and human tissues were used as controls. *β*-Actin was measured as well to determine fluorescence intensity. The results from the western blot analysis on hES-MP cells are shown in figures 9-11.

Figure 9 shows the expression of Nanog. The figure consists of two different photos of the same gel, where the upper shows *β*-Actin bands and the lower shows Nanog bands. This had to be done due to the size resemblance between *β*-actin (43 kDa) and Nanog (42 kDa). Not all samples fitted to just one gel so after the membrane was scanned, they were put together with the image-processing program Gimp. The ladder on the second gel was also removed so the membrane was put together between MSC (D3) FBS #2 and MSC (D1) HPLO #1. No hES-MP samples expressed Nanog and P27 was the only control that came back positive.

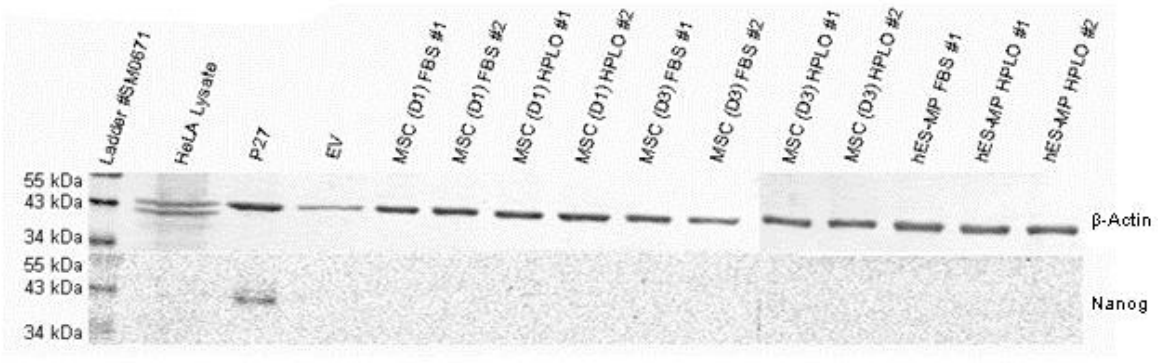

Figure 9 hES-MP cells expression of Nanog

hES-MP samples were negative for Nanog and the only control that was positive was P27. All controls and samples expressed β-Actin. HeLA Lysate expressed two bands of β-Actin and one of them was expressed in the right size.

Figure 10 displays the expression of Brachyury. Like with Nanog, not all samples could fit on one gel so the membrane was scanned and it fixed by removing the two ladders on the second gel and put together between MSC (D3) HPLO #2 and hES-MP FBS #1. All hES-MP samples expressed Brachyury at approximately 49 kDa. *β*-Actin was also positive in all samples. A549 lysate and MSC samples were positive as well. A549 lysate expressed four bands with one of them having the right molecular weight for Brachyury expression.

When the fluorescence intensity was examined in the expression of Brachyury (figure 11), there was a distinct difference in Brachyury expression between MSC and hES-MP cells. Nevertheless, the difference was so little that there was not a statistical significance between these groups of cells in HPLO or FBS.

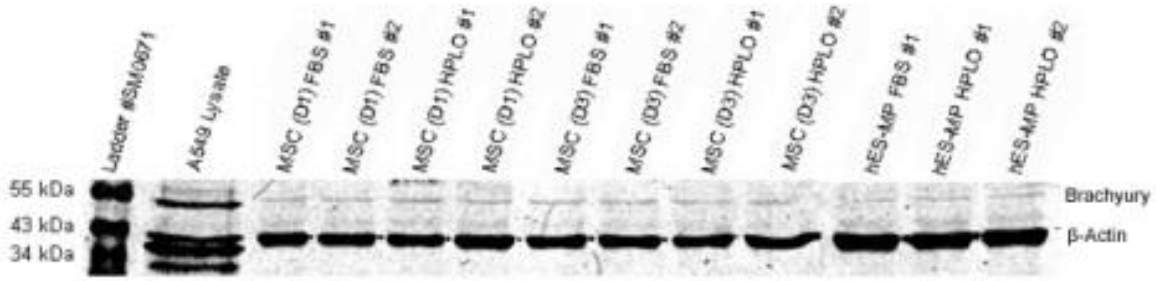

Figure 10 hES-MP cells expression of Brachyury

All hES-MP samples were positive for Brachyury along with MSC samples and the A549 lysate. All samples and controls expressed β-Actin. A549 lysate expressed four different bands, one having the right molecular weight to match Brachyury.

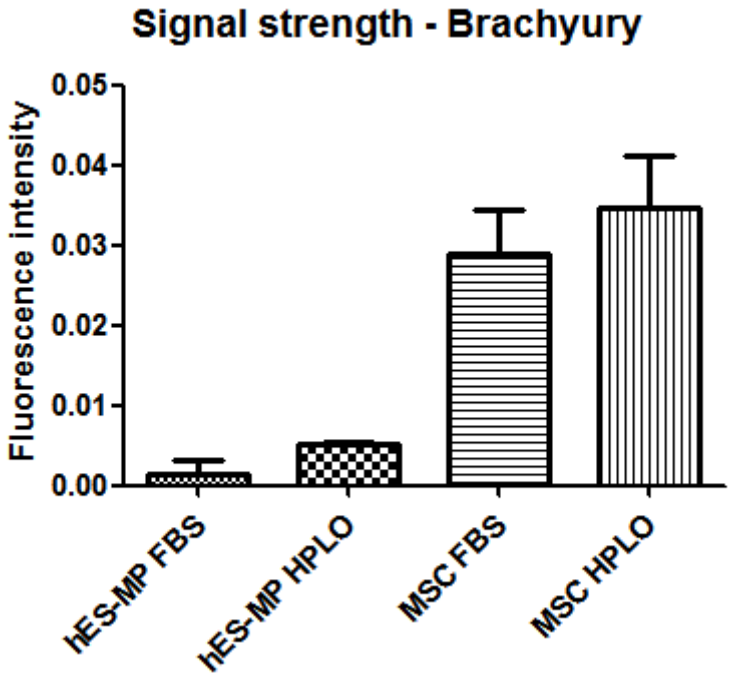

Figure 11 Expression of Brachyury in hES-MP cells, expanded in HPLO and FBS

Expression of Brachyury in hES-MP cells is presented with SEM as error bars. There was a definite difference between hES-MP cells and MSC but without any statistical significant expression, except for between hES-MP–FBS and MSC–HPLO ($p<0,05$). There was not a statistical significance between the expression of hES-MP cells and MSC in HPLO or FBS ($p>0,05$).

4 Discussion

The immunophenotype of hES-MP cells, that had been expanded in 10% HPLO expansion media or 10% FBS expansion media were examined and compared with two kinds of assays. First, the expression of CD10, CD13, CD29, CD44, CD45, CD73, CD105, CD184 and HLA-DR surface markers were analysed in hES-MP cells from both media in passages 4, 6 and 10 with a fluorescence-activated cell sorting (FACS) assay. Then the expression of the embryonic marker Nanog and the mesodermal marker Brachyury were analysed in hES-MP cells (P5) and MSC (P4) from both media with western blot analysis.

The expression of surface markers CD10, CD13 and CD105 were significantly different between media where CD29, CD44, CD45, CD73, CD184 and HLA-DR did not differ between media. The hES-MP cells also expressed Brachyury but did not express Nanog.

4.1 Immunophenotyping

The surface markers CD29, CD44 and CD73 were positive in both mediums and the surface markers CD45, CD184 and HLA-DR were negative in both mediums. However, the expression of surface markers CD10, CD13 and CD105 was significantly different between mediums. hES-MP cells were positive for the presence of CD105 and CD13 in both mediums but CD10 was expressed negative in FBS in contrast to cells expanded in HPLO, where CD10 was expressed positive. These results indicate that hES-MP cells have the same immunophenotype as MSC when expanded in FBS. However, CD10 expression was positive in HPLO, which does not fit with the immunophenotype of MSC but CD10 is not present in mesenchymal stem cells.

hES-MP cells were positive for CD13 in both mediums, which fits with the CD13 expression in MSC. The expression of CD13 was however different in HPLO, in contrast to FBS. CD13 expression rose in correlation with the age of the hES-MP cells (Figure 7C). CD13 (Aminopeptidase N, (APN)) is a zinc-dependent metallopeptidase and a membrane-bound glycoprotein (Gabrilovac et al., 2005). It has been shown that human APN cDNA sequence is identical to the coding of the myeloid marker CD13 so it has also been referred to as CD13/APN. It is a marker for normal and malignant myeloid lineages but the physiological function in myeloid and lymphoid cells is still a kind of a mystery (Gabrilovac et al., 2005). Bhagwat et al. demonstrated that an up-regulation of endogenous CD13/APN can be caused by numerous phenomenona, for example angiogenic growth factors and in response to hypoxia (Bhagwat et al., 2001). TGF-β is an angiogenic growth factor but HPLO contains high amount of TGF-β. Another study, done by Kim et al., showed that an increased CD13 expression in liver cancer cells can be associated with TGF-β-induced phenomenon, similar to EMT (epithelial mesenchymal transition) (Kim et al., 2012). Yet another study demonstrated that TGF-β_1 triggers up-regulation of CD13 on a human myelo-moncoytic cell line HL-60 at the membrane protein level and at the mRNA level (Gabrilovac et al., 2005). These studies indicate that TGF-β may be the reason why expression of CD13 is higher in HPLO than in FBS. However, these are only speculations and this

does not explain the escalated expression between passages. There is a need for more decisive tests to determine the cause of CD13 up-regulation in these cells.

hES-MP cells were tested positive for the presence of CD105 in both mediums. However, the expression CD105 is barely positive in cells from P4 and P10 that had been expanded in HPLO (Figure 7B). This positive expression correlates with the MSC immunophenotype, but MSC are positive for the presence of CD105. CD105 (Endoglin) is a type I membrane and is present on cellular lineages within the connective tissue and vascular system. It is expressed as a disulphide-linked homodimer at the cell surface (Fonsatti et al., 2001; Pérez-Gómez et al., 2010). It works as auxiliary receptor alongside betaglycan for the TGF-β receptor complex and binds TGF-β_1, TGF-β_3 and other proteins due to TβRI and TβRII signalling (Pérez-Gómez et al., 2010). Thus, Endoglin is a member of the ligand-TβRI/TβRII complex and plays a possible role in down-regulation of TGF-β signalling and seems to modulate binding of the TGF-β receptor (Pérez-Gómez et al., 2010; Redondo et al., 2012). Several studies have reported about the regulation of Endoglin. Jin et al. demonstrated that expression of CD105 has negative correlation over the time course of multi-lineage differentiation in human umbilical cord blood-derived mesenchymal stem cells. Whereas Li et al. showed that TNFα (Tumour necrosis factor-α) down-regulates CD105 in human dermal microvascular endothelial cells but TGF-β_1 up-regulates CD105 expression. (C. Li et al., 2003). Hypoxia can also cause up-regulation in CD105 expression (Dallas et al., 2008). However, there is not a great deal of TNFα in HPLO so it is not known whether TNFα is in fact down-regulating CD105 here. It is essential to perform tests that could provide more decisive results to identify what is causing this down-regulation.

hES-MP cells expanded in FBS were negative for CD10 which is consistent with the immunophenotype in MSC. However, hES-MP cells expanded in HPLO were positive for CD10. These results indicate that there is a substance present in the HPLO supplementary media and not in the FBS supplementary media, which causes this major up-regulation of CD10. CD10 (MME (Membrane metallo-endopeptidase)) is a part of the membrane-bound zinc-dependent endopeptidase family (Maguer-Satta et al., 2011). Function of CD10 can be divided into two parts. Firstly, CD10 is present on the cell surface and cleaves peptides by their extracellular enzymatic activity. Residues formed during this segmentation then contribute to stem cell regulation by being activated or inhibited. Secondly, CD10 is a part of intracellular signaling pathway and mediates signals between cells. CD10 is therefore an important marker that is able to mediate signals from both cell microenvironment and between different cells (Maguer-Satta et al., 2011). Due to CD10's ability to cleave substrate, there is a possibility that some kind of a substrate in HPLO up-regulates CD10. Substrates such as bradykinin, endothelins and atriopeptin are few of the substrates that CD10 controls *in vivo* and CD10 is known to act on neuropeptides such as tachykinin peptides (Substance P (SP)) (Maguer-Satta et al., 2011; Xie et al., 2011). Recent studies have shown that ligands and receptors of the tachykinin peptides are present in platelets (Gibbins, 2009). These agents are thought to contribute to positive feedback regulation, where they are released from the platelet in an inactivated state and activated outside of the platelet which causes them to bind to their receptors on the platelet surface and mediate their function (Gibbins, 2009). Substance P promotes the production of cytokines such as interleukin-1 (IL-1), IL-2, TNF-α and stem cell factor (SCF) and has been thought to be an important pathogenic factor

in inflammatory skin diseases such as psoriasis and atopic dermatitis (Xie et al., 2011). SP function in haematopoiesis is well known due to its supporting role in haematopoiesis but SP has a promoting effect on haematopoietic stem cells (Nowicki et al., 2007). As stated earlier, the relations between CD10 and their controlling aspects of stem cells has been established so CD10 function in the haematopoietic microenvironment may concern cleaving Substance P and thus maintain haematopoietic stem cell dormancy. If SP exists in HPLO, it may be a natural response in hES-MP cells to increase CD10 expression, which down-regulates SP in their environment. This process is known in CD10 positive human fibroblasts (Xie et al., 2011). However, these are only speculations about the up-regulation of CD10 in hES-MP cells expanded in HPLO. It is necessary to explore SP and other tachykinins in HPL and what effects they have on CD10 in hES-MP cells.

Similar to the mesenchymal stem cell immunophenotype, the hES-MP cells were positive for the presence of CD29, CD44 and CD73, CD13 and CD105 but CD45, CD184 and HLA-DR were negative. These results support the hypothesis that hES-MP cells are differentiating towards the mesenchymal lineage. However, it is difficult to clarify the difference of CD13, CD105 and especially CD10 expression between the HPLO and FBS expansions in this study so there is a need for more specialized assays to determine this alteration.

4.2 Western blot

hES-MP cells express the mesodermal marker Brachyury but not the embryonic marker Nanog (Figures 9 and 10). There is a slight difference in the fluorescence intensity between hES-MP cells and MSC but the expression was not significantly different. In addition, there was hardly any difference in fluorescence intensity between cells expanded with HPLO and cells expanded with FBS (Figure 11). Thus, the results indicate that hES-MP cells are differentiating towards a mesenchymal lineage.

hES-MP cells were positive for Brachyury in HPLO and FBS. Brachyury is a T-box transcription factor and is an important factor in early differentiation and determination of mesoderm in vertebrates (Technau, 2001). In fact, Brachyury is one of the main genes that regulate notochord formation, and connects embryonic structure with the development of notochord (Vujovic et al., 2006).

As stated before in chapter 1.2.3, Nanog is a transcription factor and is a marker for all pluripotent cell lines. Nanog is an important factor in maintaining undifferentiation (Cavaleri & Schöler, 2004). The negative Nanog expression in the hES-MP cells was anticipated due to the analysis done by Karlsson et al. who examined typical embryonic markers, for example Oct-4 and Nanog, in hES-MP cells. hES-MP cells were negative for those markers (Karlsson et al., 2009).

These results indicate that hES-MP cells are no longer equipped with the undifferentiation potential, which is the main characteristic for hESC. Thus, making them differentiated stem cells, like mesenchymal stem cells.

4.2.1 Experimental limitations

The western blot assay is a very delicate procedure and it has to be performed with precision for it to produce accurate results. Even though every protocol step was followed precisely, there were complications during this assay, as can be seen in Appendix B.

First, antibodies from Cell Signaling Technology Inc. were used (Figure 12). A Brachyury membrane was soaked in Brachyury #12312 antibody and a Nanog membrane in Nanog (D73G4) XP Rabbit mAb antibody. β-Actin was expressed in all wells but no bands for Brachyury or Nanog were visible. No cell lysates were used as controls in this assay but MSC should express Brachyury so when no bands were visible in those wells, it was obvious that the Brachyury antibody was not working. Due to these results, other antibodies from Santa Cruz Biotechnology Inc. were used along with cell lysate controls from the same company. Figure 13 displays the results where a Brachyury membrane was soaked in Brachyury (N-19): sc-17743 antibody (1:200) and a Nanog membrane in Nanog (H-155): sc-33759 (1:200). The antibody was non-selective to Brachyury, which caused a formation of numerous bands. One band in all wells was strongly expressed but it had the molecular weight of 34 kDa approximately, which is not the correct molecular weight for Brachyury (49 kDa according to the work sheet). However, the cell lysate A549 expressed bands in two places, one approx. 34 kDa, similar to the bands expressed in samples and then one band with a correct molecular weight for Brachyury. This implies that the antibody works but the cells do not seem to respond sufficiently to it. The Nanog membrane was intriguing. Bands were visible in wells that contained cells that had been expanded with HPLO but not with FBS. However, those bands did not have the correct molecular weight (40 kDa according to the worksheet). The molecular weight was >55 kDa and no band was visible in HeLA cell lysate, so it was concluded that these bands were not Nanog. That same membrane was added into the antibody from Cell Signaling (1:2000) to test whether any bands would form in the cell lysate, but no bands were visible (Figure 14).

The Cell Signaling antibodies (1:2000) were tried once more (Figure 15). This time the cell lysates P27 and EV were added to the Nanog gel because it was known that they expressed Nanog. Similar to previous assays, there was no expression of Brachyury, not even in the A549 cell lysate. When the Nanog membrane was examined, it was clear that one of the cell lysates were positive for Nanog. P27 expressed a band in with a correct molecular weight, which was 42 kDa according to the worksheet. No sample or other cell lysates were positive for Nanog. This positive expression in P27 confirmed that the antibody worked so the conclusion was that Nanog was not expressed in hES-MP cells. This was used as conclusive result for this thesis. However, no expression of Brachyury was visible, which was peculiar, especially because MSC should express Brachyury. The same membranes from figure 15 were added to the antibodies from Santa Cruz with the dilution 1:400 instead of 1:200 (Figure 16). Here 5% skim milk powder was used in the secondary antibody instead of 5% BSA. Once again, there was no positive expression of Brachyury in the cells or in the A549 lysate. The Nanog membrane did express something positive in cells expanded with HPLO, just like in figure 13. However, shadow bands were visible in the wells containing cells expanded with FBS and EV expressed something positive as well. P27 still expressed the Nanog band but also a band that had a much higher molecular weight than the molecular weight of Nanog, similar to the other bands (>55 kDa). Finally, a new

Brachyury membrane was added in another antibody from Santa Cruz Biotechnology Inc. (Brachyury (H-210): sc-20109) (Figure 17). Similar to the assay described by figure 16, the secondary antibody contained 5% skim milk powder instead of 5% BSA. When the membrane was examined, the A549 cell lysate expressed a Brachyury band and one sample (MSC (D1) HPLO#1), expressed something that had a higher molecular weight (<55 kDa) than the molecular weight of Brachyury. Some shadows were also visible so the membrane was dried and then scanned again. At last, an expression of Brachyury was visible in the MSC samples as well as hES-MP cells and the Brachyury expression of the A549 cell lysate confirmed this expression. The Nanog membrane in figure 15 and the dried Brachyury membrane in figure 17 were used as results in this thesis.

Clearly, it is pivotal to use the right antibody when working with western blot. In addition, it is important to handle the antibodies and whole cell lysates accordingly. As it shows in the results and Appendix B, the whole cell lysates from Santa Cruz Biotechnology Inc. did either not work or expressed more than one band. There is a possibility that they were damaged due to improper handling when they arrived to the Bloodbank. They were not stored in -20°C after as instructed. Instead, they were stored in 4°C for some time before being moved to proper storage. This could play a big part in why they did not work as well they are supposed to, but it is not truly known and is still an enigma.

After reviewing these unsuccessful assays, it is clear that membranes need to be scanned both wet and dry but signal strength could be greater on a dry membrane as was shown in figure 17. All membranes were scanned dry after the western blot analysis was completed. There were no modifications between the dry and wet membranes, except for the Brachyury membrane from figure 13. Weak Brachyury bands were visible in all MSC and hES-MP samples (data not shown).

There is not a definite explanation as to why the Cell Signaling antibody on Brachyury did not work on the MSC and hES-MP cells even though protocols were followed accordingly. However, both Santa Cruz antibodies worked when the membranes were scanned dry. There is a possibility that the Brachyury signal strength in the hES-MP cells is typically low and the Cell Signaling antibody had to be more concentrated to detect it but that does not explain why no expression was captured in MSC. Even though both Santa Cruz antibodies worked, the non-selective element raises speculations about the accuracy and specificity of these antibodies. As described before in figure 13, expression of Brachyury was detected in all samples, but bands with high expression of Brachyury had the molecular weight <34 kDa instead of 49 kDa, which is the correct molecular weight for Brachyury. The A549 cell lysate expressed that same signal. This expression is rather atypical and results that are more conclusive are needed to comprehend what this expression stands for.

It was necessary to perform the Nanog assay this often until at least one control expressed Nanog. The most interesting result regarding these western blot assays on Nanog was the positive expression that was only present in cells that had been expanded in HPLO and had a molecular weight of >55 kDa, which indicates that they are not Nanog (Figures 13 and 16). Some shadows are however visible in the cells that had been expanded in FBS in figure 16. The element that causes this expression is unknown but both P27 and EV express similar expression in figure 16. Therefore, this expression

cannot be linked explicitly to the HPLO supplementary media but results that are more conclusive are needed to figure out what is causing this expression.

4.3 Future directions

According to these results, using HPLO as a supplementary media instead of FBS is an option. Nevertheless, the immunophenotype difference between HPLO and FBS regarding the expression of CD10, CD13 and CD105, raises questions about the platelet lysate contents.

Further research is needed on the connection between CD13 and TGF-β complex and possibly expanding hES-MP cells over a longer period, and inspect the CD13 expression in additional passages and if the expression keeps rising. Substance P content in HPLO needs to be examined and the possibilities of CD10 down-regulating SP in stem cells. This can be done by using a knockout procedure, inhibiting either SP or CD10 *in vitro* and analysing the amount of these two substances. This same procedure may be used when looking at the connection between CD105 and TNFα.

It cannot be confirmed whether HPLO can be used as a supplementary media instead of FBS until every aspect of these differences has been researched and analysed more thoroughly.

5 Conclusion

This thesis shows that there is a possibility for using human platelets derived from expired platelet rich plasma (HPLO) as a supplementary media for the expansion of hES-MP cells instead of using fetal bovine serum (FBS) for hES-MP expansion, and hES-MP cells do express a similar immunophenotype as mesenchymal stem cells. Thus, they are derived to a mesodermal lineage. However, even though HPLO is a considerable better choice for hES-MP cell expansion than FBS due to both ethical issues and the xeno-free environment, it is unknown what causes the immunophenotype difference in HPLO supplemented media. Further studies have to be executed to determine what is affecting the expression of these surface markers before HPLO can be implemented as a potential alternative for FBS in *in vitro* cell expansion.

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Appendix A

Tables 4 and 5 demonstrate how samples and lysates were added to the gels for western blot analysis.

Table 5 Setup of samples and lysates in gels for Brachyury

Brachyury was loaded for electrophoresis in two separate gels. 2 µl of Ladder was added to define the size of bands, 20 µl of A549 lysate was added as a positive control and 15 µl of samples were then added to each well.

Brachyury (H-210): sc-20109			
Well	**Gel #1**	**Well**	**Gel #2**
1	Ladder	**1**	Ladder
2	A549 Lysate	**2**	Ladder
3	MSC (D1) FBS #1	**3**	hES-MP FBS #1
4	MSC (D1) FBS #2	**4**	hES-MP HPLO #1
5	MSC (D1) HPLO #1	**5**	hES-MP HPLO #2
6	MSC (D1) HPLO #2		
7	MSC (D3) FBS #1		
8	MSC (D3) FBS #2		
9	MSC (D3) HPLO #1		
10	MSC (D3) HPLO #2		

Table 6 Setup of samples and lysates in gels for Nanog

Nanog was loaded for electrophoresis in two separate gels. 2 µl of Ladder was added to define the size of bands, 20 µl of HeLA lysate and 30 µl of P27 and EV were added as positive controls and 15 µl of samples were then added to each well.

Nanog (D73G4) XP			
Well	**Gel #1**	**Well**	**Gel #2**
1	Ladder	**1**	Ladder
2	HeLA Lysate	**2**	MSC (D3) HPLO #1
3	P27	**3**	MSC (D3) HPLO #2
4	EV	**4**	hES-MP FBS #1
5	MSC (D1) FBS #1	**5**	hES-MP HPLO #1
6	MSC (D1) FBS #2	**6**	hES-MP HPLO #2
7	MSC (D1) HPLO #1		
8	MSC (D1) HPLO #2		
9	MSC (D3) FBS #1		
10	MSC (D3) FBS #2		

Appendix B

The following figures demonstrate unsuccessful attempts of western blotting. Table 6 shows a list of sample and cell lysates to clarify their positions in the following figures.

Table 7 List of samples and lysates

List of all specimen used in Western blot to clarify their positions in figures 12-17.

Contents in wells			
1	Ladder #SM0671	**7**	MSC (D1) HPLO #2
2a	A549 lysate	**8**	MSC (D3) FBS #1
2b	HeLa lysate	**9**	MSC (D3) FBS #2
3a	P27	**10**	MSC (D3) HPLO #1
3b	EV	**11**	MSC (D3) HPLO #2
4	MSC (D1) FBS #1	**12**	hES-MP FBS #1
5	MSC (D1) FBS #2	**13**	hES-MP HPLO #1
6	MSC (D1) HPLO #1	**14**	hES-MP HPLO #2

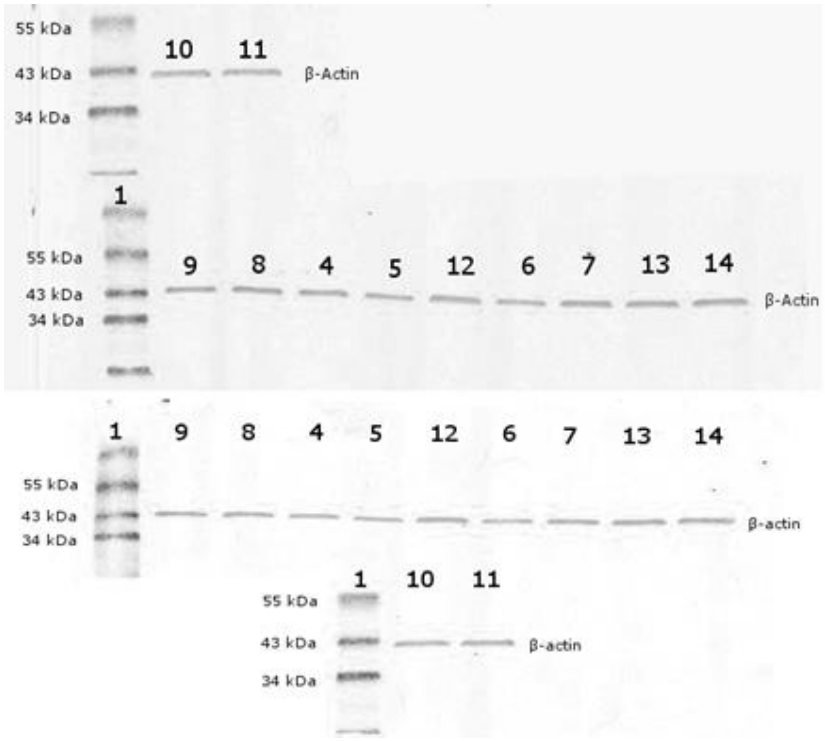

Figure 12 Brachyury and Nanog membranes in antibodies from Cell Signaling (1:2000)

Nanog membrane (upper figure) was added to Nanog (D73G4) XP Rabbit mAb antibody and the Brachyury membrane (lower figure) was added to Brachyury #12312 antibody from Cell Signaling Technology Inc. β-Actin was expressed in all wells but no Nanog or Brachyury bands were visible.

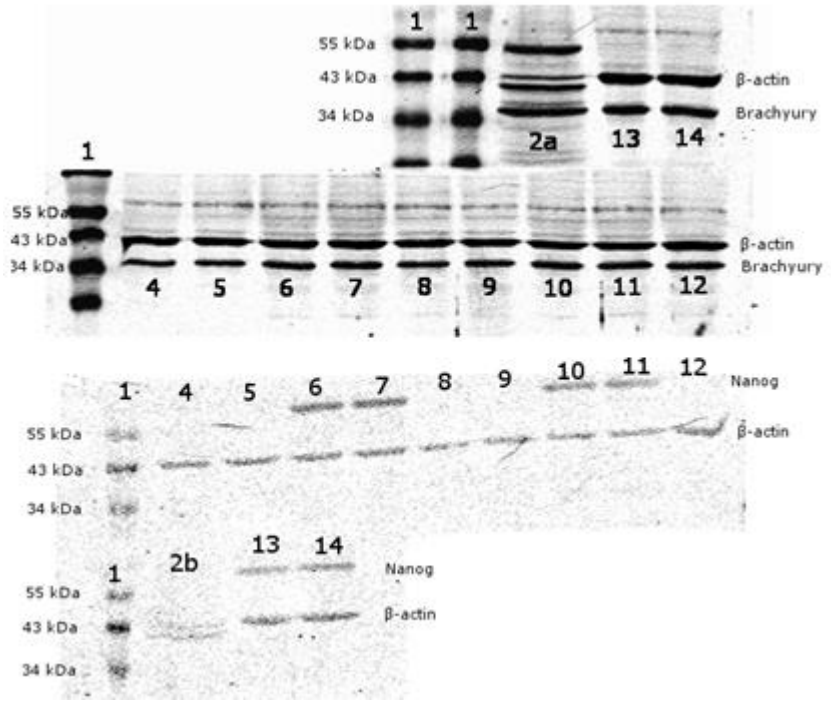

Figure 13 Brachyury and Nanog membranes in antibodies from Santa Cruz (1:200)

Brachyury membrane (upper figure) was soaked in Brachyury (N-19): sc-17743. The antibody was non-selective to Brachyury so numerous bands are visible. The Nanog membrane (lower figure) was soaked in Nanog (H-155): sc-33759. Nanog bands were visible in all samples in HPLO supplemented media but their molecular weight was >55 kDa instead of 40 kDa like Santa Cruz states. The secondary antibodies for both membranes contained 5% BSA.

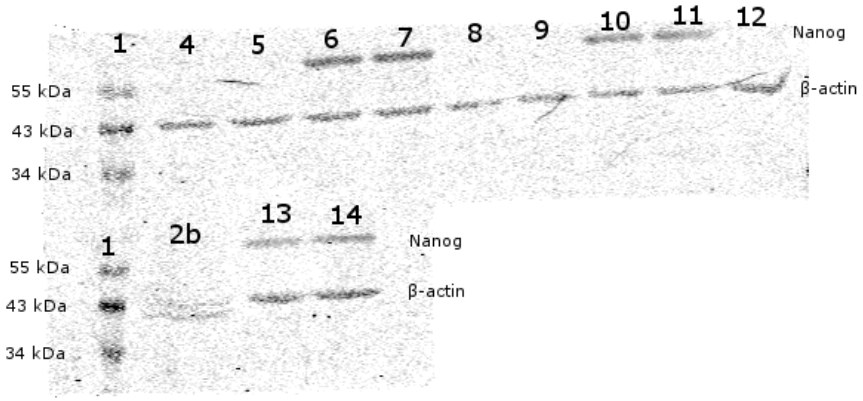

Figure 14 Nanog membrane in antibody from Cell Signaling (1:2000)

The same Nanog membrane from figure 13 was soaked in Nanog (D73G4) XP Rabbit mAb. No distinctive variation was seen between these figures. The secondary antibody contained 5% BSA.

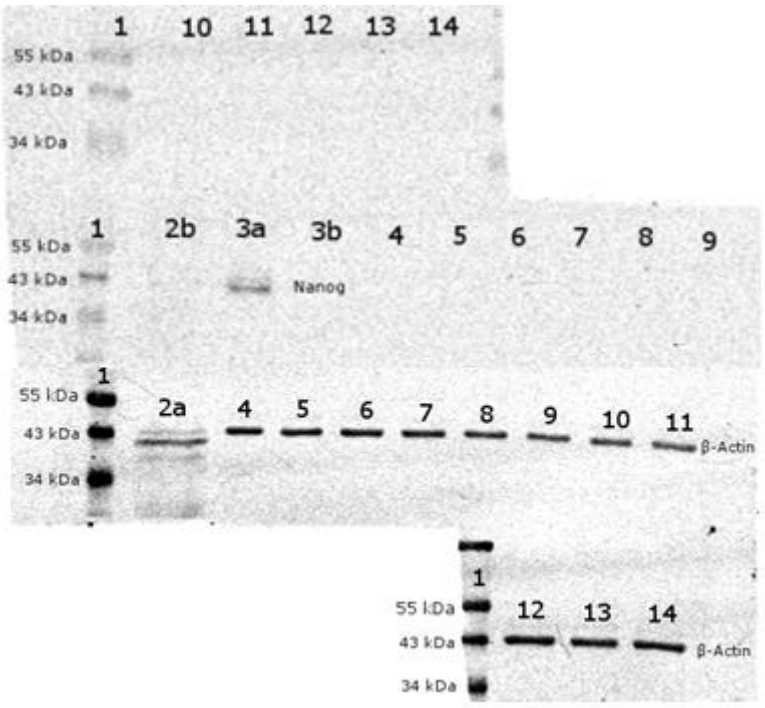

Figure 15 Brachyury and Nanog membranes in antibodies from Cell Signaling (1:2000)

The Nanog membrane (upper figure) was soaked in Nanog (D73G4) XP Rabbit mAb. Here, a Nanog band appears in the P27 well with the right molecular weight. β-Actin was removed from the figure to visualize the Nanog band better. The Brachyury membrane (lower figure) was soaked in Brachyury #12312. β-Actin was well expressed but Brachyury was not expressed. The secondary antibodies contained 5% BSA.

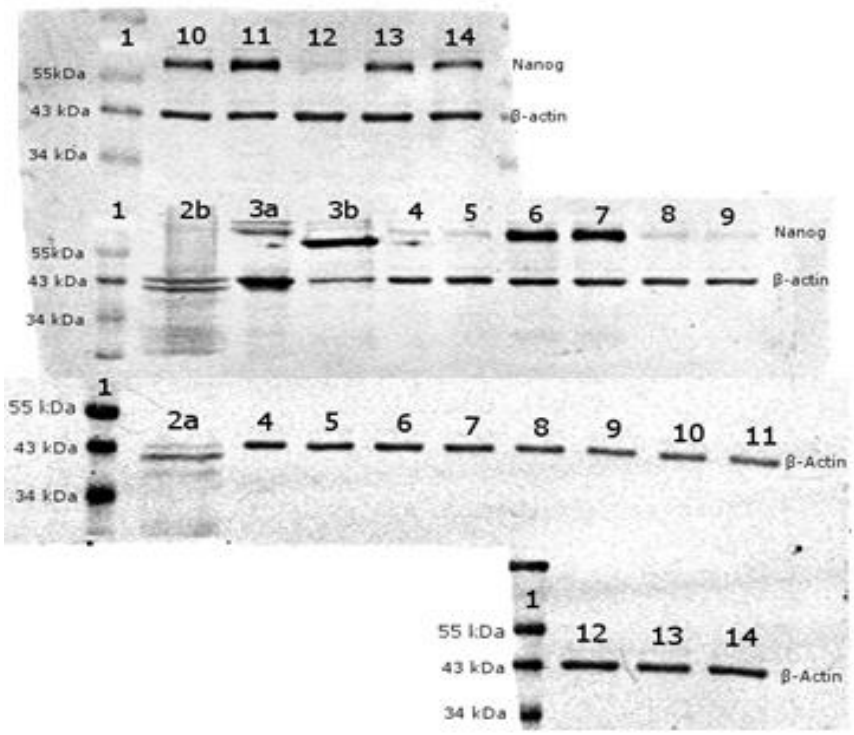

Figure 16 Brachyury and Nanog membranes in antibodies from Santa Cruz (1:400)

The same membranes from figure 15 were soaked in antibodies from Santa Cruz, the same antibodies as the ones described in figure 12 but with the dilution 1:400 instead of 1:200. The Nanog membrane (upper figure) expressed Nanog like in figure 12, in all samples with HPLO supplemented media with the wrong molecular weight, but EV and P27 also expressed Nanog. P27 was the only lysate that expressed Nanog with the right molecular weight. The Brachyury membrane (lower figure) did not alter. The secondary antibodies contained 5% skim milk powder.

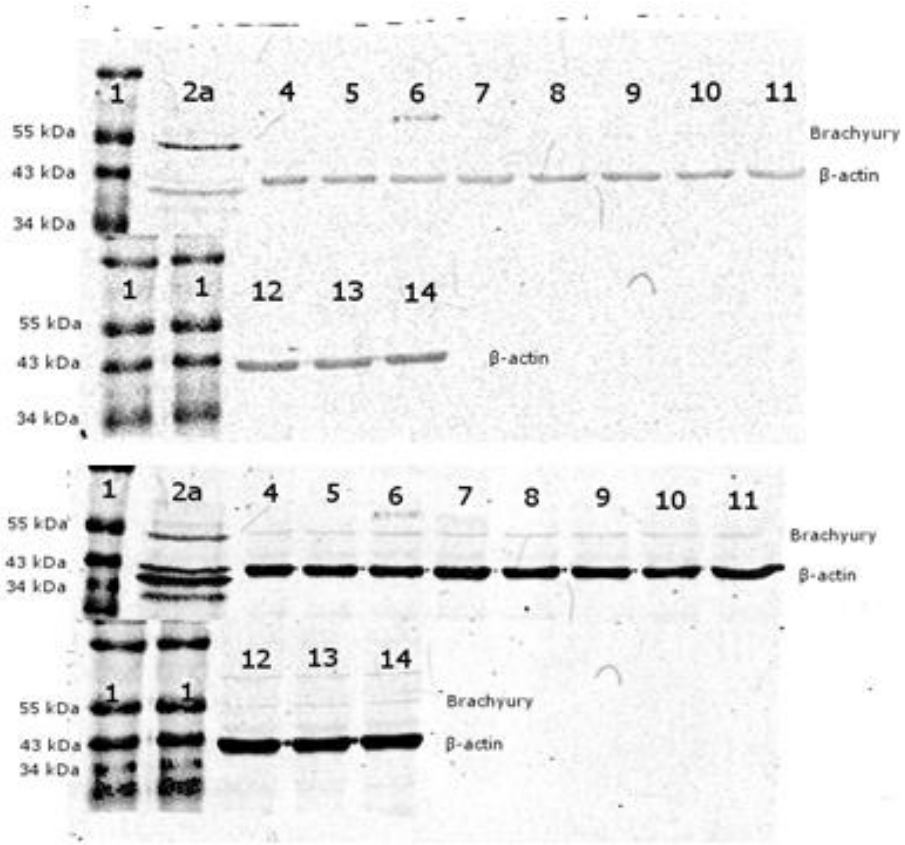

Figure 17 Brachyury membrane scanned wet and then dried in antibodies from Santa Cruz (1:1000)

Just one band (well 6) was visible in the wet Brachyury membrane (upper figure) but the band did not have the right molecular weight. When the Brachyury membrane was scanned again dried (lower figure), weak bands were visible in all wells with the correct molecular weight. Shadows of other bands were also visible. The secondary antibodies contained 5% skim milk powder.