UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN BIOTECHNOLOGY

Vid JAN

SERUM REDUCTION APPROACHES IN MESENCHYMAL STEM CELL MEDIA

M. SC. THESIS
Master Study Programmes

Ljubljana, 2015

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PRISTOPI ZNIŽEVANJA SERUMSKE KONCENTRACIJE V GOJIŠČIH ZA MEZENHIMSKE MATIČNE CELICE

MAGISTRSKO DELO

Magistrski študij – 2. stopnja

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AB Human mesenchymal stem cells (hMSCs), also known as mesenchymal stromal cells are currently being clinically tested for their therapeutic potential in treating a variety of diseases, disorders and injuries. Because hMSCs normally represent just a small fraction of human mononuclear cells (MNCs), it is essential that we expand them *in vitro*, before they can be used for research or therapeutic purposes. Nowadays hMSCs are still mostly cultivated in different basal culture media that are supplemented with human or animal-derived serum (such as FBS). Because of many disadvantages of serum supplementation (e.g. undefined composition, inconsistent lot-to-lot performances, high retail price, possible negative effect on therapeutic effectiveness of cultivated hMSCs), many researchers are trying to develop chemically defined media for serum-free cultivation of hMSCs and approaches of serum-reduction processes. In our thesis we have designed 3 separate gradual serum reduction experiments that were tested on cells, cultivated in 3 chemically defined media (Hektor, TurboDoma and SCM-1). These were compared in performance to cells, propagated in control medium (α-MEM + 10 % of human serum). Even though we have managed to grow hMSCs in lower serum concentrations (at 3% HS for Hektor, TurboDoma; at 1 % HS for SCM-1), the serum depletion process had a drastic effect on morphology, viability, growth rates and metabolic activty of hMSCs. Moreover none of the 3 investigated chemically defined media could support completely serum-free cultivation of hMSCs. Although we were not succesful in complete serum elimination, SCM-1 culture medium stood out as a potential candidate for further evaluation, which could eventually lead to it supporting hMSC growth in completely serum-free conditions.

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AI Človeške mezenhimske matične celice (hMSC), poznane tudi pod imenom mezenhimske stromalne celice, so trenutno vključene v številne klinične študije zaradi njihovega terapevtskega potenciala pri zdravljenju različnih bolezni, okvar in poškodb. Ker hMSC predstavljajo le majhen delež človeških mononuklearnih celic (MNC), jih moramo pred njihovo uporabo v klinične ali raziskovalne namene, nujno namnožiti v *in vitro* pogojih. Dandanes hMSC najpogosteje gojimo v bazalnih gojiščih z dodanim serumom, človeškega ali živalskega izvora (npr. fetalni goveji serum). Ker pa ima serum kot dodatek gojiščem številne pomanjkljivosti (npr. nedefinirana sestava, nekonsistentna učinkovitost od serije do serije, visoka cena, morebiten negativen vpliv na terapevtsko učinkovitost gojenih hMSC), se številni raziskovalci trudijo razviti pristope zniževanja serumske koncentracije v gojiščih in kemijsko definirana gojišča, ki bi omogočala brezserumsko gojenje hMSC. Tekom našega magistrskega dela smo razvili 3 različne pristope postopnega zniževanja serumske koncentracije, ki smo jih preiskusili na hMSC, gojenih v 3 kemijsko definiranih gojiščih (Hektor, TurboDoma and SCM-1). Učinek zniževanja serumske koncentracije na celice smo nato primerjali s celicami, ki so bile ves čas gojene v kontrolnem gojišču s konstantnimi pogoji (α-MEM + 10 % človeškega seruma). Čeprav nam je uspelo gojiti hMSC pri nižjih koncentracijah seruma (3 % Hektor in TurboDoma, 1 % SCM-1), pa je postopek zniževanja serumske koncentracije imel izrazite učinke na morfologijo, viabilnost, hitrost rasti in metabolno aktivnost hMSC. Razen tega pa nam ni v nobenem izmed 3 kemijsko definiranih gojišč uspelo gojiti hMSC v popolnoma brezserumskih pogojih. Čeprav smo bili pri prilagoditvi hMSC na brezserumske pogoje neuspešni, pa se je gojišče SFM-1 izkazalo za potencialnega kandidata za nadaljnjo proučevanje. Obstaja verjetnost, da bi lahko omenjeno gojišče z drugačnimi pogoji zniževanja serumske koncentracije in določenimi prilagoditvami v prihodnosti omogočalo popolnoma brezserumsko gojenje hMSC.

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ABBREVIATIONS AND SYMBOLS

% (v/v)	Volume percent
AT	Adipose tissue
bFGF	Basic fibroblast growth factor
DMEM	Dulbecco's Modified Eagle's Medium
EGF	Epidermal growth factor
FBS	Foetal bovine serum
Fe	Iron
FGF	Fibroblast growth factor
GvHD	Graft-versus-host disease
HCl	Hydrochloric acid
HS	Human serum
K	Potassium
mAb	Monoclonal antibody
MNCs	Mononuclear cells
MSCs	Mesenchymal stem cells
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na	Natrium
NADH	Nicotinamide adenine dinucleotide hydride
RBCs	Red blood cells
SDS	Sodium dodecyl sulfate
SFM	Serum-free media
SSM	Serum-supplemented media
UCB	Umbilical cord blood
ucMSCs	Umbilical cord-derived mesenchymal stem cells
Zn	Zinc
α-MEM	Minimum essential medium alpha

1 INTRODUCTION

Mesenchymal stem cells (MSC) were first characterized by Friedenstein et al. (1970), as clonal, plastic-adherent cells, and serving as a source of osteoblastic, adipogenic and chondrogenic cell lines (Chen et al., 2014). MSCs were first found in bone marrow in 1970 (Friedenstein et al., 1970), but have been later also isolated from other tissues including peripheral blood, umbilical cord blood (UCB), placenta and adipose tissues (AT) (Kogler et al., 2004; Lee et al., 2004). Since their first identification in 1970s, a lot of focus has been placed on the use of MSCs for cell-based therapies and more recently also for the use of MSCs for paracrine support and immune modulation, including the prevention of graft-versus-host disease (GvHD).

As the population of MSCs in foetal or especially adult tissue is always low compared to other nucleated cells, it is essential that we efficiently expand them *in vitro* for later research or clinical application purposes. Historically, MSCs culture medium has constituted a basal culture medium (Dulbecco's Modified Eagle's Medium (DMEM) or Minimum Essential Medium alpha (α-MEM)), which was supplemented with foetal bovine serum (FBS) or human serum (HS), with or without additional growth factors (e.g. basic fibroblast growth factor (FGF)). Although these traditional formulations provide robust undifferentiated MSC expansion, the ill-defined composition of serum, high retail prices, unethical manufacturing processes (FBS), inconsistent lot-to-lot performance and contamination risks have pushed academic groups and industry to develop new chemically-defined media (Chase et al., 2010). This media should enable experiments in serum-free conditions, which will reduce variability in qualitative and quantitative culture medium composition, reduce risk of microbial contamination (mycoplasma, viruses, prions), ease the isolation of cell culture products (down-stream processing) and reduce or completely avoid suffering of animal foetuses and adult animals (FBS) (Brunner et al., 2010).

Although there are many serum-free media on the market today, it is still a golden standard to supplement stem cell and cell culture media with serum. In this thesis, we analysed key biological characteristics of human umbilical cord-MSCs (ucMSCs) during a prolonged *in vitro* cultivation with a gradual serum reduction approach. Biological features included possible morphological changes, viability, growth curve characteristics, senescence and metabolic activity.

1.1 RESEARCH OBJECTIVES

In our experiment we tried to adapt primary human mesenchymal stem cells (hMSC), isolated from umbilical cord, to cultivation in serum-free media. Our plan was to gradually reduce serum concentration in our media and achieve cultivation in serum-free conditions. Serum reduction process was performed in 3 different chemically defined media, while 1 batch of cells was passaged in control medium (α-MEM) with 10 % of HS for later comparison in performance. During serum reduction process we monitored the cells for

any morphological changes, which could occur as a consequence of lowering serum concentration, observed growth parameters, metabolic activity and viability of our cells and performed analyses to detect senescence in cells, which could occur as a result of a high number of passages. Three different reduction approaches were attempted to reach serum-free cultivation. In the so called slow reduction approach we lowered serum concentration once per week, while leaving cells at the same serum concentration for 1 more passage before reducing serum concentration again. That gave hMSCs more time to adapt to a lower serum concentration. In our fast reduction process we lowered serum concentration twice per week, which meant that serum concentration was reduced every passage. The third approach was designed in a way that cells were only passaged, when they reached the desired confluence (preferably higher than 60 %) and serum concentration was lowered at the same time, during each cell splitting. The objective of our experiment was to determine, if our media could be used for stem cell cultivation without any serum content and which one of the 3 was the most suitable for MSCs, isolated from umbilical cord blood.

1.2 HYPOTHESIS

In the beginning of our experimental work we had established the following hypothesis:

- It is possible to wean primary mesenchymal stem cells from serum-supplemented medium and cultivate them in serum-free medium.
- If the correct approach is used to lower the serum-concentration, cells will adapt to lower serum concentration and eventually grow without any serum supplementation.
- If the chemically defined media we used are designed correctly, they should enable our MSCs to survive serum reduction approach and later proliferate in serum-free conditions.
- Our serum reduction process and chemically defined media will enable our cells to adapt to gradual lower serum concentration without imposing stress and morphological changes, while maintaining cell growth, viability and metabolic activity.

2 LITERATURE REVIEW

2.1 MESENCHYMAL STEM CELLS

MSCs are one of the somatic stem cell populations that possess asymmetric self-renewal potential and ability to differentiate into mesodermal lineage upon induction with appropriate differentiation-inducing factors (Sato et al., 2015). MSCs or MSC-like cells can be expanded from numerous compartments, including human bone marrow, skeletal muscles, adipose tissue, umbilical cord, synovium, dental pulp, amniotic fluid, human embryonic stem cells, and numerous other sources (Figure 1) (Phinney and Prockop, 2007; Lian et al., 2007). Human MSCs (hMSCs) isolated and expanded in classical FBS-containing media are mostly spindle-shaped (or fusiform) and cuboidal fibroblast-like cells (Jung et al., 2012). Sekiya et al. (2002) also observed that hMSCs go through a time-dependent morphological conversion from thin, spindle-shaped cells (which are considered to be stem cells or early progenitors) to wider (larger), spindle-shaped cells (in appearance more similar to mature cells) when cells are plated at 1 to 1000 cells/cm^2.

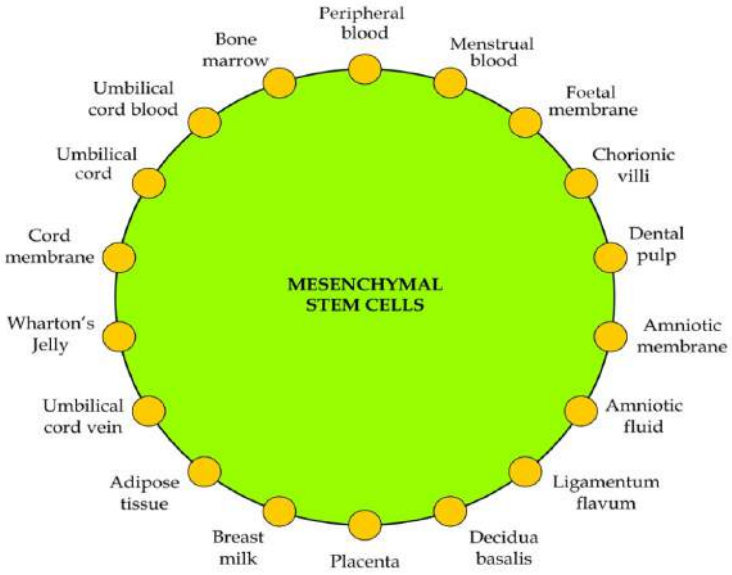

Figure 1: Sources of MSCs. MSCs can be derived from many different infant or adult tissues (Phuc Pham, 2011).

Slika 1: Viri mezenhimskih matičnih celic (MMC). MMC lahko izoliramo iz številnih različnih otroških ali odraslih tkiv (Phuc Pham, 2011).

Because the MSCs from different sources are distinct in their characteristics (e.g. proliferation rate, surface antigen expression, differentiation potency) and functional capacity (Shetty et al., 2010) the International Society of Cellular Therapy (ISCT) has established the minimum criteria for characterization of MSCs. Included are adhesion ability to plastic vessel, expression of cell surface antigens, such as CD73, CD90 and CD105, and absence of cell surface antigens, CD14, CD34, CD45 and HLA-DR. MSCs

also have to maintain an ability of *in vitro* differentiation into osteoblast, adipocyte and chondrocyte cell lines (Dominici et al., 2006).

As a consequence of their great potential as therapeutic agents in regenerative medicine, MSCs have become a subject of many research projects. Clinical trials are underway for using MSC therapies for a variety of disorders that include Crohn's disease, multiple sclerosis, graft - versus - host disease, type 1 diabetes, bone fractures and cartilage defects, just to name a few (Figure 2).

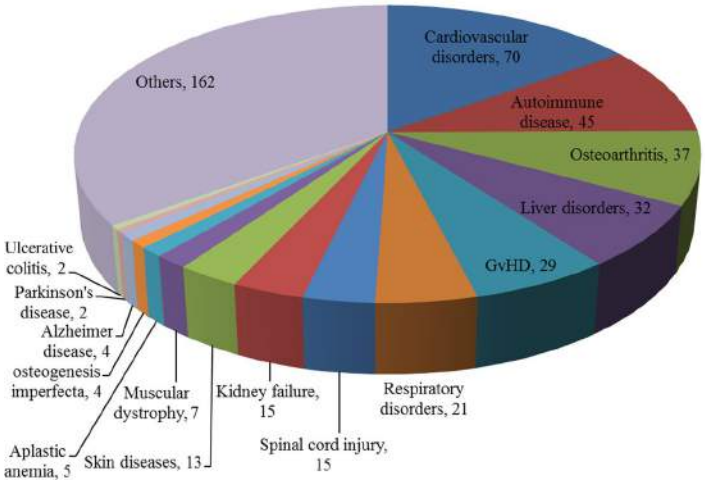

Figure 2: Number of common diseases registered for MSCs based therapy (Ullah et al., 2015)

Slika 2: Število znanih obolenj, registriranih za terapije z MMC (Ullah in sod., 2015)

The number of MSCs in any tissue where they are found is very small. Kern et al. (2006) reported in their study that hMSCs accounted for 557 (stromal vascular fraction of adipose tissue) and 83 (bone marrow) clones per 1×10^6 mononuclear cells (MNCs), while Bieback et al. (2004) describe even smaller numbers, when dealing with MSCs in umbilical cord blood (0 to 2.3 clones per 1×10^8 MNCs) (Table 1). That is why it is absolutely necessary to expand MSCs *in vitro* for any possible research or clinical use. For *in vitro* expansion of MSCs we need a culture medium, which is in most cases comprised of basal medium like Dulbecco's Modified Eagle's Medium (DMEM), α-minimum essential medium (α-MEM) or Ham's F12. In addition of basal medium, culture media for MSCs normally also contain serum of animal or human origin and growth factors (Chen et al., 2014).

Table 1: Isolation frequency of colony forming units (CFU-F) from different tissues per 1×10^6 of MNC (Kern et al., 2006)

Preglednica 1: Pogostost fibroblastov, ki tvorijo kolonije (CFU-F), izoliranih iz različnih tkiv na 1×10^6 mononuklearnih celic (MNC) (Kern in sod., 2006)

Tissue	CFU-Fs per 1×10^6 MNC
Stromal vascular fraction of adipose tissue	557
Bone marrow	83
Umbilical cord blood	0,00 to 0.023

2.2 SERUM'S ROLE IN STEM CELL CULTIVATION

Serum is the clear portion of blood obtained after removing cells, platelets and clotting factors (Venes and Taber, 2009). It contains amino acids, proteins, growth factors, hormones, vitamins, inorganic substances, nutrients and metabolites (Freshney, 2010: 110) (Table 2) and is often used in mammalian cell culture as a supplement to culture media (added in the range of 5-15 % (v/v)) to promote and sustain cell growth as well as provide buffering and protection to cells.

Table 2: Constituents of serum (Freshney, 2010: 110)

Preglednica 2: Sestavine seruma (Freshney, 2010: 110)

Constituent	Range of concentration[a]	Constituent	Range of concentration[a]
Proteins and polypeptides	40-80 mg/mL	Polyamines:	0.1-1.0 µM
Albumin	20-50 mg/mL	Putrescine,	
Fetuin[b]		Spermidine	
Fibronectin	10-20 mg/mL		
Globulins	1.0-10 µg/mL	Urea	170-300 µg/mL
Protease Inhibitors:	1.0-15 mg/mL		
α1-antitrypsin	0.5-2.5 mg/mL	Inorganics	0.14-0.16 M
α2-macroglobulin		Calcium	4.0-7.0 mM
Transferrin		Chlorides	100 µM
	2.0-4.0 mg/mL	Iron	10-50 µM
		Potassium	5.0-15 mM
Growth factors:		Phosphate	2.0-5.0 mM
EGF, PDGF, IGF1 and	1.0-100 ng/mL	Selenium	0.01 µM
2, FGF, IL-1, IL-6		Sodium	135-155 mM
		Zinc	0.1-1.0 µM
Amino acids	0.01-1.0 µM		
		Hormones	0.1-200 nM
Lipids	2.0-10 mg/mL	Hydrocortisone	10-200 nM
Cholesterol	10 µM	Insulin	1.0-100 ng/mL
Fatty acids	0.1-1.0 µM	Triiodothyronine	20 nM
Linoleic acids	0.01-0.1 µM	Thyroxine	100 nM
Phospholipids	0.7-3.0 mg/mL		
		Vitamins	0.01-10 µg/mL
Carbohydrates	1.0-2.0 mg/mL	Vitamin A	10-100 ng/mL
Glucose	0.6-1.2 mg/mL	Folate	
Hexosamine[c]	0.6-1.2 mg/mL		
Lactic acid[d]	0.5-2.0 mg/mL		
Pyruvic acid	2.0-10 µg/mL		

[a]The range of concentrations is approximate and is intended to convey only the order of magnitude
[b]In foetal serum only
[c]Highest in human serum
[d]Highest in foetal serum

2.2.1 Advantages and disadvantages of serum-supplemented media

Serum-supplemented media have been and still are used in stem cell cultivation for a reason. Arora (2013) listed following advantages of serum in culture media:

- Serum supplies cultured cells with basic nutrients (both in solution as well as bound to the proteins).
- It provides several growth factors and hormones involved in growth promotion and specialized cell function.
- Serum contains several binding proteins like transferrin and albumin, which can transport other molecules into the cell (e.g. albumin can carry lipids, vitamins, hormones, etc. into cells).
- It supplies proteins, like fibronectin, that help cells attach to the surface of culture vessels.
- Serum also includes protease inhibitors, which protect cells from proteolysis.
- It provides minerals, like Na+, K+, Zn2+, Fe2+, etc.
- It increases medium viscosity and consequently protects cells from shear stress during agitation of suspension cultures or during cell splits.
- It acts like a buffer.
- Because of serum's complex composition it can be used as a supplement with basal media for almost any cell type cultivation.

Serum's complex composition is beneficial and harmful at the same time. It contains both growth factors and inhibitors. FBS-based media remain a common standard in generating hMSCs for basic research and clinical studies as well, however the use of FBS is not desirable, because of several safety and other concerns. Potential problems associated with the ill-defined FBS and other animal-derived supplements can be found in table 3.

Table 3: Advantages and disadvantages of using serum in culture media (Arora, 2013; Jung et al., 2012)

Preglednica 3: Prednosti in slabosti uporabe seruma v gojiščih (Arora, 2013; Jung in sod., 2012)

Advantages of serum in media	Disadvantages of serum in media
Growth factors and hormones that stimulate cell growth and functions	Lack of uniformity in the composition of serum (problems with standardization, due to batch-to-batch variations
Helps in attachment of the cells	Risk of contamination
Acts as a spreading factor	High content of xenogeneic proteins
Acts as a buffering agent which helps in maintaining the pH of culture media	Presence of growth inhibitors, cytotoxic substances and/or differentiation agents
Functions as a binding protein	Requirement of a set of strict quality controls to minimize the risk of contamination
Minimizes mechanical damages or damages causes by viscosity	Interference of unidentified factors on the effect of hormones, growth factors, or other additives under investigation
	Limited availability and high cost
	Ethical issues
	Downstream processing

If we consider advantages and disadvantages of serum supplementation, we can conclude that despite strict selection and testing for safety and growth-promoting capacity, the use of FBS represents a major obstacle for the wide implementation of hMSC-related therapies. Although relatively safer than FBS for human therapeutic application, the use of human-sourced supplements is still a matter of debate. Even if we use HS, plasma, platelet-derivatives and cord blood serum, we still come across some similar problems as with animal-derived supplements. There is a possibility that the allogeneic human growth supplements are contaminated with human pathogens, which are not detected with routine screening of blood donors (Jung et al., 2012). Furthermore, these blood derivatives are also poorly defined and suffer from similar consequent problems as FBS, like batch-to-batch variation, therefore their ability to maintain hMSC growth and therapeutic potential could vary immensely. Because of great variability between different batches, implementation of clinical-scale production of hMSCs could be more difficult to implement (Jung et al., 2012). The biggest problem is that with big variations in serum supplements, it would be very hard to obtain cells retaining desired qualities in a consistent and predictable manner, which is very important, when you are trying to minimize treatment failures (Jung et al., 2012). That is why a lot of scientists agree, that the best solution in a long run is a design of chemically defined serum-free media, with which we would be able to eliminate all the problems, connected with serum supplementation.

2.3 DEVELOPMENT OF CHEMICALLY-DEFINED SERUM-FREE MEDIA

Chemically defined serum-free media development or optimization for a specific cell type can be a very challenging process, because multiple variables, affecting the maintenance, growth, and characteristics of cells are interconnected (Jung et al., 2012). Designing serum-free media for adherent cells like hMSCs is even more difficult, because compared to cells, grown in suspension culture, we also need to understand the interaction of adherent cells with the substrate on which they attach and spread prior to growth. Medium development studies should consist of rational approaches:

- To select suitable factors (e.g. basal medium formulations and attachment/growth proteins)
- To screen them in a stepwise, systematic manner for their impact on cell characteristics and growth.

There are a few general approaches to serum-free media development for a specific cell line or primary culture and 3 of them are listed below.

2.3.1 Ham's approach

In first one we take a familiar recipe for a related cell type, which can be supplemented with 10 – 20 % of dialyzed serum, and then alter the components one by one or in groups, against a variety of serum concentrations (as well as 0%) (Freshney, 2010: 124). Clonal growth analyses will then demonstrate any frugal outcomes of a possible serum

replacement (i.e. in the presence of the replacement, we will need less serum to acquire the same clonal growth). We can then determine the ideal concentration of the compound at the lowest serum supplementation, which would still allow clonal growth. This approach was embraced by Ham (1984) and will basically provide optimal conditions. When a group of compounds demonstrate efficacy in reducing serum supplementation, we can identify the active ingredient by the systematic omission of a single component and then optimize the concentrations of the essential components (Ham, 1984). Unfortunately the above-mentioned method is a very time-consuming and laborious process. It may take us at least 3 years to develop a new medium formulation for a new cell type (Freshney, 2010: 129).

2.3.2 Sato's Approach

Compared to Ham's approach, which is analytical in nature, Sato et al. (1980) developed a synthetic method for the replacement of serum in culture media. In this approach the researchers have supplemented well known media such as RPMI 1640 (Carney et al, 1981) or combination of media such as DMEM with Ham's F12 (Barnes and Sato, 1980) and manipulated only a shorter list of substances. Once again, the ideal concentrations were determined at a limiting serum supplementation. Some of the most regularly analysed substances are transferrin, selenium, albumin, estrogen, insulin, hydrocortisone, triiodothyronine ethanolamine, phosphoethanolamine, growth factors (epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), endothelial growth supplement, etc.), prostaglandins (prostaglandin E_1, prostaglandin F2α), and any other substances that may have special importance (Freshney, 2010: 129). Barnes and Sato (1980) identified various key essential supplements for many cell types, such as hormones, binding proteins, lipids, trace elements and attachment factors, required for addition to basal medium. Most importantly, they have discovered that transferrin, insulin and selenium are essential for the growth of most cells, whereas EGF and hydrocortisones have to be added for certain types of cells. With this approach Barnes and Sato (1980) managed to replace serum with specific supplements for a number of different cell types, without significantly changing the basal medium. Even though this approach was quite effective, it still takes a lot of time and work (Jung et al., 2010).

2.3.3 Top-down and Bottom-up approaches

Top-down and bottom-up approaches can also be used for the formulation of new serum-free medium formulation and are considered more practical (Butler and Burgener, 2005).

In Top-down approach we make use of an existing medium formulation, which is already used for a similar type of cells, and then identify stimulatory constituents in the presence of serum for the growth of investigated cells (Jung et al., 2010). The process continues, while the serum content is slowly reduced. This approach was developed through a hypothesis that a cell type that belongs to a group of cells with similar characteristics, often need the same or comparable mixture of growth factors for growth.

In the Bottom-Up method we select an appropriate basal medium (generally a medium that is used for the proliferation of a related cell type) and then screen selected exogenous factors for their influence on the stimulation of growth (Jung et al., 2010). Because the medium is only supplemented with active components, which are required for an adequate growth of our cell type, the final composition will embody a compact and easily adaptable medium. This method is considered to be quite labour-intensive and time-consuming, and it also has to be taken into account that screening of factors is executed in serum-free conditions. That is why, it is necessary that critical functions of serum, required to see the effects of our screened factors, are carefully evaluated and satisfied by different means (e.g., treatment of culture surface, well-controlled physiochemical parameters, trypsinization and passage protocols) (Jung et al., 2010). Serum-free basal medium does not normally provide such functions.

2.3.4 Serum-free design in practice

Because none of the before-mentioned approaches is perfect and without its faults, it is very important for each researcher to know and understand disadvantages and advantages of any one of them and then use the best qualities of them all. When Jung et al. (2012) were developing a new hMSC serum-free medium, they chose different medium ingredients, such as basal medium, binding proteins, extra nutrients, hormones, buffering agents, vitamins and growth and attachment factors, built on their understanding of the requirements of cell culture (including the role of serum ingredients). Chosen factors were then investigated in a consecutive manner, using the best characteristics of each method, described earlier. In doing so Jung and his colleagues determined chronological influence on proliferation, attachment and isolation of hMSCs (Figure 3).

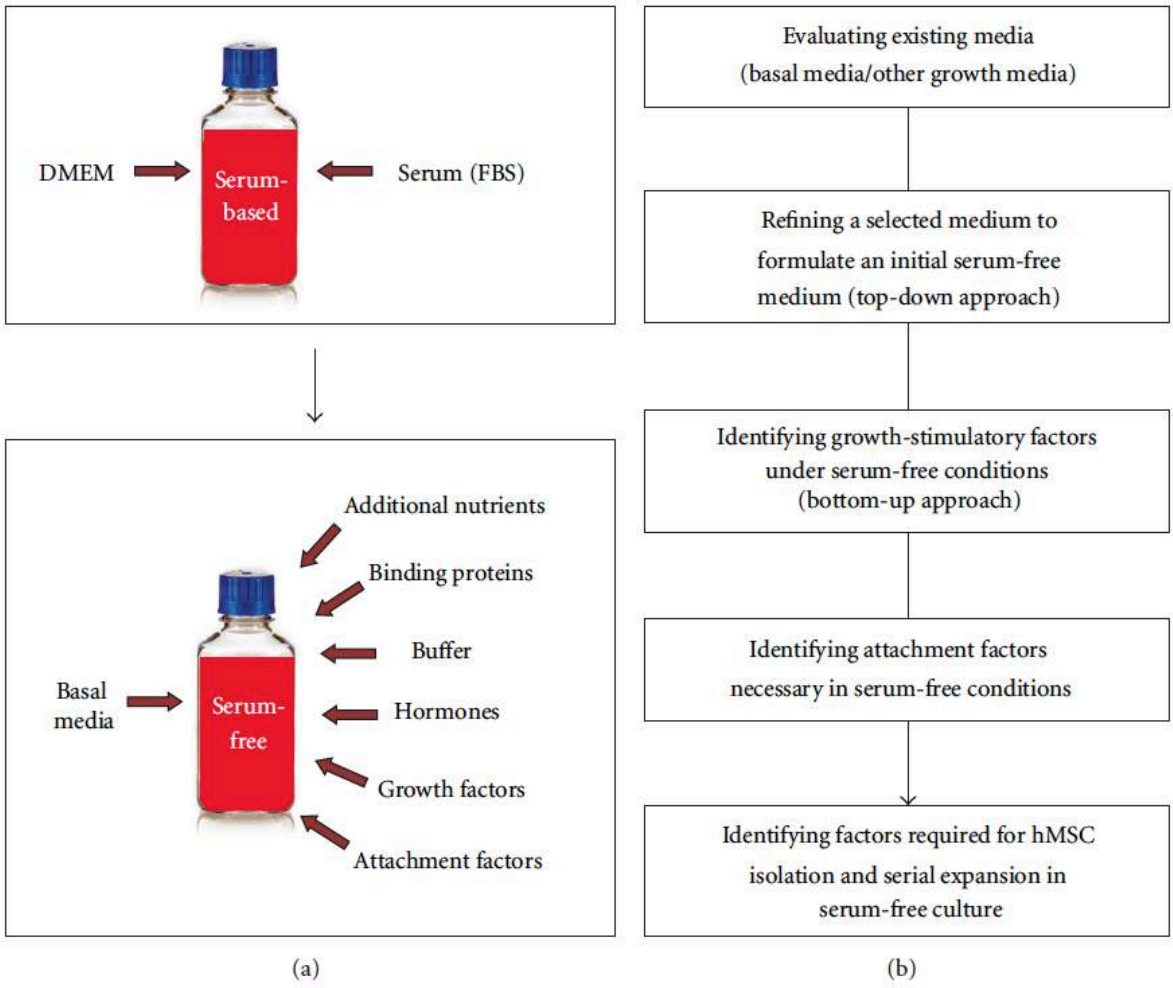

Figure 3: Outline representing a procedure for the development of a defined serum-free medium for hMSCs. (a) Ill-defined serum was substituted with chosen well-defined supplements, which included a selection of medium components, such as basal media, additional nutrients (e.g. lipids and vitamins), binding proteins, hormones, physiochemical reagents (e.g. buffer), growth factors and attachment factors. (b) Sequential strategy was planned for efficient screening of chosen basal media and medium supplements to develop a defined serum-free condition that supports the isolation and proliferation of hMSCs (Jung et al., 2010).

Slika 3: Shema prikazuje proces razvoja definiranega brezserumskega gojišča za človeške MMC. a) Slabo definirani serum je bil zamenjan z izbranimi dobro definiranimi dodatki, ki so vključevali izbor komponent gojišč, kot so bazalno gojišče, dodatna hranila (npr. lipidi in vitamini), vezavni proteini, hormoni, fiziokemični reagenti (npr. pufer), rastni dejavniki in vezavni dejavniki. b) Sekvenčna strategija je bila načrtovana za bolj učinkovito presejanje bazalnih gojišč in gojitvenih dodatkov, kar bi nam omogočilo vzpostavitev definiranih brezserumskih pogojev, ki bi podpirali izolacijo in proliferacijo človeških MMC (Jung in sod., 2010).

2.4 ADAPTATION OF MESENCHYMAL STEM CELS TO SERUM-FREE MEDIUM

There are many different approaches to adapting mammalian cells to serum-free cultivation. When dealing with cell cultures, gradual weaning processes are normally used, that involve progressive adaptation to lower serum concentrations until serum-free

conditions are reached (van der Valk et al., 2010). Cultures, planned for the adaptation should be in the logarithmic phase of growth and should display viability, higher than 90 %. Van der Valk et al. (2010) also state, that because of the adaptation process, an unwanted selection of a change in the population of cells can occur, by indirectly selecting cells capable of growth in serum-free media. Hence it is essential to monitor cellular morphology and function during the weaning process and check the performance of cultures. A few of adaption protocols are presented below (Figure 4).

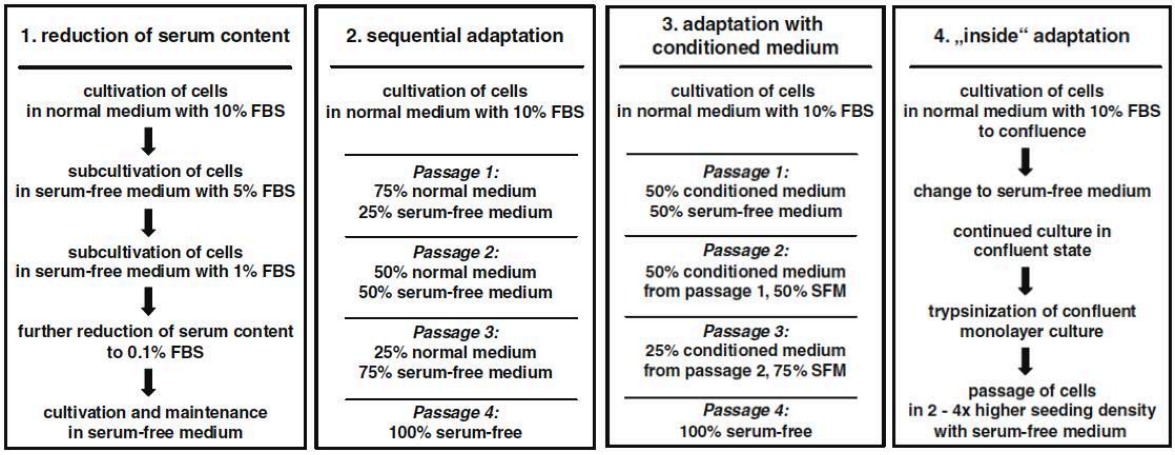

Figure 4: Adaptation of cultures to serum-free medium. A comparison of the most common adaptation protocols (FBS: foetal bovine serum and SFM: serum-free medium) (van der Valk et al., 2010)

Slika 4: Prilagoditev celičnih kultur na brezserumska gojišča. Primerjava najbolj običajnih adaptacijskih protokolov (FBS: fetalni goveji serum; SFM: brezserumsko gojišče) (Van der Valk in sod., 2010)

2.4.1 Reduction of serum content

In this approach serum content is reduced in every passage, until we reach 0.1 %. We start with propagation in normal medium with 10 % of serum, then move the cells to serum-free medium with 5 % of serum, after which we subcultivate cells in 1 % serum, etc. Serum-free medium can be supplemented with hormones.

2.4.2 Sequential adaptation

Quite related to the first approach, cells in sequential adaptation method are split into combinations of serum-supplemented and serum-free media, until complete serum-free cultivation is achieved. If the last step, when cells are transferred from 75 % to 100 % serum-free medium, imposes too much stress on cells, it is suggested to add another step with 10 % of serum-supplemented and 90 % serum-free medium for 2-3 passages, before moving to a completely serum-free conditions (van der Valk et al., 2010).

2.4.3 Adaptation with conditioned medium

This method keeps to similar steps than Sequential adaptation approach, but uses reducing combinations of conditioned media from past passages.

2.4.4 Inside adaptation

In this method freshly harvested cells are weaned in serum-free medium, and then cultures are propagated to confluence. The confluent monolayer is then split into serum-free medium. With this approach it is recommended to passage cells in 2 to 4x higher cell density.

2.4.5 Approaches, preferred for hMSCs

There have been many attempts by different research groups to develop serum-free media for human or animal MSC growth, but many of them achieved inadequate performance (Lennon et al., 1995; Liu et al., 2007; Marshak and Holecek, 1999; Parker et al., 2007). Media compositions, designed in the referred studies only supported cell expansion for single-passage cultures or at slow rates through multiple passages. Jung et al. (Jung et al., 2012) propose, that this could be a consequence of serum supplementation in initial isolation/expansion phases. Serum-derived contaminants could be transferred with the cells when they are being adapted to serum-free conditions after exposure to serum, hence the serum supplementation in any stage of cell handling could ultimately limit their therapeutic use (Jung et al. 2012). That is why many studies agree, that the best approach is to exclude the use of serum even in the isolation steps (Sato et al., 2015; Chen et al., 2014). Those studies also demonstrated best performance of serum-free media. In fact in studies, where serum-free media were used from the very beginning (including isolation), MSCs even outperformed MSCs in serum-supplemented media in cell growth, viability at isolation and potential for bone repair (Sato et al., 2015; Al-Saqi et al., 2014), and had a comparable biological stability to cells, cultured in SSM.

If our source material was already isolated and cryopreserved, before we start with our experiment (as was the case in our experiment), we can decide to move cells straight into serum-free conditions (inside adaptation approach) or gradually reduce serum concentration and give the cells time to adjust to lower serum concentrations. We decided to adopt the latter.

2.5 CELL CULTURE ASPECTS IN SERUM-FREE CONDITIONS

Generally the cells in serum-free media and during the adaptation process are more sensitive to any kind of extremes, such as low or high temperature, pH variations, and

changes in osmolality and shear force (Jung et al., 2012). We also have to be more careful with enzyme treatment. They should be handled with great care to minimize cell damage that could immensely lower the viability of our cell population. The following points should be considered, before starting the adaptation procedure or serum-free cultivation.

2.5.1 Antibiotics

It is highly recommended to avoid using antibiotics in serum-free cultivation. Because there are no serum proteins to bind antibiotic, the levels of antibiotic in cell culture medium may be toxic to the cells. If they are added to the culture medium, they should be used in concentrations that are 5 to 10 times lower than in serum-supplemented media (ThermoFisher Scientific, 2015).

2.5.2 Buffer System of Media

Because serum also plays a buffering role in cell culture media (modulates pH), it is recommended to add a chemical buffer, such as HEPES, in addition to bicarbonate-CO_2 system, in order to increase the buffering capacity of the medium (Jung et al., 2012). HEPES can be added to concentrations, higher than 15 mM without becoming toxic to the cells, but it may be necessary to adjust osmolality of the medium accordingly (Barnes and Sato, 1980).

2.5.3 Lack of detoxifying Substances and Protease inhibitors

Because there are no serum proteins to bind and neutralize toxic contaminants, medium's protective and detoxifying activity is compromised. That is why, it is even more essential, that water, reagents and culture techniques are selected with great care. The addition of serum to cells, exposed to trypsin, also neutralizes any residual proteolytic activity (Jung et al., 2012). In absence of serum, we can use protease inhibitors to the same effect, or choose a different dissociation solution that allows the dislodging without the use of enzymes (e.g. Sigma non-enzymatic dissociation solution).

2.5.4 Higher density

Before we start with the adaptation, we should check that cells are in mid-logarithmic phase of growth and are highly viable (>90 %). Cells should also be seeded at a higher concentration, so that the appropriate numbers of cells are grown to perform further passages.

2.5.5 Clumping and morphology

One of the common problems that occur during adaptation to SFM is cell clumping. Clumping normally becomes apparent during the dissociation step. Longer incubation time and gentle trituration is proposed to break down clumps and obtain separate single cells. Small changes in cellular morphology are not uncommon during and after adaptation to SFM, but as long as doubling times and viability stay in normal values, it should not be a reason for concern. It is recommended to have an adequate amount of frozen cell stock before we start with the adaptation, so we can repeat the procedure, if something goes wrong.

3 MATERIALS AND METHODS

3.1 COURSE OF THE EXPERIMENTS

We have lined out the course of all 3 different gradual serum reduction approaches in tables 4, 5 and 6.

Table 4: Course of the slow reduction approach through passages

Preglednica 4: Potek dela pri počasnem zmanjševanju serumske koncentracije v različnih pasažah

Passage	Assessments, performed during each passage	Main procedures	Weekly assessments
P1		Thawing of the hMSC	
P2 (10% HS)	Cell counting, trypan blue staining, morphology assessment	Transfer of hMSCs to 4 different media	Seeding of cells for MTT viability assay
P3 (10% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting	
P4 (5% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay
P5 (5% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting	
P6 (3% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay
2%, 1% 0.5%, 0,3 % P7-P15	. . .	. . .	. . .
P16 (0.1% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay
P17 (0.1% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting	
P18 (0.0% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay

Table 5: Course of work for fast reduction experiment

Preglednica 5: Potek dela pri hitrem zmanjševanju serumske koncentracije

Passage	Assessments, performed during each passage	Main procedures	Weekly assessments
P1		Thawing of the hMSC	
P2 (10% HS)	Cell counting, trypan blue staining, morphology assessment	Transfer of hMSCs to 4 different media	Seeding of cells for MTT viability assay
P3 (5% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	
P4 (3% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay
2%, 1% 0.5%, 0,3 % P7-P15	. . .	. . .	. . .
P9 (0.1% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	
P10 (0.0% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction	Seeding of cells for MTT viability assay

Table 6: Course of work for adjusted reduction experiment

Preglednica 6: Potek dela pri prilagojenem zmanjševanju serumske koncentracije

Passage	Assessments, performed during each passage	Main procedures	Weekly assessments
P1		Thawing of the hMSC	
P2 (10% HS)	Cell counting, trypan blue staining, morphology assessment	Transfer of hMSCs to 4 different media	Seeding of cells for MTT viability assay
P3 (5% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction (only at confluence >60 %)	
P4 (3% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction (only at confluence >60 %)	Seeding of cells for MTT viability assay
2%, 1% 0.5%, 0,3 % P7-P15	. . .	. . .	. . .
P9-14 (0.1% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction (only at confluence >60 %)	
P10-15 (0.0% HS)	Cell counting, trypan blue staining, morphology assessment	Cell splitting + serum reduction (only at confluence >60 %)	Seeding of cells for MTT viability assay

3.2 MATERIALS

3.2.1 Chemicals

In our work we used the following chemicals: MTT powder (Sigma Aldrich, USA), PBS (Life Technologies, USA), Gentamyicine (Biozym, Germany), human serum (Blood Bank Linz, Red Cross Austria, Austria), Accutase (PAA Laboratories, Austria), Cell Dissociation Solution Non-enzymatic 1x (Sigma Aldrich, USA), N,N-Dimethylformamide (Sigma Aldrich, USA), glycerol (Sigma Aldrich, USA), Trypan Blue solution (Life Technologies, USA). 1% GlutaMAX™ medium supplement (Life Technologies, USA), ethanol (Carl Roth Gmbh & Co. KG, Germany), SDS (Sigma Aldrich Gmbh, Germany)

3.2.2 Media

In our research we used the following media:

- α-MEM medium: 10 % (v/v) HS
- TurboDoma® medium: 10 % (v/v), 5 %, 3 %, 2 %, 1 %, 0.5 %, 0.3 %, 0.1 % HS, 1% GlutaMAX™
- Hektor® medium: 10 % (v/v), 5 %, 3 %, 2 %, 1%, 0.5 %, 0.3 %, 0.1 % HS, 1% GlutaMAX™
- Stemcell Cell Medium 1 (SCM-1): 10 % (v/v), 5 %, 3 %, 2 %, 1 %, 0.5 %, 0.3 %, 0.1 % HS, 1% GlutaMAX™

The company called Cell Culture Technologies supplied all 3 chemically defined media.

Stem Cell Medium 1 (SCM-1) was created as a basal minimal medium for the cultivation of stem cells, and turned out to support proliferation of immortalized hMSC_TERT cells with no supplements as demonstrated by the Technische Hochschule Mittelhessen in Giessen, Germany. Such minimal medium exclusively consists of small molecules and contains no growth factors, no proteins and no peptides. Currently, SCM-1 is used to grow stem cells of various origins when properly supplemented with selected growth factors. The medium is buffered with HEPES.

The TurboDoma® media were developed in the 90's to grow hybridomas. The TP-6 version is a production medium currently used by life science companies to produce monoclonal antibodies (mAb) for diagnostic purposes. Compared to SCM-1, the TP-6 medium contains more nutrients to ensure high cell density and productivity.

Hektor® S medium was developed for the transient transfection of 293T and 293EBNA cells. Such medium was developed in collaboration with Zisch et al. (2003). The same medium was reported to sustain serum-free proliferation of several kidney-derived mammalian cell lines. The nutrient strength of the Hektor S medium is comparable to SCM-1.

3.2.3 Buffers, solutions and reagents

Table 7: Solutions and reagents for Senescence beta-Galactosidase Cell Staining Protocol

Preglednica 7: Raztopine in reagent za β-galaktozidazni test celične senescence

Solution or reagent	Composition of the solution or reagent
10X Fixative Solution	20% formaldehyde, 2% glutaraldehyde in 10X PBS
10X Staining Solution	400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM $MgCl_2$
X-Gal	5-bromo-4-chloro-3-indolyl-βD-galactopyranoside powder
100X Staining Solution Supplement A	500 mM potassium ferrocyanide
Staining Solution Supplement B	500 mM potassium ferricyanide

3.2.4 Cell type

We used umbilical cord-derived mesenchymal stem cells from a single donor, labelled ucMSC130613 for all our experiments. Cells were isolated from umbilical cord Wharton's jelly. Label describes the type and isolation date of cells and is used for group's Liquid Nitrogen Log. General information of the cells is as follows:

- Cells were isolated on the 13th of June, 2013 in-house from umbilical cord tissue according to an SOP routinely used in the research group.
- Biological material was obtained from a blood bank of Linz Red Cross under valid ethical approval.
- Cells grow plastic adherent and present important MSC surface markers (analysis based on commercial flow cytometry kit (Miltenyi).
- Cells have been tested for differentiation potential towards osteogenic, adipogenic and chondrogenic lineage using commercial media (Miltenyi). Differentiation was verified by staining with Calcein, Alizarin Red, von Kossa, Oil Red O, Alcian Blue.
- Cells have normal human female karyotype 46, XX (analysis performed by prof. Neesen's group at the Institute of Medical Genetics at Medical University of Wien.
- Under standard cultivation conditions, the cells had an average doubling time of 35 h in the first 10 doublings (37 °C/5 % CO_2 in α-MEM medium with 10 % of HS).
- The cells can be proliferated (at standard conditions) for more than 20 passages before they start to become senescent around passage 24-26.

3.2.5 Laboratory equipment

The following equipment was used during our practical work: Neubauer counting chamber (Brand, Germany), centrifuge for conical tubes (Eppendorf AG, Germany), ultra-pure water system Arium 611 (Sartorius AG, Germany), microscope Leica DMIL LED (Leica Camera AG, Germany), incubator Heracell 150i (Thermo Scientific Gmbh, Germany), Thermo Scientific Multiskan™ FC Microplate photometer (Thermo Scientific Gmbh, Germany), suction pump biovac 106 ILMVAC (ILMVAC Gmbh, Germany), clean bench MSC-Advantage (Thermo Scientific Gmbh, Germany), water bath (GFL Gmbh, Germany), refrigerator -80 °C Hera freeze basic (Thermo Scientific Gmbh, Germany), cryo tank -165 °C Cryotherm Biosafe MD (Cryotherm Gmbh & Co. KG, Germany), Autoclave Varioklav (Thermo Scientific, Germany), Scale (Sartorius AG, Germany), Vortex (Aigner Unilab Gesmbh, Austria), cam Leica ICC50 HD (Leica Camera AG, Germany), refrigerator HITECH ARTIS (AEG), magnetic stirrer MSH BASIC (IKA Werke Gmbh & Co KG, Germany), pipetting aid Pipetboy acu 2 (INTEGRA Biosciences AG, Switzerland), cell culture flasks T25, T75, T175 (Sarstedt AG & Co., Germany), cell culture plates 6-, 12-, 24-. 96-wells (Sarstedt AG & Co., Germany), 15 and 50 ml conical tubes (Corning Inc., USA).

3.3 METHODS

3.3.1 Isolation of MSCs from umbilical cord

Even though other researchers in the group did the isolation of MSCs beforehand, we are adding a short description of the method, which was described by Moretti et al. (2010). Cells were isolated from umbilical cord's Wharton's jelly, which is a gelatinous connective tissue of umbilical cord that represent a rich source of multipotent stem cells.

In the days before the isolation, the appropriate amount of cell culture medium was prepared and the instruments were steam sterilized. On the day of isolation sterile and syringe filtered (0.2 µm pore size) culture medium was prewarmed. Umbilical cord was removed from buffered transport medium and then perforated via tweezers. Afterwards it was flushed with PBS to get rid of any blood still present in vessels. Using scissors the umbilical cord was cut in pieces (preferably smaller than 0.5 m^3 in size), which were arranged into 10 small equal piles (approximately 2 spoons). Each pile was then transferred into separate culture flask with a spoon. Shortly afterwards, 25 mL of culture medium was supplied to each culture flask and the pieces of umbilical cord were evenly distributed over the bottom of the flasks. Photographs of the flasks were taken to document the amount and size of tissue pieces, after which the flasks were incubated at 37 °C / 5 % CO_2. Half of the flasks were incubated in normoxic conditions (21% O2), while the other half was kept in hypoxia (5% O2) incubator. We used the cells that were incubated in normoxia incubator, because all our experiments were also conducted under normoxic conditions.

Cell outgrowth and medium colour was checked every day and microscopic pictures were taken for documentation. Medium was changed every 24-72 h. In case there were a lot of red blood cells (RBCs), 1 or 2 initial medium changes were performed in 24 h intervals to reduce RBCs.

Medium change was performed for a maximum of 5 flasks at a time (to prevent drying of the outgrown cells due to lengthy procedure). If RBCs settled to the flask bottoms, each flask was tilted several times to resuspend the RBCs (to ease their removal). The flasks were then put in upright positions. After the tissue pieces sedimented in one corner of the flask, the supernatant was removed with a large (25 mL) serological pipette. The pipette tip was gently placed on the bottom/edge of the flask to avoid sucking in tissue pieces and the supernatant was collected in a (sterile) waste bottle. After the supernatant removal the flasks were refilled with 25 mL of fresh medium each. Tissue pieces were again evenly distributed over the flask bottoms, before the flasks were put back into the incubator.

Cell outgrowth from the tissue pieces is normally observed after about 5 to 7 days. Once many cells (ca. 70 %) have grown out of the tissue pieces (after 10 to 14 days), the pieces were aseptically transferred to fresh T175 flasks. Most of the supernatant was removed and the tissue pieces tipped into fresh flasks. New flasks were labelled, supplemented with 25 mL of fresh medium and cultivated appropriately.

Adhered cells from the old flasks were harvested and cryopreserved (passage 0) to create a working cell bank. Cryopreservation of MSCs was performed under standard protocol, routinely used in the research group. If a larger number of cells is needed, the new flasks are incubated the same way as the first ones, where cell outgrowth is normally visible after about 24-48 h. Once the 2nd round of flasks is ready for harvesting, the tissue pieces can be discarded.

We started our experiment with cryopreserved vials of our cells.

3.3.2 Thawing of MSC and transferring the cells to chemically-defined media

To thaw cryopreserved MSCs we used the adapted procedure, proposed by Freshney (2010: 327). Firstly the cell culture medium was prewarmed in water bath in the laboratory for Tissue engineering. After the cell sample was located and signed out on Liquid Nitrogen Log (online excel sheet), we quickly transferred the cryopreservation vial from the liquid nitrogen storage tank (- 180 °C), submerged it into 37 °C water bath and swirled it for approximately 1 to 2 min. While the vial was still cool to the touch but completely thawed, it was removed from the water bath, sprayed with ethanol (70 %) and placed inside a flow hood. We added 1 mL of cold α-MEM to the sample vial and while keeping the pipette in our hand, waited for 2 minutes. After 2 minutes the content in the sample vial was transferred to 50 mL tube. Sample vial was additionally washed with 2 mL of α-MEM and the content was again added to the 50 mL tube with cells. After 2 minutes we topped up the tube to 10 mL with α-MEM. Afterwards we centrifuged the tube at 300xg for 5 min. Supernatant was suctioned away with sterile Pasteur (glass) pipette. The pellet was flicked and then resuspended in 3 mL of cell culture medium. A small fraction was removed for cell counting under haemocytometer. After we calculated the cell number, we topped up

the cell suspension to 12 mL with cell culture medium and transferred it to T-75 cell culture flask. Cells were then incubated at 37 °C/5 % CO_2 overnight. After 24 hours we changed the medium.

3.3.3 Cell splitting

We designed our cell splitting procedures on a method, proposed by Todaro and Green (1963). Before each cell splitting we checked the confluence of our cells under the microscope and took photographs for later morphological analysis. We wrote down the approximate confluence and examined the cells for any possible morphological changes. All the reagents were prewarmed to 37 °C in a water bath.

We used 2 separate protocols; 1 was used for the first split after the thawing of the cells and the second one was used for all the following passages. After we had changed the medium in culture flasks, the cells were incubated in the incubator at 37 °C/5 % CO_2, until they reached the appropriate confluence (above 60 %). We then took them out of the incubator and transferred them to the clean bench. All the reagents that were needed in our cell splitting were prewarmed to 37 °C, before the start of our procedure. We started cell splitting with the removal of culture medium from each of our 2 T-75 culture flasks. The flasks were then rinsed with 10 mL of PBS (1x). After a few seconds we removed PBS from the flasks and added 4 mL of Sigma dissociation solution. Flasks were incubated for approximately 5 min at 37 °C and then tilted gently to force cells to detach. After the incubation and tilting we checked the level of detachment under the microscope. If the cells remained adhered, we incubated our flasks for additional 2 min at 37 °C and repeated the procedure. When the cells were completely detached (~98%), we sterilized outer flask surface and transferred them back in sterile environment. We added 6 mL of culture medium to each flask and then transferred whole content of each flask (10 mL) into one 50 mL tube (total volume = 20 mL). After making sure that cells were evenly distributed, we split the volume into 4 50 mL tubes (each with 5 mL of cell suspension) and centrifuged them for 5 min at 300g. Supernatant from each tube was discarded using Pasteur pipettes and suction device and the pellets were resuspended with gentle snipping. Afterwards the pellets were additionally resuspended in one of our 4 culture media (Control, TurboDoma, Hektor or SCM-1) with 10 % HS and in case of our 3 examined chemically defined media (TurboDoma, Hektor and SCM-1) also with 1% GlutaMAX™. In order to get the appropriate concentration of cells for the next passage we also performed a cell count under the haemocytometer. Consequently we mixed the cell suspension and transferred 4000 viable cells/cm^2 to a fresh cell culture flask (T-25). Fresh culture medium was added to each flask to get the total volume of 5 mL and distributed evenly over flask bottom. Flask were labelled and moved to the incubator, where they stayed until the next cell splitting (at 37 °C and 5 % CO_2).

When cells were moved into 4 different media, we started using smaller T-25 flasks to save on material (we had 3 biological replicates (3 flasks) for each examined medium). We performed the whole procedure separately for each examined medium, to avoid any mistakes or mix-ups. After prewarming reagents to 37 °C, checking confluence of our cells and taking photographs under the microscope, we moved our culture flasks in a clean

bench. Culture medium was removed from the culture flasks and transferred to 50 mL tubes. We then added 5 mL of PBS to wash the cells, and then PBS was also transferred to 50 mL tubes. After the removal of PBS we added 1.5 mL of Sigma dissociation solution to each flask and distributed it evenly over the entire cell layer by tilting the culture vessel. Cells were then incubated at 37 °C for approximately 5-10 min and regularly checked for cell detachment using a microscope (incubation times lengthened as the serum concentration declined). As soon as cells were round and started floating, we added 3.5 mL of culture medium in the sterile environment. We used the culture medium to rinse cells from the bottom of the flask, and then transferred the whole content to our 50 mL tubes, where we already had the residue from the old culture medium and PBS, used for washing the cells. After we moved all the cells from T-25 flasks, 50 mL tubes were centrifuged for 5 min at 300 g. When the centrifugation was finished, supernatant was discarded and the pellets were resuspended with gentle snipping. We resuspended the pellets with the appropriate amount of culture medium and then performed cell counting with a haemocytometer. When we calculated our cell number, we diluted our cell suspension to get the right seeding concentration with our medium. Cell seeding concentration was adjusted from passage to passage according to cell growth rate in the previous passage. We made sure that our cell suspension was evenly distributed over the whole flask bottom. Flasks were then labelled (date, cell strain name, passage number, seeding concentration, replicate number) and moved to the incubator. Every second passage we also seeded our cells onto a 24-well plate for MTT viability test. Cells were seeded at a concentration of 3500 cells/cm^2 (V=1 mL). It was always very important to make sure that the cells very evenly distributed through the whole well bottom surface, otherwise the cells accumulated in the middle of the well, which could consequently distort the results of the viability test. In the second 24-well plate we seeded blanks for each medium. For the blanks we added 1 mL of each culture medium per well, without any cells. Seeding layout can be seen in Figure 5. Cells were incubated for 4 days at 37 °C and 5 % CO_2. After the incubation time, we performed the MTT viability test as described later in this thesis.

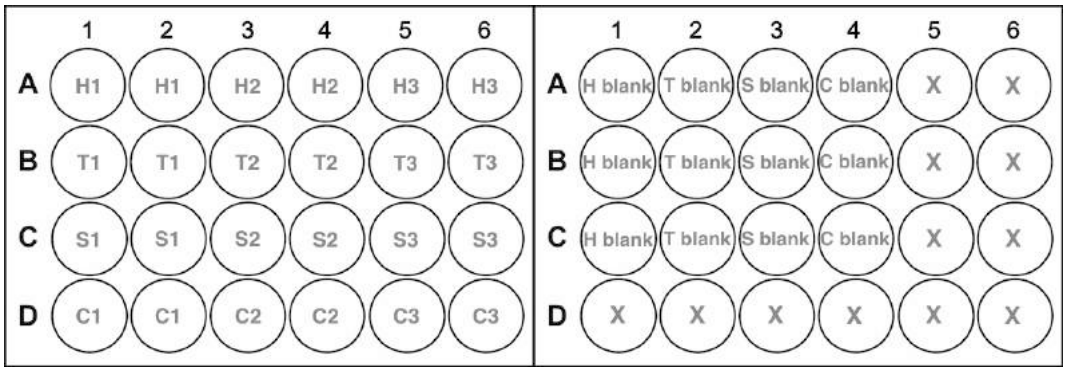

Figure 5: Layout for MSCs, seeded on 24-well plates for MTT analysis. H1, H2, H3 – biological replicates, grown in Hektor medium, T1, T2, T3 – biological replicates, grown in TurboDoma medium, S1, S2, S3 – biological replicates, grown in SCM-1 medium, C1, C2, C3 – biological replicates for control medium, H, T, S, C – wells with blanks for each investigated medium, X – unused wells.

Slika 5: Razporeditev MMC, nacepljenih na gojilni posodici s 24 vdolbinicam za kasnejši viabilnostni test MTT. H1, H2, H3 – biološke ponovitve za gojišče Hektor, T1, T2, T3 – biološke ponovitve za gojišče TurboDoma, S1, S2, S3 – biološke ponovitve za gojišče SCM-1, C1, C2, C3 – biološke ponovitve za kontrolno gojišče, H, T, S, C – luknjice s slepo probo za vsakega izmed 4 preiskovanih gojišč, X – neuporabljene luknjice.

3.3.4 Cell counting and viability check

During each centrifugation, we assembled haemocytometer. When the centrifuge had stopped, we took 20 µL aliquot from our resuspended cell suspension. We made sure the cell suspension was evenly distributed before the aliquot was taken. 20 µL of cells suspension was mixed with 20 µL of trypan blue staining solution. The stained cell suspension was then transferred to haemocytometer (10 µL/side). We counted cells in 4 large squares on both sides. Viable (light) and dead (dark) cells were both counted to determine cell viability. Once our cells were counted, we calculated cell number, cell concentration, viability, population doubling level and the required amount of medium, we needed to dilute our cell suspension, if our cell concentration was too high for next passage.

3.3.5 Calculation of cell number, population doubling times, population doublings and Population Doubling Level

As noted beforehand we needed to calculate the cell number (Equation 1) for each culture flask of each cell culture to determine the volume in which we would dilute the cells to obtain the correct seeding concentration for the next passage.

$$Cell\ number = \frac{counted\ cells}{\#large\ squares} \times volume\ factor \times dilution \times volume \quad \ldots(1)$$

Equation cell number in cell suspension. Volume factor – 10000, dilution – 2, volume (of total cell suspension).

From equation for cell growth (Equation 2) we can calculate population-doubling time (Equation 3), which is defined as the time (usually in hours) it takes for a certain quantity of cells to double in numbers.

$$N = N_0 \times 2^{\frac{t}{t_D}} \quad \ldots(2)$$

$$t_D = \frac{t}{\log_2\left(\frac{N}{N_0}\right)} \quad \ldots(3)$$

Equation for cell growth. N – cell number after cultivation time t, N_0 – starting cell number, t – cultivation time, t_D – population doubling time.

We also calculated population-doubling level (PDL), which is the standardized way to communicate the age of primary cells and is one of the most important parameters of clinical cell culture. It gives us the information on how many times the cells divided since isolation (Equation 4).

$$PDL = 3.32 \times (logN - \log N_0) + S \quad \ldots(4)$$

Equation for population-doubling level. PDL – population doubling level, N – cell number at the end of the incubation time, N_0 – cell number at the beginning of the incubation time, S – starting PDL.

PDL is very useful parameter in MSC culture, because of the following:

- MSC growth varies significantly between donors and preparations, therefore we should use more precise tools to measure it.
- PDL values of MSCs have a direct correlation to genomic instability (Pan et al., 2014).
- PDL number of MSCs directly correlates with replicative senescence, which is linked to the loss of potency (Carlos Sepúlveda et al., 2014).
- If PDL would be set as a standard in the research of MSCs it would enable better comparison and analysis of different studies.

3.3.6 MTT viability test

The MTT assay is a photometric method which enables the determination of metabolic activity and thereby the viability of cells. Within the mitochondria, the water-soluble yellow colourant MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to violet coloured Formazan by nicotinamide adenine dinucleotide hydride (NADH)(Figure 6). The amount of Formazan crystal formation reflects the metabolic activity of the cells and therefore the viability. For photometric detection of the water insoluble product Formazan we have to lyse the cells and dissolve the crystals with SDS, diluted with HCl.

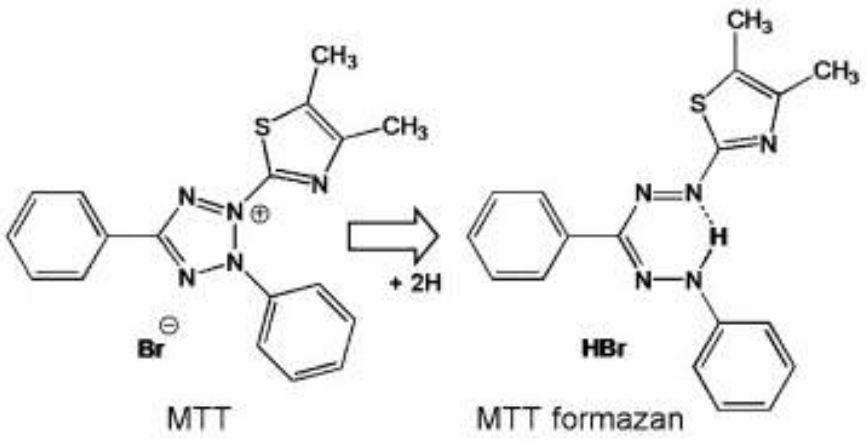

Figure 6: Chemical structure of MTT and its reduced formazan product (Stockert et al., 2012)

Slika 6: Kemijska struktura molekule MTT in njegove reducirane oblike formazan (Stockert in sod., 2012)

We used the procedure, designed by Denizot and Lang (1986). Firstly we prewarmed PBS and basal medium in water bath at 37 °C. We removed the medium from the cells (using pump and Pasteur pipettes) and then added 500 µL of PBS per each well. 10 % MTT working solution was prepared by adding 60 µL of MTT stock solution to 540 µL of basal medium per each well. After the MTT working solution was made, we removed PBS from cells (using suction device and Pasteur pipettes). 600 µL of MTT working solution was added to each well, then 24-well plate was incubated for 4 h at 37 °C and 5 % CO_2. After 4 h we added 90 µL of SDS solution per well. Cells were then incubated for the subsequent 24 h, again at 37 °C and 5 % CO_2. When 24 h elapsed, we transferred coloured solution from each well to a 96-well plate (from each well on 24-well plate to 4 wells on 96-well plate; V=100 µL). Absorbance of the coloured solution was determined with a spectrophotometer at 570 nm (measurement) and at 630 nm (reference) to exclude media effects. We also measured a probe with all reagents but without cells as blank for further calculations.

3.3.7 Senescence β-Galactosidase Cell Staining Assay

Senescence β-Galactosidase Staining kit is designed to detect β-galactosidase activity at pH 6, a known characteristic of senescent cells not found in presenescent, quiescent, or immortal cells (Dimri et al., 1995). Limited capacity to replicate is a defining characteristic of most normal cells and culminates in senescence, an arrested state in which the cells remain viable (Goldstein, 1990). Senescent cells are not stimulated to divide by serum or passage in culture, and senescence invokes a specific cell cycle profile that differs from most damage-induced arrest processes or contact inhibition (Sherwood et al., 1988). Senescent cells are further characterized by an enlarged cell size, expression of pH-dependent β-galactosidase activity (Dimri et al., 1995) and an altered pattern of gene expression (Cristofalo et al., 1998; Linskens et al., 1995).

We followed the protocol, designed by Debacq-Chainiaux et al. (2009). We performed Senescence β-Galactosidase Cell Staining on cells that were cultivated in control medium (α -MEM + 10 % HS). We tested the cells from passages 4, 24 and 26. We wanted to make sure that cells would not reach senescent state before we would finish our serum reduction. As the whole purpose of serum reduction in MSCs is their later use, either in academic research, regenerative medicine, etc., it is necessary that we acquire presenescent cells at the end of our experiment. As we have to split the cells many times to reach complete serum deletion (18 passages in slow reduction, 10 passages in fast reduction, 10-15 passages in adjusted reduction), it is reasonable to check if the cells are still presenescent. If the cells already became senescent during the serum reduction process, then we would have to look for another approach to serum reduction or exclusion in culture media.

Before we started with our procedure, we prepared at least 6 ml of 1x PBS per 35 mm well. Staining Solution was firstly re-dissolved by heating to 37 °C with agitation, then diluted with distilled water from 10X concentrated stock Staining Solution to 1x working Staining Solution. We needed 930 µL of the 1x staining solution per 35 mm well. We diluted 10x Fixative solution to 1x solution with distilled water (1 m of the 1x solution per well). 20 mg of X-gal was dissolved in 1 mL of DMF to prepare a 20x stock solution.

After we had prepared all the ingredients, we could combine them in a polypropylene container to make β-Galactosidase Staining Solution. For each 35 mm well we needed to mix 930 µL of 1x Staining Solution, 10 µL of Staining Supplement A, 10 µL of Staining Supplement B and 50 µL of 20 mg/ml X-gal in DMF. Due to variations in water pH we had to make sure that the solution had a final pH of 5.9-6.1, because the cells only stain at that pH value. Once we prepared all our reagents, we removed the growth media from the cells and rinsed the plates once with 1x PBS (2 mL for each 35 mm well). 1 mL of 1x Fixative solution was added to each well and the cells were allowed to fix for 10-15 min at room temperature. We then rinsed the plate twice with 1x PBS (2 mL for each 35 mm well) and added 1 ml of the β-galactosidase Staining Solution to each well. Subsequently the plate was incubated at 37 °C overnight in a dry incubator (without CO_2). After 24 h and while the β-galactosidase Staining Solution was still on the plate we checked the cells under a microscope (200x total magnification) for the development of blue colour.

4 RESULTS

In this chapter we will present all the results that have been obtained during our experimental work. We have designed 3 separate experiments. Firstly we started off with 2 experiments simultaneously. In the first one (slow reduction) we performed 2 cell splits per week and 1 serum reduction step, while in the other (fast reduction) serum reduction step was carried out during each cell split (2 serum reductions/week). Since we had problems with uneven cell growth, due to serum reduction process, we designed another experiment (adjusted reduction), in which we only split the cells when the confluence of our cell population was high enough (preferably over 70 %). During each experiment we monitored cell morphology, viability and cell growth parameters and performed MTT viability. On our control cell population we also performed senescence test to determine how many passages we can achieve with our uc-MSCs, before they become senescent. All the results can be found in the ensuing subchapters.

4.1 ASSESSMENT OF CELLULAR MORPHOLOGY

Before each cell splitting we checked our cells under the microscope to assess any possible changes in morphology, estimate the confluence and take photographs for later comparison of different passages and between different culture media.

4.1.1 Cells in control medium (α-MEM, supplemented with 10 % HS)

We did not observe any drastic changes in cellular morphology in cells that were kept as a control in α-MEM with 10 % HS until very late passages (beyond passage 20), when they displayed more flattened, characteristic fibroblast-like morphology (Figure 7). After the 20^{th} passage, cells grew in size dramatically and it took them much longer to reach confluence.

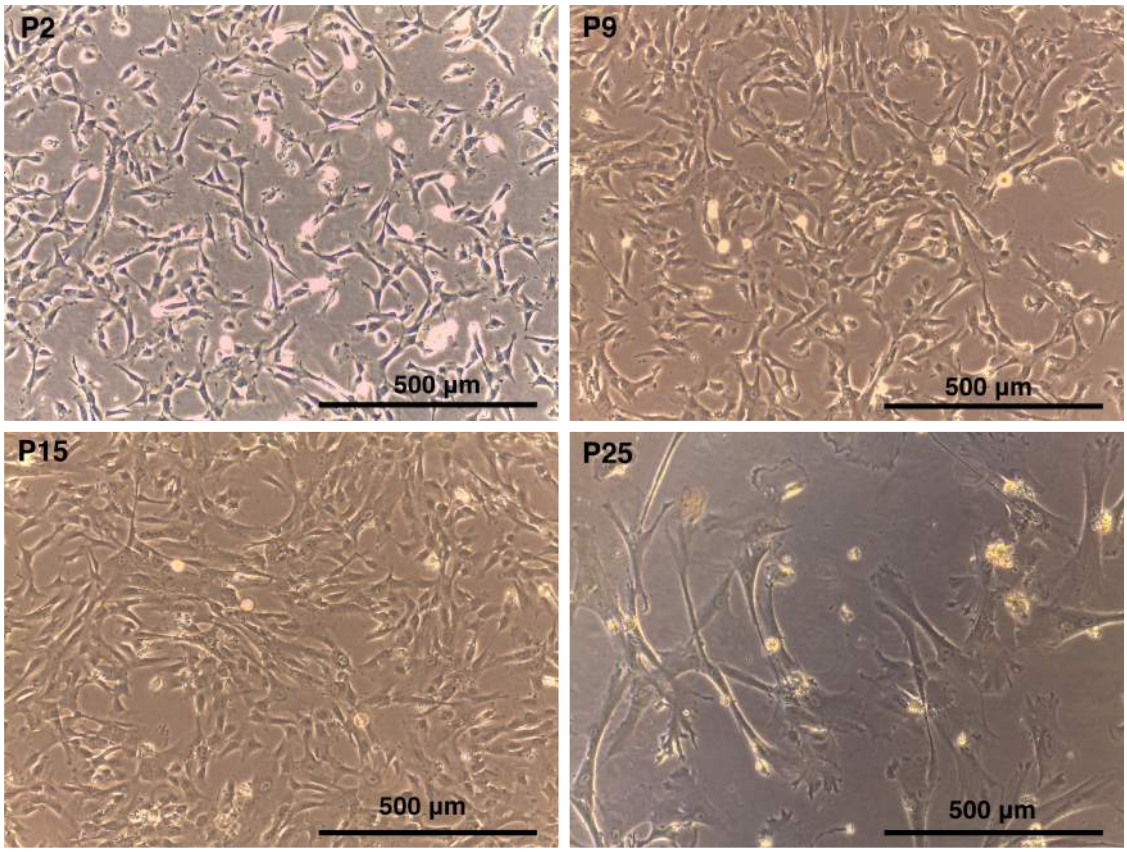

Figure 7: Microscopic observation of the morphology of MSCs in control culture medium α-MEM with 10 % HS at passages 2, 9, 15 and 25. Apart from passage 25, cells display fibroblast-like morphology with no aging-induced morphological changes.

Slika 7: Mikroskopsko opazovanje morfologije MMC v kontrolnem gojišču α-MEM z 10 % človeškega seruma (HS) v pasažah 2, 9, 15 in 25. Celice so z izjemo pasaže 25 izkazovale fibroblastom podobno morfologijo brez morfoloških sprememb, povezanih s celičnim staranjem.

4.1.2 Cells, cultured in chemically-defined culture medium SCM-1

In higher serum concentrations, MSCs, which were cultivated in SCM-1 culture medium, demonstrated similar cell morphology than the cells that were kept in control conditions. When the cells were moved to serum concentrations, lower than 2 %, they became bigger in size and also started to change shape (Figure 8). When we lowered serum concentration, MSCs also started to grow in distinct bundles of cells, compared to cells in control medium, which grew in more-flattened, uniform monolayer. Cells in SCM-1 medium were also more difficult to detach, when splitting in lower serum concentrations. When cells were cultivated in conditions with less than 2 % of HS, it was impossible to keep our schedule that planned 2 passages per week. In the end we even had to resort to cultivate cells in 6-well plate, because we could not harvest enough cells for T-25 flasks. At 0.3 % HS step we did not harvest even enough cells for 6-well plates.

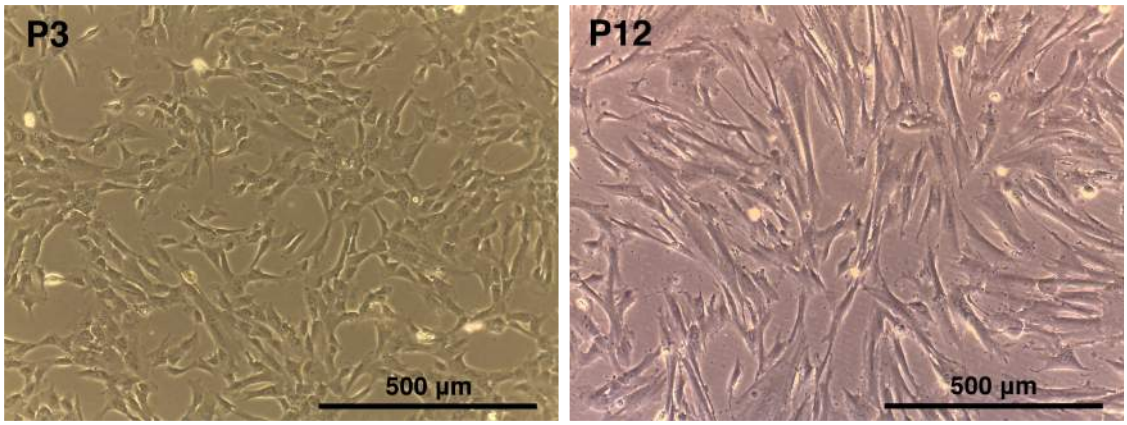

Figure 8: Microscopic observation of the morphology of MSCs in SCM-1 culture medium at passage 3 (10 % HS) and 12 (0.5 %). In early stages (at serum content >2 % HS) cells displayed normal cellular morphology, comparable to cells in control culture medium, but at serum concentration of 1% and below, cells' appearance changed drastically.

Slika 8: Mikroskopsko opazovanje morfologije MMC v gojišču SCM-1 v 3. (10 % HS) in 12. pasaži (0,5 % HS). V zgodnjih korakih zmanjševanja serumske koncentracije (pri koncentracijah nad 2 % HS) smo pri celicah opažali normalno celično morfologijo, primerljivo kontrolnim celicam. Ko pa smo delež seruma znižali pod 1 %, smo zaznali drastične spremembe v izgledu celic.

4.1.3 Serum reduction in TurboDoma medium

In passage 3, when cells were firstly cultivated in TurboDoma and supplemented with 10 % HS, they still displayed normal cellular morphology of hMSCs. They also looked similar to cells, cultivated in control culture medium and SCM-1 and Hektor. When we checked the cells under the microscope, before we transferred them to fresh medium with 2 % HS (P6 to P7), they have already grown in size and displayed unidentified secretions, which were monitored on top of cell bundles (Figure 9). We did not manage to cultivate cells in lower serum concentrations than 3 %.

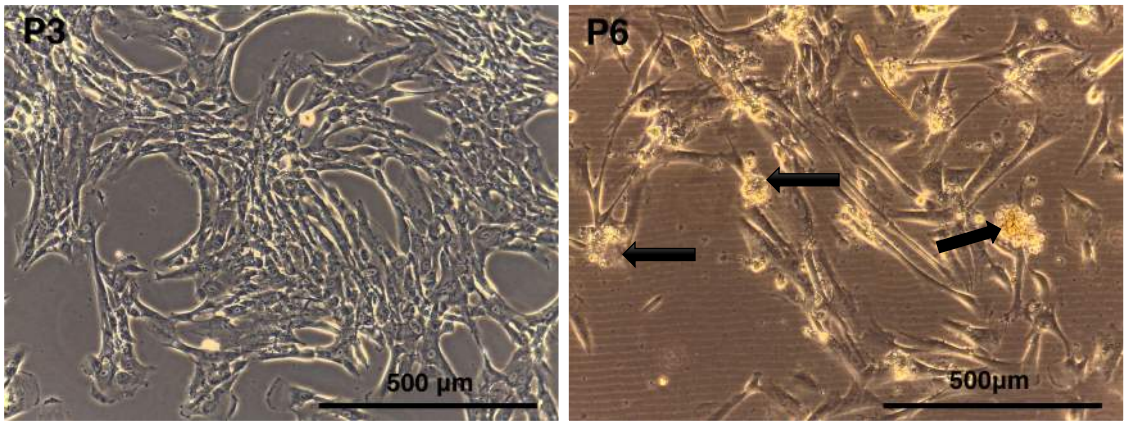

Figure 9: Microscopic observation of the morphology of MSCs in TurboDoma culture medium at passage 3 (10 % HS) and 6 (3 % HS). In the first step of serum reduction process, when cells were cultivated in TurboDoma culture medium, supplemented with 10 % HS, cells displayed normal cellular morphology, comparable to cells in control culture medium, but at passage 6 (3 % of HS) cells displayed a much larger size and extreme change in shape. At passage 6 it is also possible to see unidentified secreted compounds (black arrows).

Slika 9: Mikroskopsko opazovanje morfologije MMC v gojišču TurboDoma v 3. (10 % HS) in 6. pasaži (3 % HS). Ko smo gojišču dodajali 10 % HS so celice v gojišču TurboDoma še izkazovale normalno celično morfologijo, podobno kontrolnim celicam. Pri 3 % HS pa so bile celice izrazito večje od kontrolnih celic, zaznali pa smo tudi drastično spremembo oblike. V 6. pasaži so s črnimi puščicami označene tudi neidentificirane izločene spojine.

4.1.4 Cells, cultured in Hektor culture medium

Cells that were cultured in Hektor culture medium reacted similarly to serum reduction than cells in TurboDoma medium. They grew normally, when supplemented with 10 % of HS, but started to show changes in size and shape when moved to fresh medium with lower serum concentrations (Figure 10). Cells performed a bit better than those, cultivated in TurboDoma medium, but still could not be cultivated in conditions, with less than 3 % of HS. Also noticed was that when serum concentration was lowered, cells started to grow in bundles, in contrast to cells, grown in control medium, which grew in more-uniform monolayer.

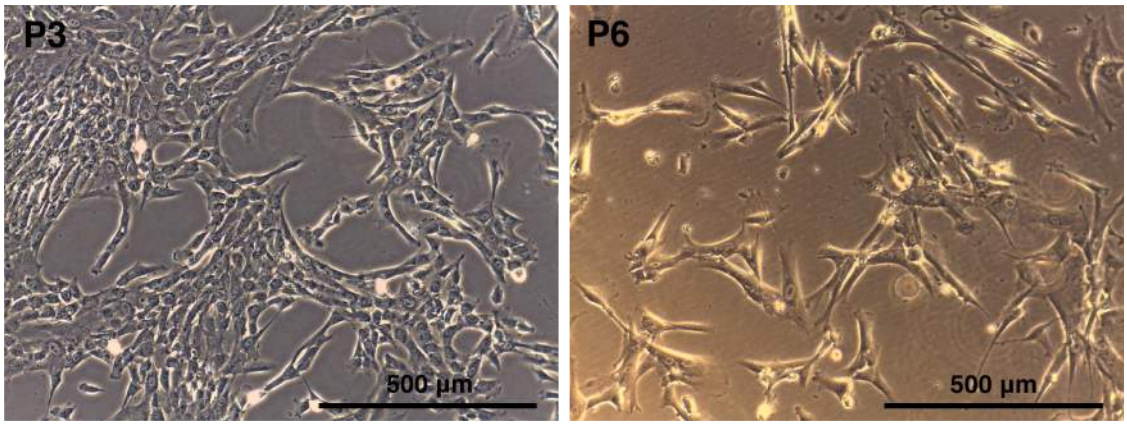

Figure 10: Comparison of cellular morphology of MSCs, cultivated in Hektor culture medium at passage 3 (10 % HS) and 6 (3 % HS). As seen from microscopic photographs, cells change in size and shape when moved to conditions with lower serum concentration.

Slika 10: Mikroskopsko opazovanje celične morfologije MMC, gojenih v gojišču Hektor v 3. (10 % HS) in 6. (3 % HS) pasaži. Kot je dobro razvidno iz mikroskopskih fotografij, so se celice po prenosu v gojišče z nižjo vsebnostjo seruma povečale in spremenile obliko.

4.1.5 Cellular morphology of cells, cultivated in different chemically defined media, supplemented with 3 % of HS

A more clear comparison of cellular morphology of MSCs, cultivated in different chemically defined media and control culture medium can be seen in Figure 11. Cells in chemically defined media are displayed at passages with 3 % of HS, while cells in culture medium were kept at 10 % of HS. Here we can see, that cells in SCM-1 medium still display normal cellular morphology, similar to those kept in control culture medium, while cells in TurboDoma and Hektor already show extensive changes in shape and size. Throughout the whole culture surface cells in TurboDoma and Hektor culture media grew in bundles, with big empty areas, where no cells were present. We also saw distinctive contrast intracellular structures in cells that were cultivated in Hektor and TurboDoma media. Some cells seemed to have 2 nuclei.

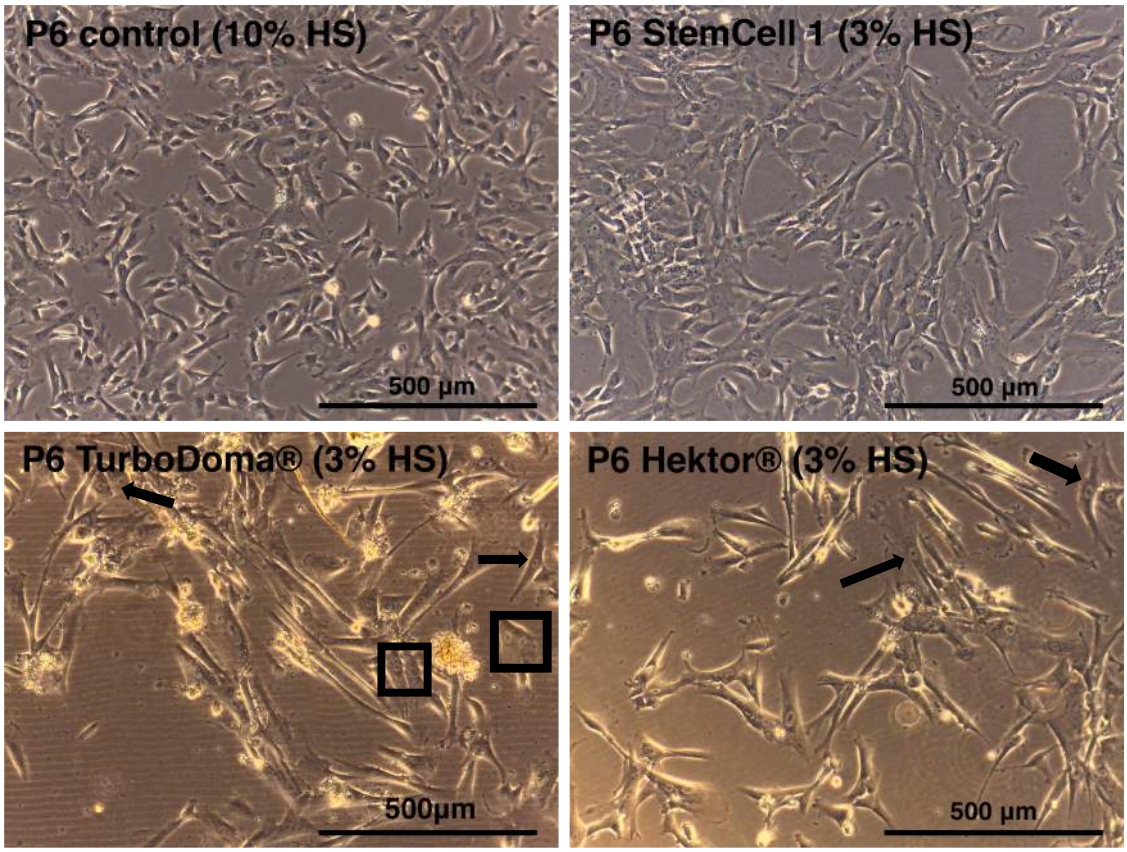

Figure 11: Comparison of cellular morphology of MSC at passage 6. Cells in chemically defined media (SCM-1, TurboDoma and Hektor) were supplemented with 3 % HS, while cells in control medium were cultured with 10 % HS. While cells, cultured in SCM-1 medium, still display similar morphology to cells, kept in control medium, cells that were cultivated in TurboDoma and Hektor media already show distinct changes in shape and size. It is also possible to see distinctive contrast intracellular structures (black arrows) and some cells, cultured in TurboDoma seem to divide their nuclei, but do not divide in whole.

Slika 11: Primerjava celične morfologije MMC v 6 pasaži. Pri tej pasaži smo celicam v kemijsko definiranih gojiščih (SCM-1, TurboDoma in Hektor) dodajali 3 % HS, medtem ko smo kontrolnim celicam še vedno dodajali 10 % HS. Medtem ko smo pri tej koncentraciji pri celicah v gojišču SCM-1 še vedno zaznali morfologijo, podobno kontrolnim celicam, pa so celice v gojiščih TurboDoma in Hektor že izkazovale izrazite spremebe v velikosti in obliki. Pri teh celicah lahko opazimo tudi izrazite kontrastne intracelularne strukture (označene s črnimi puščicami), medtem ko smo pri določenih celicah v gojišču TurboDoma opazili celice, ki so podvojile svoja jedra, a niso zaključile celične delitve.

4.2 GROWTH CURVE ANALYSES

During each cell splitting we calculated the number of cells and population doubling levels (PDLs) for each passage. We used PDL values of each passage to draw charts that show cell doublings through the whole length of cell cultivation. Figures 12, 13 and 14 display population-doubling levels for slow, fast and adjusted reduction, respectively. As we can see from all 3 charts, the only chemically defined medium that supported cell growth for longer than 20 days was SCM-1 culture medium. As seen from both slow and fast reduction approaches, transfer to TurboDoma and Hektor culture media had a negative

effect on MSCs growth, even before we started to significantly decrease serum concentration. Population doubling levels dropped as early as at 5 % HS. Cells, cultivated in SCM-1 medium showed positive growth till passage 8 (2 % HS) and then slowed down. As it was already mentioned before, we stopped passaging cells in SCM-1 medium at 0.5 % HS, because we could not harvest enough cells for the next split. If we compare all 3 approaches on the basis of population doubling levels, we can see that PDL values were lowest in fast reduction approach (Figure 13), while slow reduction approach and adjusted approach performed similarly (Figures 15). In adjusted approach growth curve for hMSCs, grown in control culture medium (α-MEM + 10 % HS) looks more uniform, without any unusual reduction of growth from day 0 to 60. In slow reduction experiment cells, grown in StemCell 1, displayed higher PDL values. PDL values for cell, which were grown in TurboDoma and Hektor culture media are missing, because we could not harvest enough cells to split the cells in passages with reduced serum content.

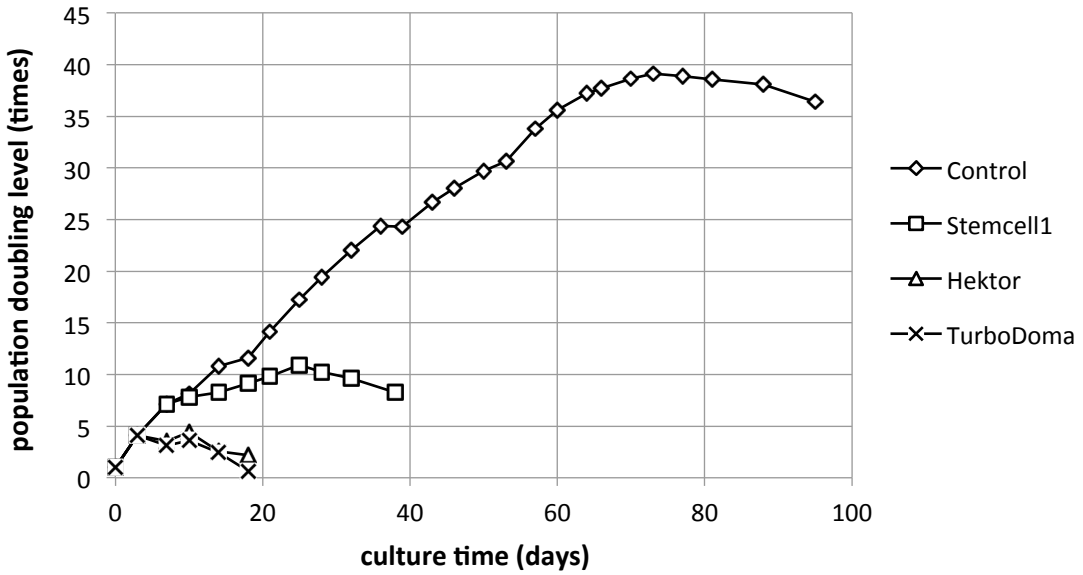

Figure 12: Population doubling levels for slow reduction cells, grown in control culture medium (α-MEM + 10 % HS), SCM-1, Hektor and TurboDoma.

Slika 12: Populacijski podvojevalni nivoji za celice tekom počasnega zniževanja serumske koncentracije v gojiščih SCM-1, Hektor in TurboDoma v primerjavi s kontrolnim gojiščem (α-MEM + 10 % HS).

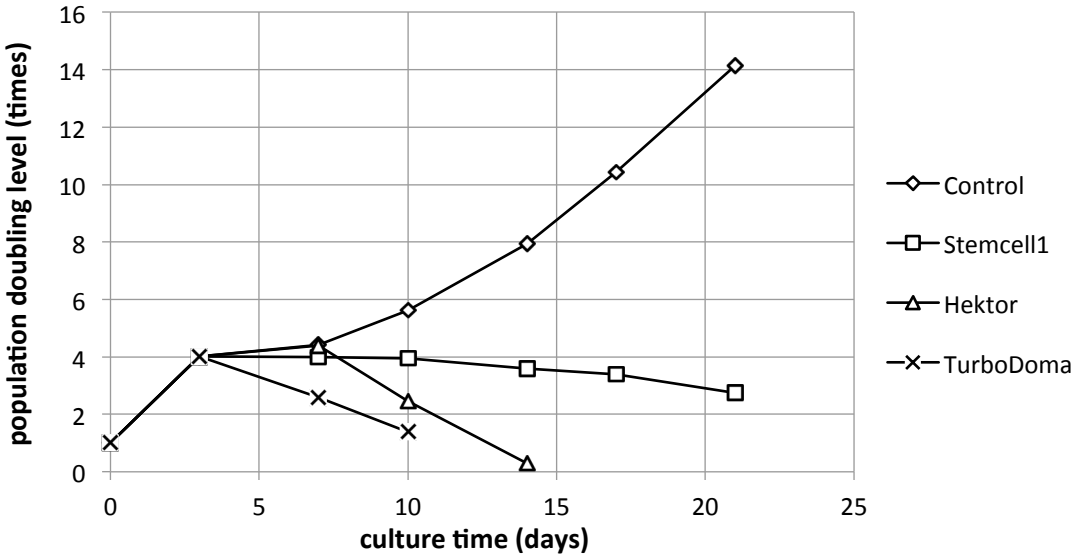

Figure 13: Population doubling levels for fast reduction cells, grown in control culture medium (α-MEM + 10 % HS), SCM-1, Hektor and TurboDoma.

Slika 13: Populacijski podvojevalni nivoji za celice tekom hitrega zniževanja serumske koncentracije v gojiščih SCM-1, Hektor in TurboDoma v primerjavi s kontrolnim gojiščem (α-MEM + 10 % HS).

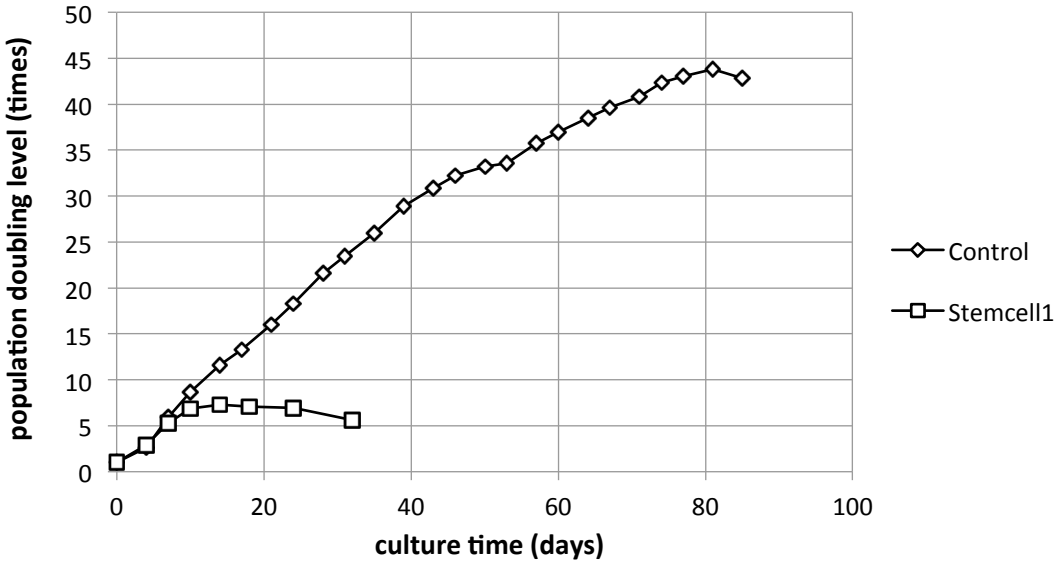

Figure 14: Population doubling levels for adjusted reduction cells, grown in control culture medium (α-MEM + 10 % HS) and SCM-1.

Slika 14: Populacijski podvojevalni nivoji za celice tekom prilagojenega zniževanja serumske koncentracije v gojišču SCM-1 v primerjavi s kontrolnim gojiščem (α-MEM + 10 % HS).

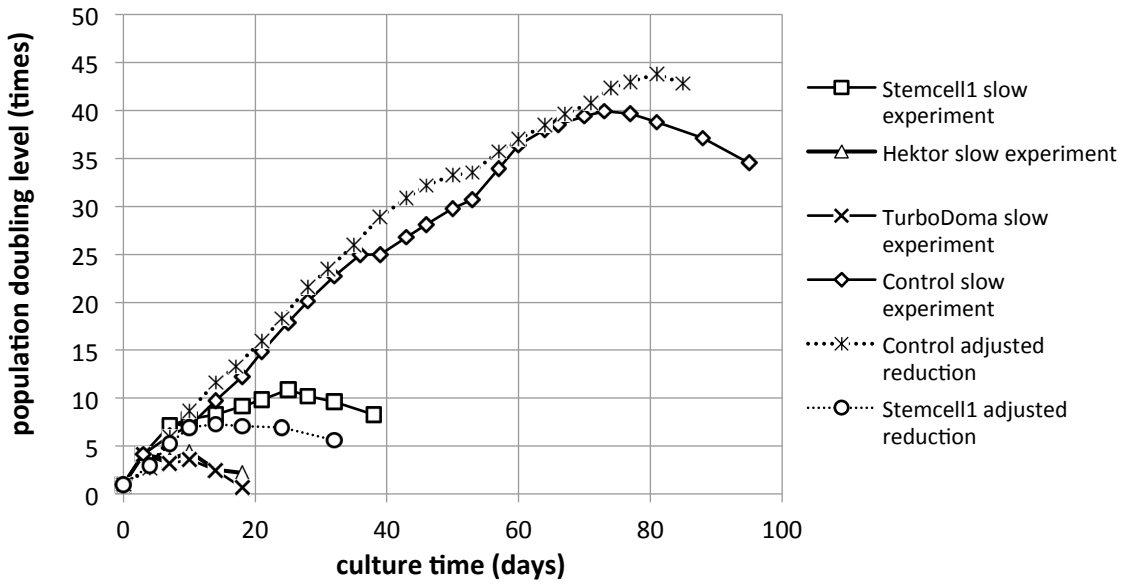

Figure 15: Comparison of PDL values between slow and adjusted reduction cells, grown in control (α-MEM + 10 % HS), SCM-1, Hektor and TurboDoma culture media.

Slika 15: Populacijski podvojevalni nivoji za celice tekom počasnega in prilagojenega zniževanja serumske koncentracije v gojiščih SCM-1, Hektor in TurboDoma v primerjavi s kontrolnim gojiščem (α-MEM + 10 % HS).

4.3 TRYPAN BLUE VIABILITY ASSAY

As it was already stated before, trypan blue viability assays were performed during regular cell counting under the microscope. Viability was determined by counting live (light) and dead (blue) cells under haemocytometer. We have calculated viability for each passage of our cell cultivation in any of our investigated 3 chemically defined media and in control medium (α-MEM + 10 % HS). After we gathered percentage of viable cells for each passage, we could calculate the average viability of cells, cultivated in each medium through all the passages, from the beginning of cultivation until the cells stopped duplicating and started dying out. Average viabilities for hMSCs during slow, fast and adjusted serum reduction experiments are presented separately and for each chemically defined medium and compared to average viability of cells, kept in control medium with constant serum supplementation (10 % HS) (Figure 16, 17 and 18).

4.3.1 Viability in slow reduction experiment

In figure 16 we can see that cells, cultivated in control conditions (α-MEM + 10 % HS) displayed highest viability through their long-term cultivation (26 passages). Cells, grown in SCM-1 culture medium during serum reduction, displayed average viability (in 12 passages) that was closest to that of control cells (80.6 % ± 8.05). Cells that were cultivated in Hektor (and TurboDoma during serum depletion displayed lower average viability (69.7 % ± 12.9 and 65.5 % ± 18.5, respectively) and varied more significantly from passage to passage (from 50 to 90 % for Hektor and from 35 to 90 % for TurboDoma). Cells were cultivated in Hektor and TurboDoma for 6 passages, before they died out.

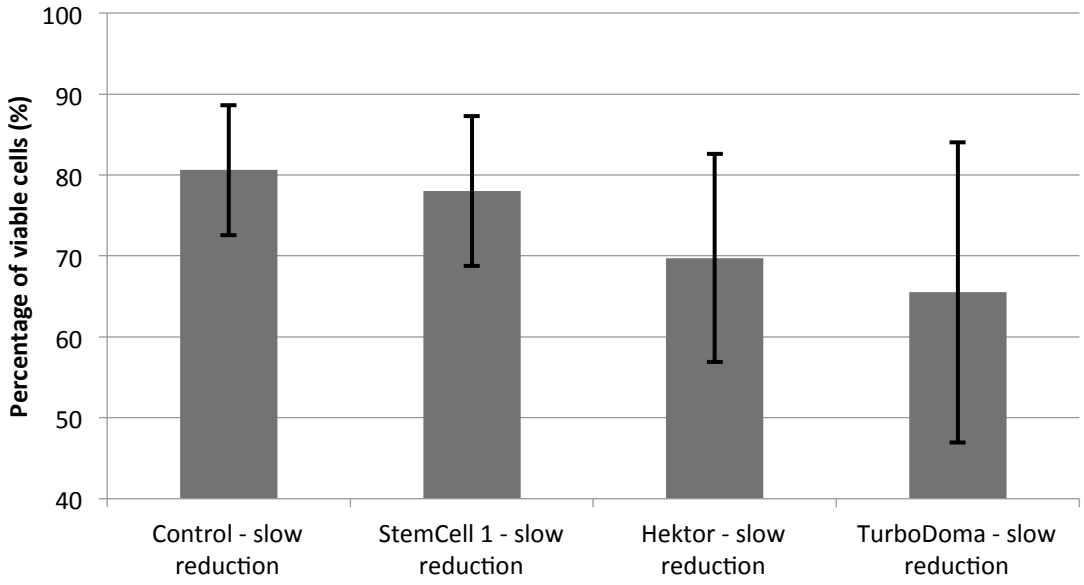

Figure 16: Average viability of hMSCs, cultivated in chemically defined media, during slow serum reduction experiment compared to an average viability of cells, kept in control medium (α-MEM) with 10 % HS. Values are represented as mean ± SD.

Slika 16: Povprečna viabilnost človeških MMC, gojenih v kemijsko definiranih gojiščih, tekom počasnega zniževanja serumske koncentracije v primerjavi s povprečno viabilnostjo celic, gojenih v kontrolnem gojišču (α-MEM) z dodatkom 10 % HS. Vrednosti so izražene kot povprečje s standardno deviacijo.

4.3.2 Viability in fast reduction experiment

In fast reduction experiment control cells displayed lower average viability (74.5 % ± 9.41) than those cultivated in SCM-1 medium during serum depletion (77.4 % ± 6.35) (Figure 17). Both cultures were processed for 7 passages. As in slow depletion approach, hMSCs that were grown in Hektor and TurboDoma during fast serum reduction showed lower viability than both control cells and cells in SCM-1 (Hektor – 67.9 % ± 10.9; TurboDoma – 69.3 % ± 10.9). We also experienced bigger variation in viability between separate

passages when serum reduction was performed in Hektor (5 passages) or TurboDoma (4 passages) culture media.

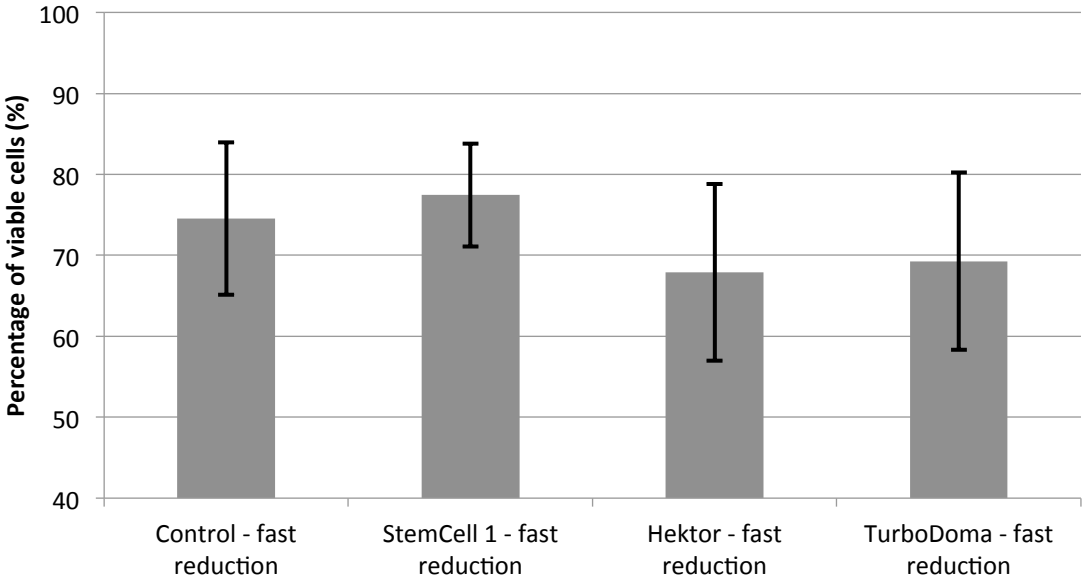

Figure 17: Average viability of hMSCs, cultivated in chemically defined media, during fast serum reduction experiment compared to an average viability of cells, kept in control medium (α-MEM) with 10 % HS. Values are represented as mean ± SD.

Slika 17: Povprečna viabilnost človeških MMC, gojenih v kemijsko definiranih gojiščih, tekom hitrega zniževanja serumske koncentracije v primerjavi s povprečno viabilnostjo celic, gojenih v kontrolnem gojišču (α-MEM) z dodatkom 10 % HS. Vrednosti so izražene kot povprečje s standardno deviacijo.

4.3.3 Viability in adjusted reduction experiment

We could only perform serum depletion in SCM-1 medium for adjusted reduction experiment, because we could not harvest enough cells for reduction in Hektor and TurboDoma culture media. As in slow reduction experiment, average viability of control cells was a few % higher than that of cells, cultivated in SCM-1 (80.1 % ± 11.3; 77.19 % ± 9.58, respectively) (Figure 18). Control cells were cultivated for 25 passages, while cells in SCM-1 medium were grown for 8 passages.

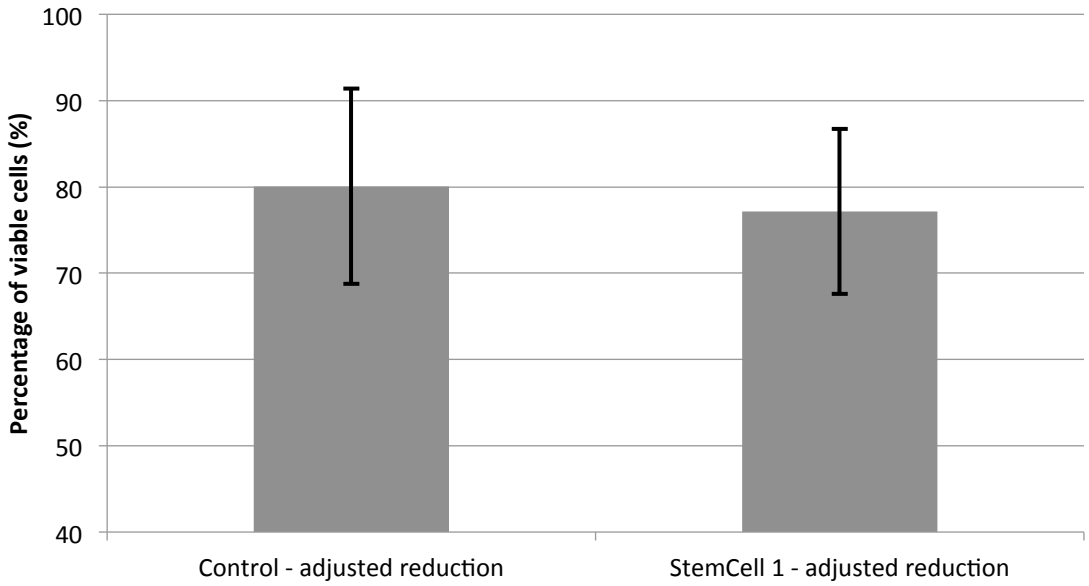

Figure 18: Average viability of hMSCs, cultivated in SCM-1 medium, during adjusted serum reduction experiment compared to an average viability of cells, kept in control medium (α-MEM) with 10 % HS. Values are represented as mean ± SD.

Slika 18: Povprečna viabilnost človeških MMC, gojenih v kemijsko definiranem gojišču, tekom prilagojenega zniževanja serumske koncentracije v primerjavi s povprečno viabilnostjo celic, gojenih v kontrolnem gojišču (α-MEM) z dodatkom 10 % HS. Vrednosti so izražene kot povprečje s standardno deviacijo.

4.4 MTT VIABILITY TEST

We performed MTT viability every second cell split to further evaluate the viability of cells during serum reduction process through their metabolic activity. Cells were seeded on 24-well plate at 3500 cells/cm^2. As we can see from figure 5, we had 2 technical replicates per each biological replicate (3 for each investigated chemically defined medium + 3 for control culture medium). In each of the 3 experiments we have performed, we experienced a drastic drop in proliferation rates, when serum concentration was lowered (Figures 19, 20 and 21).

4.4.1 MTT cell proliferation rates in slow reduction experiments

Compared to average proliferation rate of cells, grown in control medium (0.0592 ± 0.0280; over 26 passages), cells, which were cultivated in chemically defined media and supplemented with 10 % HS, displayed higher proliferation rates (Hektor: 0,2097 ± 0.0323; TurboDoma: 0.1110 ± 0.0144; SCM-1: 0.1296 ± 0.0039) (Figure 19). Supplemented with 5 % of HS, cells in Hektor culture medium still displayed higher proliferation rate (0.0787 ± 0.0057), proliferation rate of cells in SCM-1 medium was comparable to average rate of control cells (0.0543 ± 0.0055), while proliferation rate of

cells in TurboDoma medium already declined drastically to 0.0317 ± 0.0080. When chemically defined media were supplemented with 3 % of HS, cells in SCM-1 medium were the only ones that still displayed proliferation rates (0.0517 ± 0,017), comparable to average proliferation rate of control cells. MTT values of cells in Hektor medium were already a lot lower (0.0254), whereas metabolic activity of cells in TurboDoma medium barely registered. When we lowered serum concentration to 2 % of HS, even proliferation rates of cells in SCM-1 dropped. We managed to grow cells in SCM-1 medium, supplemented with as little as 0.5 % HS, but displayed proliferation rates dropped to just a fraction of control average.

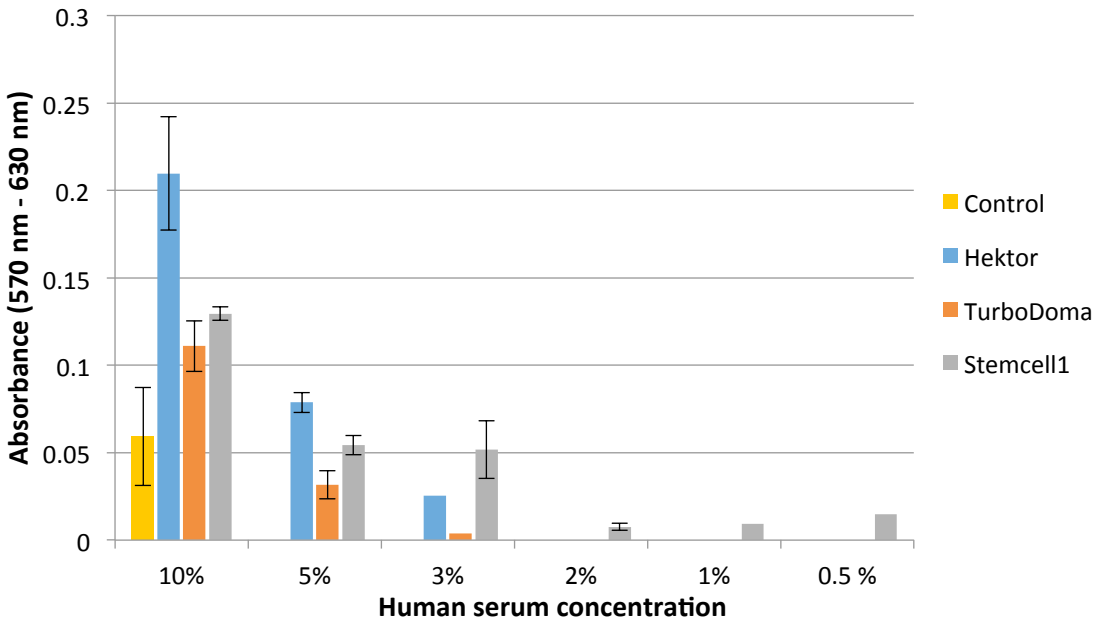

Figure 19: MTT proliferation rates in slow reduction steps for cells, cultivated in control, Hektor, TurboDoma and SCM-1 media. Proliferation rate for control medium presents an average proliferation rate over 26 passages.

Slika 19: MTT proliferacijski nivoji celic, gojenih v gojiščih Hektor, TurboDoma in SCM-1, tekom počasnega zniževanja serumske koncentracije. Proliferacijski nivo celic, gojenih v kontrolnem gojišču, je predstavljen kot povprečje 26. pasaž.

4.4.2 MTT cell proliferation rates in fast reduction experiment

In fast reduction experiment, serum concentration was reduced twice per week. Because we only performed 1 MTT viability test per week, we obtained proliferation rates for only every second reduction step (10 %, 3 %, 1 %, 0.3 %) (Figure 20). As it was already explained for the slow reduction experiment, proliferation rate for control medium is an average calculated from values, obtained during 4 weeks (8 passages) in culture. As seen in slow reduction approach, proliferation rates at 10 % HS for cells, cultivated in Hektor (0.2097 ± 0.0323), TurboDoma (0.1317 ± 0.0041) and SCM-1 (0.0991 ± 0.0232) were

higher than the average proliferation rate of cells, grown in control medium (0.0805 ± 0.0078). When media were supplemented with 5 % HS, cells in Hektor (0.0483 ± 0.0033), SCM-1 (0.0342 ± 0.0029) and TurboDoma (0.0354) all demonstrated lower metabolic activity than cells, grown in control medium. As seen in figure 20, at 1 % HS supplementation we could only obtain proliferation rate for cells in SCM-1 medium. We could not harvest enough cells that were grown in Hektor or TurboDoma media to reach such low serum supplementation.

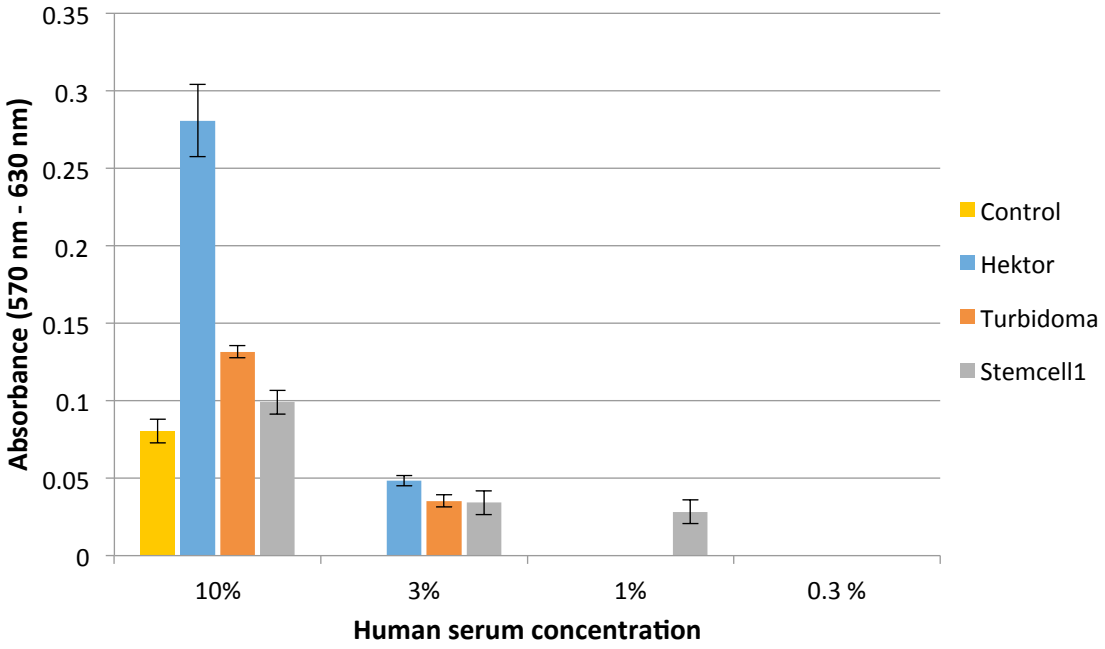

Figure 20: MTT proliferation rates in fast reduction steps for cells, cultivated in control, Hektor, TurboDoma and SCM-1 media. Proliferation rate for control medium presents an average proliferation rate over 8 passages.

Slika 20: MTT proliferacijski nivoji celic, gojenih v gojiščih Hektor, TurboDoma in SCM-1, tekom hitrega zniževanja serumske koncentracije. Proliferacijski nivo celic, gojenih v kontrolnem gojišču, je predstavljen kot povprečje 8. pasaž.

4.4.3 MTT cell proliferation rates in adjusted reduction experiment

In adjusted reduction experiment we could only compare MTT proliferation rates for cells, cultivated in control and SCM-1 medium, since cells in TurboDoma and Hektor culture media reacted really badly to serum reduction. We could not even seed them at 5 % HS supplementation. As for the cells that were grown in SCM-1 medium, they displayed higher proliferation rate at 10 % of HS (0.1116 ± 0.0164) compared to an average proliferation rate of cells in control medium through 16 passages (0.0724 ± 0.0105) (Figure 21). When cells, cultivated in SCM-1 were supplemented with 3 % HS, their metabolic activity was almost equal to the average proliferation rate of control (0.0652 ± 0.019).

Cells, grown in SCM-1 with 1 % HS and 0.5 % HS, had proliferation rates of 0.0418 ± 0.015 and 0.0208 ± 0.006, respectively.

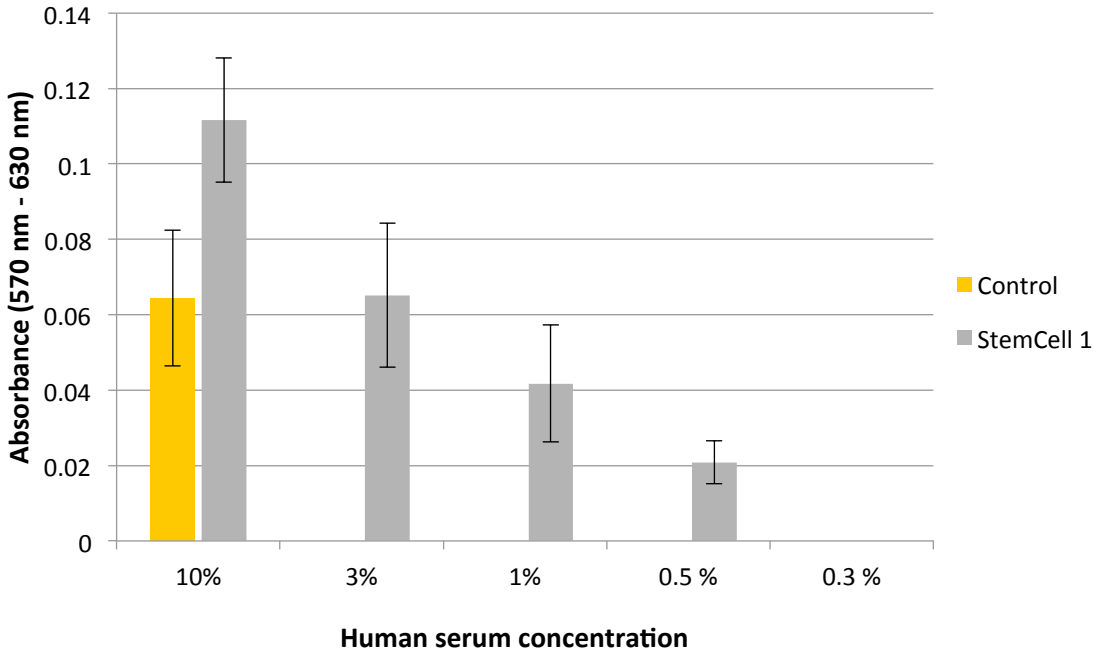

Figure 21: MTT proliferation rates in adjusted reduction steps for cells, cultivated in control and SCM-1 media. Proliferation rate for control medium presents an average proliferation rate over 16 passages.

Slika 21: MTT proliferacijski nivoji celic, gojenih v gojišču SCM-1, tekom počasnega zniževanja serumske koncentracije. Proliferacijski nivo celic, gojenih v kontrolnem gojišču, je predstavljen kot povprečje 16. pasaž.

4.5 SENESCENCE β-GALACTOSIDASE ASSAY

As we have already mentioned beforehand, senescence β-galactosidase assays were performed to determine if our hMSCs are still in presenescent state after the completion of our serum-reduction processes or do they become senescent even in passages during our serum reduction approaches. We used hMSC (code ucMSC130613) in α-MEM culture medium with 10 % HS and without antibiotics (control medium in serum depletion experiments). The assay was performed on cells from passages 4, 24 and 26, which already displayed notable differences in cell size, morphology and growth (Figure 22).

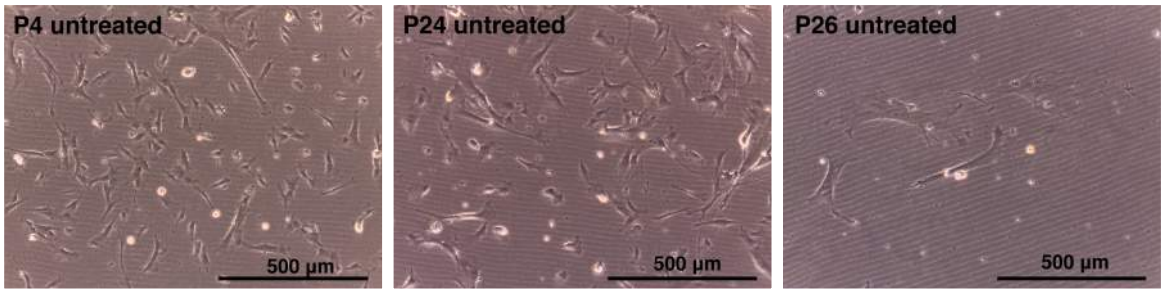

Figure 22: hMSCs, cultivated in α-MEM with 10 % HS from passages 4, 23 and 26 before β-Galactosidase assay was performed.

Slika 22: Človeške MMC, gojene v gojišču α-MEM z 10 % HS v 4., 23. in 26. pasaži pred β-galaktozidaznim testom.

We performed senescence staining with 2 Senescence β-Galactosidase Staining Kits #9860 (from Cell Signaling Technology) with different reference dates (03/2013 and 09/2014). Staining was performed according to kit's protocol, which has been described in the Methods section of this thesis. We tested old and new kit on separate plates. After we had added β-Galactosidase staining solution, we incubated them overnight (37°C, no CO2) and then observed the development of blue colour.

By mistake the pH value of β-galactosidase staining solution was not adjusted. In kit's protocol it is cited that the pH value should be adjusted to 5.9 to 6.1, but our solutions had pH values of 6.6 (old kit) and 7.0 (new kit) respectively. Non-adjustment of pH showed in the results we obtained (Figure 23).

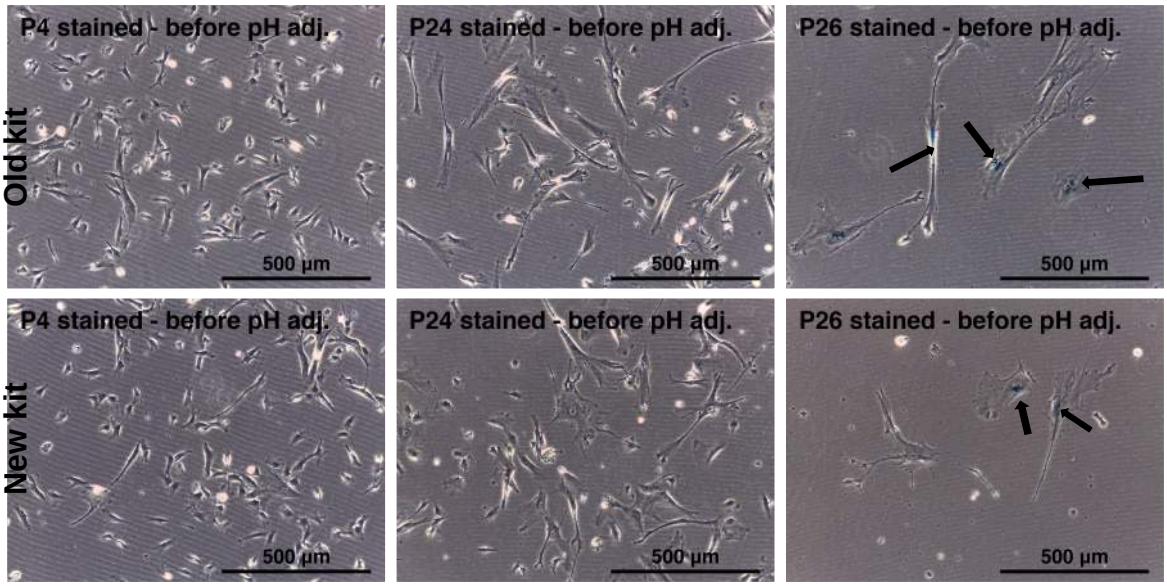

Figure 23: hMSCs stained with β-Galactosidase staining solution without the pH adjustment. Black arrows point to clear β-Galactosidase staining (blue) in cytoplasm around nuclei in P26 cells, which confirm senescence. Cells in the first row were stained with the old kit, while the ones in second row were stained with the new kit.

Slika 23: Človeške MMC, obarvane z β-galaktozidaznim testom, pred uravnanjem pH vrednosti. Črne puščice označujejo jasno obarvane predele citoplazme okrog celičnih jeder v celicah 26. pasaže, kar potrjuje pojav senescence. Celice v prvi vrsti so bile obarvane s starim testom, celice v drugi pa z novim.

Positive β-Galactosidase staining was observed only in P26 cells in old and new kit. Not all P26 cells showed staining. With the old kit majority of P26 cells stained positive (~70%), while only a few (~10%) P26 cells stained positive with the new kit (Figure 24).

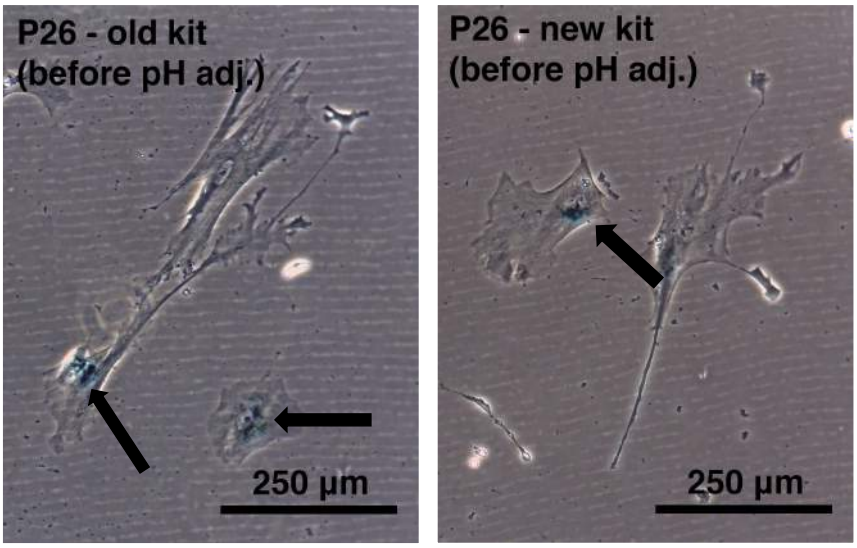

Figure 24: hMSCs stained with β-Galactosidase staining solution without the pH adjustment. Black arrows point to clear β-Galactosidase staining (blue) in cytoplasm around nuclei in P26 cells, which confirm senescence.

Slika 24: Človeške MMC, obarvane z β-galaktozidaznim testom po prilagoditvi pH vrednosti. Črne puščice označujejo jasno obarvanje citoplazme okrog celičnih jeder v celicah 26. pasaže, kar potrjuje pojav senescence v teh celicah.

When we realised that pH values were not adjusted, we further stained the identical plates with the same staining solution, but the pH values were adjusted to ~pH 6.0 by addition of 200 µL of 10 mM HCl in ddH_2O per well. As a result we obtained good staining in both old and new kit (Figure 26). P26 cells were stained in great numbers, while lighter staining was observed in P24 cells. Only a few of P4 cells were faintly stained.

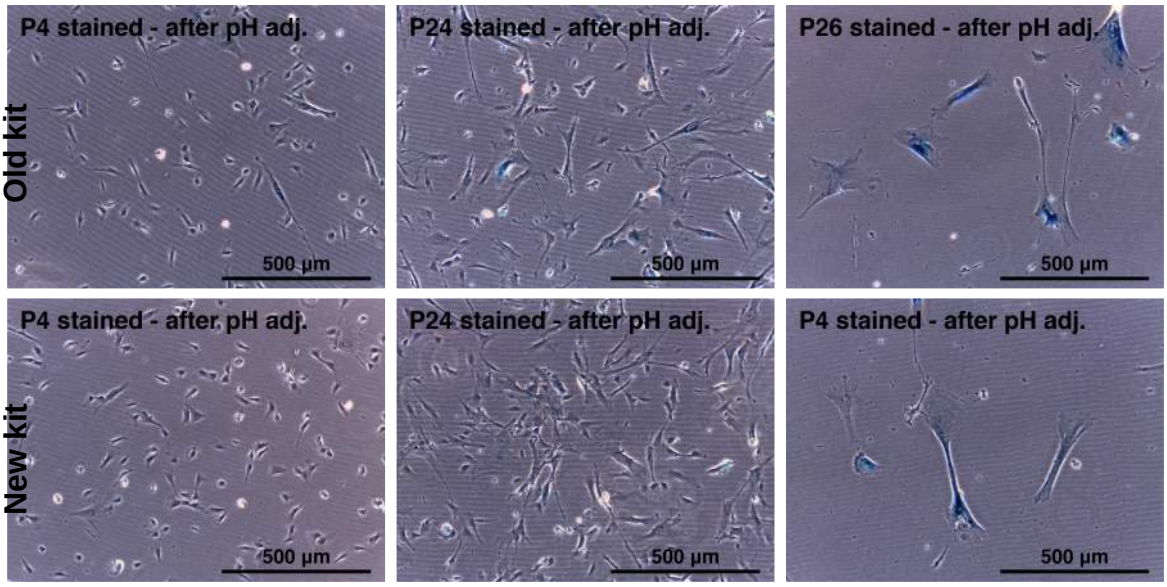

Figure 25: hMSCs stained with β-Galactosidase staining solution after the pH adjustment to 6.0.

Slika 25: Človeške MMC, obarvane z β-galaktozidaznim testom, po uravnanju pH vrednosti na 6,0.

5 DISCUSSION

Human mesenchymal stem cells (hMSCs) are surfacing as an important therapeutic tool in cell therapy. For any effective treatment that utilizes MSCs, we need a large amount of cells, which we can nowadays easily propagate in *in vitro* culture in basically any properly equipped laboratory. On the other hand the stability, regenerative effect and safety of such cells remain debatable. Therefore to further advance the role of stem cells in regenerative medicine and cell-based therapeutics it is absolutely necessary to develop new or refine the existing expansion protocols. It is also recommended to remove all undefined substrates and reagents from our cell culture, which could introduce variability in experimental results and affect the uniformity, safety and efficacy of the designed product.

The goal of our experiment was to investigate the possibility of weaning hMSCs from serum-supplemented medium to 3 different serum-free media (SCM-1, Hektor and TurboDoma) and to determine the effects of serum depletion on cellular morphology, size, viability, metabolic activity and metabolic activity. Even though we have been unable to completely remove HS from culture media, we have also obtained many interesting data, that is discussed in the following subchapters.

5.1 SERUM DEPLETION APPROACHES

We designed 3 separate serum depletion experiments. We started with slow and fast reductions that were based on gradual reduction approach. Gradual reduction approach is one of the proposed adaption protocols to aid the process of weaning (van der Valk et al., 2010). Slow reduction experiment was designed in a way that we passaged hMSCs twice each week, while serum concentration was only lowered once per week. In fast reduction experiment serum concentration was lowered twice per week (at every passage). In slow reduction, hMSCs were kept at a certain serum concentration for additional passage (2 passages per each serum reduction step), so the cells had more time to adapt to lower serum conditions. As giving the cells another passage to adapt to the adjusted conditions accumulates in longer weaning process (18 passages), hMSCs would be in a very late passage, when we could finally use them for our academic research or therapeutic purposes. Even though hMSCs from young donors can undergo between 24 to 40 population doublings (PD), which in our case correlates to passages 22-26, before their growth ceases and they become senescent (Kassem, 2006), it is advisable to expand cells in as few passages as possible. Cells from lower passages are less likely to contain genetic abnormalities and have a higher proliferative capacity. Wang et al. (2013) reports that hUC-MSCs, cultivated in long-term culture develop genomic alterations, so it is best to strive towards serum depletion protocols that do not require very long-term cell culture. That is why we also performed fast reduction experiment, which takes 10 passages before we reach 0 % of serum.

Both approaches had their positive and negative aspects. As we have already mentioned the negative aspect of slow reduction is its long procedure, during which the cells could undergo genomic and epigenetic changes, or the cells could become senescent. On the other hand, the cells did have more time to adapt to each serum reduction step, which

increased their chances of survival. The positive side of fast reduction was that as noted before we only needed 10 passages to completely remove serum from our cell culture, but as we have observed ourselves, cells did not have enough time to adapt to stressful conditions of lower serum supplementation. In accordance with our hypothesis, the added adaptation passages in slow reduction approach contributed to cells, cultivated in chemically defined media, to survive longer during serum depletion process. Those hMSCs, grown in TurboDoma and Hektor media still divided at 5 % of HS, while the cells in SCM-1 culture medium grew at 1 to 2 % of HS. In fast reduction approach negative aspects seemed to outbalance the positive ones, since the cells ceased growth almost immediately after being transferred from control medium with 10 % HS to chemically defined media.

After we had to cease the fast experiment because of dwindling cell numbers, we designed a third adjusted approach. We decided that we would not keep to a strict schedule like in the slow and fast experiments where cells were split twice a week, no matter the confluence they reached. That gave us more manoeuvrability. We could prolong the incubation time, if the cell growth was not fast enough to reach the required confluence or shorten it if the cell population doubling time was shorter than expected. Because of our experience with first 2 experiments, we could also manage seeding concentrations better and avoid over-confluent cultures, where we would get large clumps of cells, which would be impossible to properly detach and resuspend for next passage. With this approach we would also avoid inaccurate cell numbers and low viability that would arise as a consequence of filtering big clumps of cells away from our cell suspension.

We detected many practical problems that arose as a consequence of serum-depletion procedure. The biggest by far was the difficult detachment of cells during cell passaging, when serum concentrations reached lower values. While hMSCs detached normally in culture with high percentage of HS, they stuck firmly to the surface when serum concentration was lowered below 5 % for cells in Hektor and TurboDoma culture media and 2 % for cells in SCM-1. On the suggestion of chemically defined media manufacturer we used Sigma's non-enzymatic cell dissociation solution, which was supposed to be less aggressive than trypsin. From our experience we can conclude that Sigma's non-enzymatic solution did not perform as well as planned. As we have lowered serum concentration in our cell cultures, the incubation time for detachment got longer (more than 10 min). Even with longer incubation time, a large number of cells seemed to remain attached to the surface. That meant that we obtained lower harvesting numbers, which in later passages made it even more difficult to collect enough cells for the next reduction step. We can also conclude that fixed passaging schedule in fast and slow reduction was too rigid and it actually contributed to stressful conditions. Because it was very hard to determine the appropriate seeding concentration, our cell cultures were sometimes too confluent, which resulted in cell clumps and difficult detachment, or growth was too slow and our cultures were less than 40 % confluent, which might have meant that cells were still in their lag phase. As cell cultures should be in their logarithmic phase and demonstrate viability over 90 %, passaging cells in lag phase could have a cascading effect on the following serum depletion steps.

As we have already mentioned, we have solved some issues, arising during slow and fast reduction, with the design of the third adjusted reduction approach. But even though we

had solved the problem of over-confluent culture flasks and had given the cells more time to reach logarithmic phase, our chemically defined media still could not support cell growth without any serum supplementation. For cells, grown in Hektor and TurboDoma we had to stop the experiment at 3 % HS supplementation, while cells, cultivated in SCM-1 medium, survived at lower serum supplementation (0.5 %). We have to point out that at lowest possible serum supplementation that still supported cell growth, hMSCs demonstrated many signs of stress, which resulted in change of cellular morphology, size, growth rates and metabolic activity.

5.2 CELLULAR MORPHOLOGY

Serum reduction procedures had a drastic effect on cellular morphology of hMSCs in chemically defined media. While hMSCs still displayed a similar morphology to those, grown in control medium with 10 % HS, while cultivated with higher serum supplementation (5 % HS for Hektor and TurboDoma; 2 % for SCM-1), the changes became more apparent when we lowered serum concentration to 3 % for cells that were grown in Hektor and TurboDoma media, and to 1 % for cells, which were cultivated in SCM-1 medium (Figures 7, 8 and 9). In our microscopic observations we have witnessed a significant increase in cell size that correlated with gradual serum reduction procedures. We also spotted a change in shape. While at 10 and 5 % of serum supplementation, cells, cultivated in chemically defined media, still demonstrated fibroblast-like morphology, they became more elongated at lower serum concentrations and we could also spot distinctive contrast intracellular structures (Figure 10). Cells also started to grow in bundles and we could spot large areas without any cell growth in our culture flasks. At lower serum concentrations we witnessed unidentified secreted compounds that floated on top of hMSCs. The two most realistic speculations are that these were excessive matrix depositions, which occurred as a response to cell stress, or that this was debris from dying cells, trapped in matrix that was produced by the cells. Because the debris was trapped in matrix, it could not float freely but stuck with the cells. In TurboDoma cell culture with low serum supplementation we also spotted cells that undergone nuclear division but did not go through with cell division. This could be a result of missing compounds from serum, which the composition of chemically defined medium does not replace.

Changes in morphology can be attributed to the reduction of serum concentration in chemically defined culture media. We do not know the exact mechanisms that cause the changes in morphology, so further investigation into metabolic and genetic changes, caused by serum reduction, should be performed to gain more insight into the matter.

5.3 CELL GROWTH PARAMETERS

During each cell splitting we also performed cell counting and viability assessments with trypan blue staining. Each passage we calculated the number of cells, their doubling times and population doublings (PD). When the experiments were stopped we also calculated population-doubling levels (PDL). We needed the data on number of cells to determine the

right dilution volumes to set the right seeding concentrations and we needed PDL values to determine the number of doublings our cells undertook before reaching senescence.

We obtained the best results in slow and adjusted reductions, while fast reduction did not exhibit good proliferation of cells, cultivated in chemically defined media. It is important to point out that even in slow and adjusted reduction, hMSC cultures in chemically defined media, which had undergone the serum depletion process, did not perform even close to those, kept in control medium (with 10 % HS). From PDL values, calculated from cell counting, we see that control cells displayed a uniform growth, which only declined at certain passages, where we had to filter large clumps of cells away from our cell suspension. As a consequence of filtering we harvested just a small fraction of cells that actually grew in that passage. In reality growth in control cell culture only ceased at later passages (after passage 20) when cells reached senescence. The senescence of control cells at long-term cultivation was later also confirmed with senescence β-galactosidase assay.

With PDL values we obtained a very valuable data that confirmed effects of serum depletion procedures on cell doubling. Human MSCs that were cultivated in Hektor and TurboDoma hardly even divided, after they were transferred from control medium to chemically defined media with the same serum concentration. While we can say that low harvest numbers in first passage after the transfer from control medium was due to overconfluent cultures that lead to us using the filters to remove huge clumps of cells, low harvested cell number in the consequent passages can be attributed to lower serum content and composition of chemically defined media. As we have already discussed it in cellular morphology evaluation, serum depletion seemed to result in cells transferring their energy to growth in size rather than cell division.

From all 3 chemically defined media, SCM-1 seemed to be the only 1 that supported cell doublings even at lower serum concentration. In slow reduction experiment, cells in SCM-1 medium undergone 8.5 doublings before they started to die out (at passage P8 and at serum concentration of 2%), in fast reduction they barely divided after we started to reduce serum concentration, while in adjusted reduction cells performed slightly less doublings (7.6), but also divided at lower serum concentration (1 %).

Calculated viabilities of cells from all 3 experiments correlated with all other obtained data. All cells that went through serum depletion in chemically defined media displayed lower viability, which additionally dropped when cells were transferred to passages with lower serum supplementation. Cells in chemically defined media were also more susceptible to any stress that could occur during cell handling, which resulted in bigger variations through different passages. This was expected, as we had come across many articles that pointed out this drawback (Jayme and Gruber, 2006; van der Valk et al., 2010; Jung et al., 2012).

5.4 MTT VIABILITY ASSAYS

We performed MTT viability assays to obtain valuable data on metabolic activity and thereby viability of cells. MTT proliferation rates complemented the PDL values that we have obtained during cell counting with trypan blue staining. MTT viability assay is also

perfect for measuring reduction in cell viability, when metabolic events lead to apoptosis or necrosis.

Surprisingly in all 3 reduction experiments cells, grown in chemically defined media (TurboDoma, Hektor and SCM-1) with 10 % HS displayed higher proliferation rates, compared to those, cultivated in control medium. This could be explained with high nutritional values of chemically defined media, especially of Hektor and TurboDoma, which are normally used for the propagation of several kidney-derived mammalian cell lines and for the production of monoclonal antibodies with hybridomas. Nutrient strength of SCM-1 is also supposed to be comparable to that of Hektor culture medium. It seems that high nutritional value of chemically defined media and diverse composition of serum result in significant shortage of doubling time at higher serum concentration.

Nevertheless when we lowered serum concentration proliferation rates of cells in all 3 chemically defined media and in all 3 reduction experiments started to drop. Proliferation rates seemed to drop the fastest for the cells that were cultivated in TurboDoma. Even at cultivation, supplemented with 5 % of HS, their proliferation rate was half of the average rate of control cells'. At 5 % HS cells in Hektor culture medium displayed higher proliferation rate compared to average proliferation rate of control cells, but only in slow reduction experiment. The only hMSCs, that showed comparable proliferation rate to the average of control cells at 3 % HS supplementation, were those grown in SCM-1 medium. At 1 % serum concentration even the latter displayed very low proliferation rates in slow and fast reduction experiment, while in adjusted approach the proliferation rate of SCM-1 cells equaled to about two thirds of the average proliferation rate of control cells.

We can conclude that none of our 3 chemically defined media supports serum-free cultivation of hMSCs that were isolated in serum-supplemented media and undergone gradual serum reduction.

5.5 SENESCENCE β-GALACTOSIDASE ASSAY

With senescence β-galactosidase assay we wanted to determine if hMSCs from our experiments could theoretically still be useful for academic research or therapeutic purposes after the performed weaning. We performed staining on hMSCs from passages 4, 24 and 26, which were cultured in control medium with 10 % HS. Cells demonstrated differences in cellular morphology and with senescence staining we could determine the correlation of changes in morphology with the occurrence of senescence. We used two kits with different reference dates (old and new) to achieve the staining. After the first overnight incubation we achieved barely any staining and only in P26 cells. That was later attributed to our mistake, because we had missed to adjust the pH, which is essential to β-galactosidase staining. Once the pH was adjusted, we stained the same cells with the same staining solution and this time acquired strong staining in P26 cells, mild staining in P24 cells and light staining in some P4 cells. From this we can conclude that hMSCs, which have undergone serum depletion through our reduction procedures, could technically still be used for research or therapeutic purposes, but with limited capacity. In all 3 serum reduction experiments, cells had to go through more than 10 passages, before they would

get to serum-free conditions and that would only give us approximately 10 more passages before hMSCs would become senescent.

5.6 NEXT STEPS

As none of our 3 chemically defined media supported serum-free cultivation without any additional supplementation and with gradual serum reduction we have to determine next steps. We could further optimize the best of our 3 investigated media SCM-1 (Stemcell Cell Medium 1), try out the direct adaptation, which could result in smoother adaptation to serum-free conditions, determine the essential supplements (eg. bFGF) or find other better defined supplements for HS. We could also perform additional analysis to get a deeper insight into the effects of serum reduction on cell metabolic activity, changes in cellular morphology, cell growth and genomic changes.

6 CONCLUSIONS

- None of our 3 chemically defined media could support hMSC cultivation in conditions without HS supplementation.

- During all 3 serum depletion experiments we monitored cellular morphology, viability, cell growth parameters, senescence and metabolic activity. All the monitored parameters pointed towards the fact that serum depletion process puts hMSCs under great stress. As a result of stress, cells changed their morphology from small spindle-shaped to bigger fibroblast like cells, their viability dropped considerably with the reduction of serum concentration, growth also slowed down and eventually stopped (at 3 % HS for Hektor and TurboDoma and at 0.5 % HS for SCM-1) and metabolic activity dropped at lower HS supplementation.

- SCM-1 chemically defined culture medium performed best of the 3 chemically defined media. But even though we managed to cultivate cells in SCM-1 medium with as little as 0.5 % HS, changes in cellular size and shape, lower proliferation rates, lower population doubling levels and higher mortality showed that SCM-1 chemically defined medium did not alone provide enough constituents that would support cell cultivation without the presence of HS. The same can be said for the other two chemically defined media.

- A lot of practical problems arose, because of serum depletion processes. We had to be very careful when we were operating with hMSCs, since any mechanical stress on cells would results in a drop of viability.

- According to the senescence β-galactosidase assays the investigated hMSCs only became senescent around passage P26, so technically if we would manage to gradually reduce serum concentration in our chemically defined media to 0 %, the cells could still be used for academic or clinical purposes.

- Because we would eventually like to completely eliminate serum from medium formulations and since there is a possibility that serum-derived contaminants are carried over with the cells when they are transferred to serum-free conditions (Jung et al., 2012), the next step should be a direct transfer to serum-free medium after the cells have been isolated (in our case from umbilical cord blood).

- All 3 investigated media have to undergo additional optimization before they could support serum-free cultivation of hMSCs.

7 SUMMARY (POVZETEK)

7.1 SUMMARY

Human mesenchymal stem cells (hMSCs), also referred to as mesenchymal stromal cells, have been evaluated for different clinical purposes, because of their multipotentiality, secretion of bioactive factors, tropism for sites of disease, immunomodulation and immune privileged or hypoimmunogenic properties. Because the number of hMSCs in is extremely low compared to other nucleated cells in all tissues where they are found, it is crucial that we expand them in *in vitro* conditions to obtain sufficient numbers for later experimental or clinical use. In normal practice hMSCs are propagated in culture media that ordinarily consist of basal medium (e.g. α-MEM, DMEM, Ham'S F12, RPMI 1640), supplemented with different percentage of human or animal-derived serum (5-15 % (v/v)). Even though serum as a supplement provides many advantages to the media, such as universality for cultivating many different types of cells, good protection against mechanical stress and buffering, there are also many disadvantages, caused by its presence. Because of its unidentified composition, high retail price, contamination risks and lot-to-lot variability it makes it impossible for us or anyone else to reproduce our experimental results or clinical efficacy of our hMSCs. That is why it is essential that we develop chemically defined media and approaches for serum-free cultivation that will enable us to increase the reproducibility in the field of stem cell research, eliminate or lower the variability in the clinical performance and further accelerate the progress of regenerative medicine.

In our thesis we have designed 3 different approaches for serum reduction in 3 chemically defined media for hMSC cultivation, called Hektor, TurboDoma and StemCell cell medium 1 (SCM-1). The effects of serum reduction process on cell performance, morphology, growth rate, viability, size and metabolic activity was compared to hMSCs, which were cultivated in control culture medium (α-MEM) with a constant human serum (HS) supplementation (10 %) Because our cells were already isolated in serum-supplemented media, we have decided to perform gradual reduction, which would give hMSCs more time to adapt to lower serum conditions and eventually grow in serum-free environment. We have performed slow, fast and adjusted reduction experiments. In slow reduction approach we have split the cells twice a week and lowered serum concentration every second passage, while in the fast reduction experiment serum concentration was lowered every cell split (i.e. twice per week). After the first 2 experiments, we have performed the third one that was supposed to combine the best characteristics of the first two. Here we performed serum reduction step during each passaging, but cells were only split, when they reached an appropriate confluence (preferably above 60%). That would lower the danger of diluting our cells, due to hurried cell splits, before the hMSCs reached logarithmic phase and enable us to finish the serum reduction in lesser number of passages than in slow reduction experiment. During each cell split we have monitored cellular morphology and size, calculated cell numbers, population doublings and evaluated viability, while every second passage we have also performed MTT viability assays that measure viability through cellular metabolic activity.

Through all the monitored parameters we have observed that the serum reduction procedures had negative effects on hMSCs, cultivated in all chemically defined media. The

most drastic changes in analysed parameters were seen in cells, grown in TurboDoma culture medium (under 5 % HS), followed by cells, grown in Hektor (under 5 % HS), while cells in SCM-1 medium seemed to be least effected of the 3. That is of course until we have lowered serum concentration under 2 %.

In cellular morphology the effects of the reduction culminated in transition from traditional spindle-shaped (or fusiform) and cuboidal fibroblast-like cells to much larger non-uniformed cells that varied greatly in shape and size. This transition was observed after the cells were transferred to media, supplemented with 3% HS (Hektor and TurboDoma) and 1 % HS (SCM-1), respectively. In cells, grown in TurboDoma medium with 3 % HS, we have also spotted some cells, that divided their nuclei, but did not divide into 2 cells.

We calculated population-doubling levels (PDL) from cell countings under haemocytometer that were performed before each cell split. This was done for later comparison of cell doublings, our cells performed during each passage and over the whole cultivation. At the same time we also performed trypan blue staining assay to determine viability. From PDL curves, we witnessed that hMSCs in TurboDoma and Hektor medium performed most of their doublings, before serum concentration was lowered (to 5 % HS). After we lowered serum concentration, they stayed viable, but basically just grew in size. Cells, cultivated in SCM-1 medium did perform doublings until they were supplemented with less than 2 % of HS, after which cell proliferation dropped and eventually ceased at 0.5 %. From 3 reduction approaches we got the best results in slow and adjusted reduction, while the fast reduction seemed to be too stressful and did not give hMSCs enough time to adapt to lower serum concentration. Cells that were cultivated in control medium (α-MEM + 10 % HS) were the only one that displayed a constant cell proliferation till later passages (after passage 20).

From trypan blue staining we obtained average viability of cells in different media over the whole length of their cultivation. Again hMSCs that were subject to serum reduction in chemically defined media, showed lower average viability compared to control cells. Over all 3 serum reduction experiments cells in Hektor and TurboDoma displayed lowest average viability (and over shortest cultivation time) with the highest rate of variability between passages. Average viability of cells from SCM-1 medium was only a bit smaller compared to those in control medium.

MTT viability assay results correlated to those, obtained with other analytical methods. While proliferation rates of hMCS, grown in chemically defined media, were even higher at 10 % HS supplementation compared to the average proliferation rate of those, grown in control medium, they gradually reduced over serum reduction. At 5 % serum supplementation, cells, cultivated in Hektor and TurboDoma still displayed similar or slightly lower proliferation rates, compared to the average of control cells. Cells, grown in SCM-1 performed even better. Their proliferation rate was still equal to average proliferation rate of control cells at 3 % supplementation.

We also performed senescence β-galactosidase staining assay to determine if the cells reach senescence before we complete all our reduction steps. We stained cells from passages 4, 24 and 26. Cells in passage 26 displayed strong blue staining, while cells in passage 24 showed moderate staining, Cells in passage 4 remained basically unstained. Results of our senescence assay showed that if we would manage to eliminate serum from

our chemically defined media with our gradual reduction approaches, we could still perform 6-14 passages at most before hMSCs would become senescent. The number of remaining passages depends on the serum reduction approach (6 for slow reduction, 14 for fast).

All the assays that we have performed during our serum reduction procedures lead us to the conclusion that none of our 3 chemically defined media can support serum-free cultivation of hMSCs, when the serum-supplemented medium is used in the isolation and cryopreservation procedures and gradual serum reduction is performed. High proliferation rates of cells in chemically defined media at 10 % HS can be linked to high nutritional value of chemically defined media, which are in case of Hektor and TurboDoma normally used for the cultivation of several kidney-derived mammalian cell lines and for the production of monoclonal antibodies with hybridomas. Nevertheless once we started to lower serum concentration, proliferation rates, population doubling levels, viability dropped and the morphology of cells changed drastically. The cause of these changes can be attributed to the combination of factors, which include stress during serum reduction process, unsuitable dissociation solution, hasty reduction process that does not allowed cells to adapt to lower serum concentrations, unsuitable reduction approach and usage of serum-supplemented media in isolation and cryopreservation.

In our thesis we have set many cues for further investigation of serum-free cultivation in chemically defined media. Even though none of the 3 researched media could support completely serum-free proliferation, SCM-1 still showed promise that if we use an appropriate serum reduction or exclusion approach, adjust the formulation of medium or add certain growth factors that are known to support stem cell proliferation when added to serum-free media (bFGF), it could actually support serum-free proliferation of hMSCs. We could also perform additional analyses, such as evaluation of surface antigen expression with flow cytometry, karyotyping and comparative genomic hybridization to gain more insight into what happens with hMSC during serum-reduction procedures. We were planning to perform some of these methods during our experimental work, but due to very labour intensive experiment design, we had decided to focus on metabolic activity, morphology, growth rate and viability assessment that would set the foundation for further research.

7.2 POVZETEK

Človeške mezenhimske matične celice (hMSC), poimenovane tudi mezenhimske stromalne celice, so predmet številnih kliničnih raziskav zaradi njihove multipotentnosti, izločanja biološko aktivnih dejavnikov, tropizma k območju bolezni, imunomodulacije in hipoimunogenih lastnosti. Ker hMSC v vseh tkivih, kjer jih najdemo, predstavljajo le zelo majhen delež mononuklearnih celic, jih moramo pred kakršno koli kasnejšo uporabo v raziskovalne ali klinične namene predhodno namnožiti v *in vitro* pogojih do primernih koncentracij. Običajno hMSC gojimo v gojiščih, ki temeljijo na bazalnih gojiščih (npr. α-MEM, DMEM, Ham'S F12 in RPMI 1640), ki jim dodajamo različne odstotke človeškega ali živalskega seruma (najpogosteje 5-15 % (v/v)). Čeprav nam serum kot dodatek zagotavlja številne ugodnosti, kot so univerzalnost gojišča za gojenje številnih različnih

tipov celic, dobra zaščita proti mehanskemu stresu in velika puferska kapaciteta, pa nam njegova prisotnost prinaša tudi številne slabosti. Zaradi neznane sestave, visoke cene, tveganja okužb in variabilnosti od serije do serije, nam prisotnost seruma v gojiščih onemogoča ponovljivost eksperimentalnih rezultatov ali konstantnost klinične učinkovitosti hMSC. Zatorej je nujno, da stremimo k razvoju kemijsko definiranih gojišč in pristopov za brezserumsko gojenje. Gojenje v gojiščih brez vsebnosti seruma bo izboljšalo ponovljivost rezultatov na področju matičnih celic, izboljšalo klinično učinkovitost terapevtskih proizvodov, ki temeljijo na hMSC, olajšalo izolacijske procese, zmanjšalo možnost okužb, pocenilo naše raziskave in še pospešilo razvoj znanstvenega področja.

V našem magistrskem delu smo zasnovali 3 različne pristope zniževanja serumske koncentracije v 3 kemijsko definiranih gojiščih za gojenje hMSC. Uporabljali smo gojišča Hektor, TurboDoma in StemCell cell medium 1 (SCM-1), ki nam jih je priskrbelo švicarsko podjetje Cell Culture Technologies. Učinke postopkov zniževanja serumske koncentracije na učinkovitost, celično morfologijo, hitrost rasti, viabilnost, velikost in metabolno aktivnost smo primerjali s celicami, ki so bile gojene v kontrolnem gojišču (α-MEM) z dodanim človeškim serumom (HS) (v konstatni koncentraciji 10 %). Ker so bile naše hMSC že izolirane in zamrznjene v gojiščih, ki so vsebovale serum, smo se odločili za pristop postopnega zmanjševanja vsebnosti seruma. S tem smo želeli zmanjšati stresne pogoje, ki se pojavijo zaradi zmanjševanja vsebnosti seruma in dati celicam več časa, da se postopoma prilagodijo na brezserumske pogoje. Izvedli smo hitro, počasno in prilagojeno postopno zmanjševanje vsebnosti seruma. Pri počasnem zmanjševanju smo hMSC nacepljali dvakrat na teden, medtem ko smo koncentracijo seruma znižali le vsako drugo pasažo (enkrat na teden). S tem smo celicam zagotovili dodatno pasažo pri isti koncentraciji seruma in dodaten čas za prilagoditev na nižjo vsebnost seruma. Pri hitrem zniževalnem pristopu smo celice prav tako nacepljali dvakrat tedensko, vendar smo ob vsaki pasaži znižali tudi koncentracijo seruma. Pri tem pristopu smo želeli ugotoviti, če je celice možno odvaditi od vsebnosti seruma brez dodatnih pasaž za prilagoditev. Če bi celice namreč privadili na brezserumske pogoje v krajšem času, bi nam preostalo več nacepljanj (pasaž) za kasnejše raziskovalno delo ali namnoževanje celic za klinično uporabo, preden bi celice zapadle v senescenco. Za prvima dvema pristopoma smo razvili še tretjega, ki smo ga poimenovali prilagojeni pristop. S prilagojenim pristopom smo želeli zajeti prednosti prvih dveh pristopov in se izogniti slabostim, na katere smo naleteli tekom našega dela s prvima pristopoma. V prilagojenem pristopu zniževanja smo se odločili v prvih stopnjah koncentracijo seruma zniževati vsako pasažo, vendar smo celice ponovno nacepljali le, ko so te dosegle zadostno konfluenco (nad 60 %). S tem smo se želeli izogniti redčitvi in nacepljanju celic, preden te desežejo logaritemsko fazo rasti. S tem, ko smo v prvih korakih, ko je vsebnost seruma v gojiščih še visoka, koncentracijo seruma znižali vsako pasažo, pa smo se izognili prekomernemu številu pasaž, ki nam zmanjšajo vrednost in smiselnost naših hMSC za kasnejše raziskovalne ali terapevtske namene. V primeru, da bi pri nižjih vsebnostih seruma zaznali stagnacijo rasti, pa bi se še vedno lahko odločili za dodatno pasažo pri isti koncentraciji seruma, kar bi dalo celicam dodaten čas za prilagoditev. Med vsakim nacepljanjem smo preverjali:

- Morebitne spremembe celične morfologije.
- Velikost celic.
- Število celic.

- Število populacijskih podvojitev.
- Viabilnost.
- MTT viabilnostne teste, s katerimi smo merili metabolno aktivnost celic (izvedeni vsako drugo pasažo).

Preko vseh merljivih dejavnikov smo zaznali, da ima zniževanje serumske koncentracije izrazito negativne učinke na hMSC, ki so bile gojene v kemijsko definiranih gojiščih. Najbolj izrazite spremembe smo zaznali v celicah, ki so bile gojene v gojišču TurboDoma (pod 5 % HS), tem so sledile celice v gojišču Hektor (pod 5 % HS), medtem ko so celice v gojišču SCM-1 kazale še najmanjše negativne učinke od vseh 3 gojišč. Treba pa je poudariti, da so se tudi pri hMSC v tem gojišču pokazale izrazite posledice, ko smo vsebnost seruma znižali pod 2 %.

Učinki zniževanja serumske koncentracije na celično morfologijo so se pokazali pri spremembi celične oblike iz običajnih vretenastih celic, podobnih fibroblastom, v veliko večje celice z veliko variabilnostjo v obliki in strukturi. Opazili smo razpotegnjene celice z izrazito kontrastnimi intracelularnimi strukturami, ki so rasle v skupkih in ne v enakomerno razporejeni enoslojni kulturi, ki je značilna za hMSC v normalnih pogojih. Spremembo oblike smo zaznali, ko smo celice prenesli v gojišča s 3 % HS (Hektor in TurboDoma) in z 1 % HS (SCM-1). Pri celicah, ki smo jih gojili v gojišču TurboDoma, smo pri 3 % HS zaznali tudi celice s podvojenimi jedri, ki pa niso zaključile delitve.

Populacijske podvojitvene nivoje (PDL) smo izračunali iz števila celic, ki smo jih pridobili s štetjem celic s pomočjo hemocitometra. Štetje smo opravili pred nacepljanjem v sveže gojišče. Število celic je bilo potrebno tudi za določitev primerne redčitve celic za naslednjo pasažo. Ker smo celice za štetje obarvali z barvilom Tripansko modrilo, smo lahko poleg števila celic izračunali tudi viabilnost. Iz krivulj PDL smo lahko razbrali, da so celice, ki so bile gojene v gojiščih Hektor in TurboDoma, večino podvojitev opravile pred prenosom v sveže gojišče s 5 % HS. V prvotnih korakih zniževanja seruma so hMSC ostale viabilne, a so predvsem rastle v velikosti, niso pa se delile. Celice, ki so bile gojene v gojišču SCM-1, pa so se podvojevale, dokler je bila vsebnost seruma vsaj 2%. Pri nižjih koncentracijah tudi to gojišče ni zagotavljalo pogojev za normalno proliferacijo. Celice so začele odmirati pri koncentracijah seruma, nižjih od 1 %. Med 3 pristopi zniževanja serumske koncentracije smo najboljše rezultate pridobili pri počasnem in prilagojenem zniževanju, medtem ko se je pristop hitrega zniževanja izkazal za neprimernega. Pri tem pristopu se tudi celice, gojene v gojišču SCM-1, niso podvojevale, ko smo pričeli z zniževanjem seruma, kar očitno kaže na to, da je zniževanje potekalo prehitro in hMSC niso imele dovolj časa za prilagoditev. Kljub temu da so celice v gojišču SCM-1 izkazovale celično podvojevanje tudi pri nižjih koncentracijah seruma, pa so podvojevalni časi (DT) hitro postali veliko daljši od kontrolnih celic, ki so bile stalno gojene v kontrolnih pogojih (α-MEM + 10 % HS). Kontrolne celice so edine izkazovale dokaj konstantno proliferacijo preko daljšega časovnega obdobja (do pribl. 20 pasaže).

Iz obarvanj s Tripanskim modrilom smo izračunali povprečne viabilnosti celic v različnih gojiščih, ki so bile podvržene različnim pristopom zmanjševanja serumske koncentracije. Povprečne viabilnosti so bile izračunane iz posameznih vrednosti, ki smo jih pridobili preko celotnega obdobja v kulturi. Tudi na tem področju so celice, ki so bile vključene v postopke zniževanja seruma, izražale nižje povprečne viabilnosti, ki so bile posledica izrazito nižjih viabilnosti pri pasažah z nižjo vsebnostjo seruma. V vseh treh postopkih

zniževanja so najnižje povprečne viabilnosti dosegale celice, gojene v gojiščih Hektor in TurboDoma. Te povprečne viabilnosti so bile pridobljene tudi preko najmanjšega števila pasaž, saj so celice začele odmirati že pri razmeroma visokih koncentracijah seruma (3 % HS). Prav tako pa smo pri celicah, ki so bile gojene v prej omenjenih gojiščih, zaznali tudi največjo variabilnosti v viabilnosti od pasaže do pasaže (50-90 % za Hektor; 35-90 % za TurboDoma). V nasprotju s hMSC, ki so bile namnoževane v gojiščih Hektor in TurboDoma, smo pri celicah v gojišču SCM-1 pridobili povprečne viabilnosti, ki so bile primerljive s kontrolnimi celicami. Pri postopku hitrega zniževanja je bila povprečna viabilnost celic v gojišču SCM-1 celo višja od kontrolnih celic, vendar lahko to razliko pripišemo problematiki previsoke konfluence kultur. Ko so celice dosegle 100% konfluenco, je bilo nemogoče ustvariti enotno suspenzijo brez velikih skupkov celic. Zato smo morali celične suspenzije filtrirati, kar je znižalo realno število namnoženih celic in znižalo viabilnost.

Poleg klasičnega testiranja viabilnosti s pomočjo obarvanja s Tripanskim modrilom smo izvedli tudi MTT viabilnostni test. MTT je fotometrična metoda, ki nam omogoča določanje metabolne aktivnosti in s tem viabilnosti celic. V mitohondrijih se vodotopno rumeno barvilo MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolijev bromid) s strani NADH reducira v vijolično obarvan formazan. Količina formazana odraža metabolno aktivnost celic in s tem viabilnost. Za fotometrično detekcijo v vodi netopnega formazana moramo celice lizirati in kristale formazana raztopiti s SDS, razredčenim v HCl. Rezultati MTT proliferacijskih nivojev so se ujemali s podatki, ki smo jih pridobili s pomočjo ostalih analitskih metod. Edino odstopanje v vrednostih smo dobili pri proliferacijskih nivojih celic, ki so bile gojene v kemijsko definiranih gojiščih z 10 % HS. Proliferacijski nivoji so izrazito presegali povprečni proliferacijski nivo kontrolnih celic. Z zniževanjem serumske koncentracije pa so se proliferacijski nivoji po pričakovanjih znižali. Celice, ki so bile gojene v gojiščih Hektor in TurboDoma, so imele pri 5 % HS še vedno podoben ali malce nižji proliferacijski nivo kot celice v kontrolnem gojišču, pri nižjih koncentracijah seruma pa smo metabolno aktivnost zaznali le še v izrazito manjših vrednostih. Pri celicah, ki so bile gojene v gojišču SCM-1 je bil proliferacijski nivo tudi pri 3 % HS še vedno primerljiv s povprečnim nivojem kontrolnih celic. Pri prilagojenem zniževanju je bil proliferacijski nivo dokaj visok tudi pri 1 % HS, a je še vedno predstavljal le 65 % povprečnega proliferacijskega nivoja kontrolnih celic, medtem ko je pri 0.5 % HS nivo padel že na slabo tretjino povprečnega proliferacijskega nivoja kontrolnih celic.

Poleg že omenjenih analiz smo tekom našega eksperimenta izvedli tudi β-galaktozidazni test senescence. Test zaznava β-galaktozidazno aktivnost pri pH vrednosti 6, ki je značilna lastnost celic, ki zapadejo v senescenco, ne zaznamo pa je pri nesenescentnih, mirujočih ali imortaliziranih celicah. Omejena zmožnost podvojevanja je ena od osnovnih značilnosti normalnih celic, ki sčasoma vodi v senescentno stanje. Senescenca je mirujoče stanje, v katerem celice ostanejo viabilne, a se ne delijo. Ko celice zapadejo v senescenco, postanejo za raziskovalne namene hMSC in terapevtsko uporabo neuporabne. Zato smo na kontrolnih celicah pri različnih pasažah (4, 24 in 26) opravili test senescence, da smo preverili, če po prenosu v brezserumske pogoje naše hMSC teoretično še spadajo med nesenescentne celice. Pri celicah iz 26. pasaže smo zaznali močno modro obarvanje, kar pomeni, da so že zapadle v senescenco. Pri celicah iz 24 pasaže smo zaznali zmerno obarvanje, medtem ko pa so celice iz 4 pasaže pričakovano ostale večinoma neobarvane. Iz testa senescence lahko razberemo, da celice zapadejo v senescenco okrog 24 pasaže.

Teoretično bi lahko torej po končanju prenosa v brezserumsko gojišče naše hMSc v kulturi pred pojavom senescence namnoževali še najdaljnih 6-14 pasaž. Število nadaljnih pasaž je odvisno od pristopa zniževanja serumske koncentracije (6 za počasno zniževanje, 14 za hitro).

Rezultati vseh opravljenih testov kažejo na to, da nobeno izmed naših 3 kemijsko definiranih gojišč ne more podpirati gojenja hMSC brez dodatka seruma, ko so celice ob izolaciji in zamrzovanju izpostavljene gojiščem, ki vsebuje serum. Številne študije povezujejo slabe eksperimentalne rezultate ravno s tem, da so celice pred prilagajanjem na brezserumsko gojenje izpostavljene gojiščem s serumom. Obstaja namreč možnost, da se serumski kontaminanti prenašajo preko pasaž tudi ko celice prenesemo v brezserumske pogoje. Visoke proliferacijske nivoje celic, ki smo jih gojili v kemijsko definiranih gojiščih pri dodatku 10 % HS, lahko razložimo z visoko hranilno vrednostjo kemijskih gojišč. Gojišče Hektor je bilo razvito za gojenje številnih celičnih linij ledvičnih celic, medtem ko se gojišče TurboDoma uporablja za produkcijo monoklonskih protiteles s hibridomskimi celicami. Kljub dobrim proliferacijskim nivojem pri gojenju z 10 % HS, pa se z zniževanjem serumske koncentracje znižajo tudi proliferacijski nivoji vseh celic, ki so gojene v kemijsko definiranih gojiščih Hektor, TurboDoma in SCM-1. Prav tako so se znižali populacijski podvojevalni nivoji in viabilnost, pojavile pa so se tudi izrazite morfološke spremembe. Spremembe in negativne posledice lahko pripišemo različnim vzrokom, kot so:

- Izpostavitev mehanskemu stresu med nacepljanjem.
- Prehitro zniževanje serumske koncentracije.
- Neprimeren pristop zniževanja.
- Uporaba seruma v gojiščih za izolacijo in zamrzovanje celic.
- Neprimerna raztopina za odcepitev celic od gojitvene površine.
- Nizka viabilnost celic po odmrzovanju zaradi dolgega obdobja zamrznitve.
- Formulacija gojišč, ki sama od sebe ne podpira gojenja hMSC brez vsebnosti seruma.

V magistrskem delu smo zastavili številne iztočnice za nadaljnje raziskovanje kemijsko definiranih gojišč in njihove uporabe za gojenje hMSC v pogojih brez vsebnosti seruma. Čeprav nam ni uspelo prilagoditi hMSC na brezserumske pogoje v nobenem izmed 3 proučevanih gojišč, pa se je gojišče SCM-1 izkazalo kot potencialni kandidat za nadaljnjo obravnavo. Obstaja možnost, da bi s primernim pristopom zniževanja serumske koncentracije, primerno prilagoditvijo formulacije ali z dodatkom določenih rastnih dejavnikov (npr. bFGF), gojišče uspešno omogočalo proliferacijo hMSC brez prisotnosti človeškega ali živalskega seruma. Med potencialne prihodnje pristope bi tu lahko umestili tudi neposredno izolacijo hMSC v brezserumska gojišča. Čeprav se zdi pristop sprva nelogičen, saj bi pričakovali, da nemuden prenos v gojitvene pogoje brez seruma predstavlja prevelik stres za hMSC, pa določene raziskovalne skupine (Jung in sod., 2012) prav ta pristop predpostavljajo kot najbolj primeren ter najbolj učinkovit. Z izločitvijo dolgih prilagoditvenih postopkov iz našega poskusa, bi pridobili tudi številne zgodnje pasaže, ki jih v nasprotnem primeru porabimo za samo adaptacijo. Z neposrednim prenosom bi lahko torej hMSC uporabljali od samega začetka, ko so te še mlade in genetsko stabilnejše. S tem bi se povečala uporabnost in vrednost izoliranih celic, zaradi popolne izločitve seruma pa bi tudi odstranili kakršen koli dvom o morebitnih

kontaminacijah, ki bi se nam lahko pripetile zaradi uporabe seruma v izolacijskem in zamrzovalnem koraku. Poleg spremembe pristopa bi lahko v prihodnosti na temeljih rezultatov magistrskega dela opravili številne dodatne analitske metode, ki jih zaradi izrazite delovne intenzitete poskusa, v času našega poskusa nismo uspeli. S pomočjo pretočne citometrije bi lahko opravili analize izražanja površinskih antigenov na hMSC in izvedli citogenetske analize s kariotipizacijo in primerjalno genomsko hibridizacijo. Seveda so to le ene izmed možnih analiz, ki bi nam priskrbele še dodatne informacije o učinkih zniževanja serumske koncentracije na hMSC. Nekatere izmed teh metod smo želeli opraviti že tekom našega poskusa, a smo se na koncu osredotočili na analizo različnih pristopov zniževanja serumske koncentracije skozi metabolno aktivnost, celično morfologijo, hitrost rasti in viabilnost, s čimer smo postavili temelje za prihodnje raziskave.

8 REFERENCES

Arora M. 2013. Cell Culture Media: A Review. Mater Methods, 3, 175, doi: 0.13070/mm.en.3.175

Barnes D., Sato G. 1980. Methods for growth of cultured cells in serum-free medium. Analytical Biochemistry, 102, 2: 255-270

Bieback K., Kern S., Klüter H., Eichler H. 2004. Critical Parameters for the Isolation of Mesenchymal Stem Cells from Umbilical Cord Blood. STEM CELLS, 22, 4: 625-634

Brunner D., Frank J., Appl H., Schoffl H., Pfaller W., Gstraunthaler G. 2010. Serum-free cell culture: the serum-free media interactive online database. Altex, 27, 1: 53-62

Butler M., Burgener A. 2005. Medium Development. In: Cell Culture Technology for Pharmaceutical and Cell-Based Therapies. Ozturk S., Hu W. S. (ed.). CRC Press: 41-79

Carlos Sepúlveda J., Tomé M., Eugenia Fernández M., Delgado M., Campisi J., Bernad A., González M. A. 2014. Cell Senescence Abrogates the Therapeutic Potential of Human Mesenchymal Stem Cells in the Lethal Endotoxemia Model. Stem Cells, 32, 7: 1865-1877

Carney D. N., Bunn P. A., Gazdar A. F., Pagan J. A., Minna J. D. 1981. Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. Proceedings of the National Academy of Sciences of the United States of America, 78, 5: 3185-3189

Chase L. G., Lakshmipathy U., Solchaga L. A., Rao M. S., Vemuri M. C. 2010. A novel serum-free medium for the expansion of human mesenchymal stem cells. Stem Cell Research & Therapy, 1, 1: 8

Chen G., Yue A., Ruan Z., Yin Y., Wang R., Ren Y., Zhu L. 2014. Monitoring the biology stability of human umbilical cord-derived mesenchymal stem cells during long-term culture in serum-free medium. Cell and Tissue Banking, 15, 4: 513-521

Cristofalo V. J., Volker C., Francis M. K., Tresini M. 1998. Age-dependent modifications of gene expression in human fibroblasts. Critical Reviews in Eukaryotic Gene Expression, 8, 1: 43-80

Debacq-Chainiaux F., Erusalimsky J. D., Campisi J., Toussaint O. 2009. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nature Protocols, 4, 12: 1798-1806

Denizot F., Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods, 89, 2: 271-277

Dimri G. P., Lee X., Basile G., Acosta M., Scott G., Roskelley C., Medrano E. E., Linskens M., Rubelj I., Pereira-smith O. 1995. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proceedings of the National Academy of Sciences of the United States of America, 92, 20: 9363-9367

Dominici M., Le Blanc K., Mueller I., Slaper-Cortenbach I., Marini F., Krause D., Deans R., Keating A., Prockop D., Horwitz E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy, 8, 4: 315-7

Freshney R. I. 2010. Serum-Free Media. Culture of Animal Cells. John Wiley & Sons, Inc.: 732 p.

Friedenstein A. J., Chailakhjan R. K., Lalykina K. S. 1970. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinetics, 3, 4: 393-403

Friedenstein A. J., Gorskaja J. F., Kulagina N. N. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Experimental Hematology, 4, 5: 267-74

Goldstein S. 1990. Replicative senescence: the human fibroblast comes of age. Science, 249, 4973: 1129-1133

Ham R. G. 1984. Growth of human fibroblasts in serum-free media. In: Cell culture methods for molecular and cell biology. Vol. 3. Barnes D. W., Sirbasku D. A., Sato G. H. (eds.). New York, Liss: 249-264

Jayme D. W., Gruber D. F. 2006. Chapter 5 - Development of Serum-Free Media: Optimization of Nutrient Composition and Delivery Format. In: Cell Biology. Celis J. E. (ed.). 3rd ed. Burlington, Academic Press: 33-41

Jung S., Panchalingam K. M., Rosenberg L., Behie L. A. 2012. Ex Vivo Expansion of Human Mesenchymal Stem Cells in Defined Serum-Free Media. Stem Cells International, 2012: 21

Jung S., Sen A., Rosenberg L., Behie L. A. 2010. Identification of growth and attachment factors for the serum-free isolation and expansion of human mesenchymal stromal cells. Cytotherapy, 12, 5: 637-657

Kassem M., 2006. Stem cells: potential therapy for age-related diseases. Annals of the New York Academy of Sciences, 1067: 426-442

Kern S., Eichler H., Stoeve J., Kluter H., Bieback K. 2006. Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. Stem Cells, 24, 5: 1294-1301

Kogler G., Sensken S., Airey J. A., Trapp T., Muschen M., Feldhahn N., Liedtke S., Sorg R. V., Fischer J., Rosenbaum C., Greschat S., Knipper A., Bender J., Degistirici O., Gao J., Caplan A. I., Colletti E. J., Almeida-Porada G., Muller H. W., Zanjan E., Wernet P. 2004. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. The Journal of Experimental Medicine, 200, 2: 123-35

Lee O. K., Kuo T. K., Chen W. M., Lee K. D., Hsieh S. L., Chen T. H. 2004. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. Blood, 103, 5: 1669-1675

Lennon D. P., Haynesworth S. E., Young R. G., Dennis J. E., Caplan A. I. 1995. A chemically defined medium supports in vitro proliferation and maintains the osteochondral potential of rat marrow-derived mesenchymal stem cells. Experimental Cell Research, 219, 1: 211-222

Lian Q., Lye E., Suan Yeo K., Khia Way Tan E., Salto-Tellez M., Liu T. M., Palanisamy N., El Oakley R. M., Lee E. H., Lim B., Lim S. K. 2007. Derivation of clinically compliant MSCs from CD105+, CD24- differentiated human ESCs. Stem Cells, 25, 2: 425-436

Linskens M. H., Feng J., Andrews W. H., Enlow B. E., Saati S. M., Tonkin L. A., Fun, W. D., Villeponteau B. 1995. Cataloging altered gene expression in young and senescent cells using enhanced differential display. Nucleic Acids Research, 23, 16: 3244-3251

Liu C.-H., Wu M.-L., Hwang S.-M. 2007. Optimization of serum free medium for cord blood mesenchymal stem cells. Biochemical Engineering Journal, 33, 1: 1-9

Marshak D. R., Holecek J. J. 1999. Chemically defined medium for human mesenchymal stem cells. US patent application US 46459995 A-1999/06/01

Moretti P., Hatlapatka T., Marten D., Lavrentieva A., Majore I., Hass R., Kasper C. 2010. Mesenchymal Stromal Cells Derived from Human Umbilical Cord Tissues: Primitive Cells with Potential for Clinical and Tissue Engineering Applications. In: Bioreactor Systems for Tissue Engineering II. Kasper C., van Griensven M., Pörtner R. (eds.). Heidelberg, Springer Publishing: 33-35

Pan Q., Fouraschen S. M., de Ruiter P. E., Dinjens W. N., Kwekkeboom J., Tilanus H. W., van der Laan L. J. 2014. Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells. Experimental Biology and Medicine, 239, 1: 105-115

Parker A. M., Shang H., Khurgel M., Katz A. J. 2007. Low serum and serum-free culture of multipotential human adipose stem cells. Cytotherapy, 9, 7: 637-646

Phinney D. G., Prockop D. J. 2007. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair--current views. Stem Cells, 25, 11: 2896-2902

Phuc Pham V. 2011. Stem Cell Therapy for Islet Regeneration. In: Stem Cells in Clinic and Research. Dr. Ali Gholamrezanezhad (ed.). Rijeka, InTech: 551-578

Sato K., Itoh T., Kato T., Kitamura Y., Kaul S., Wadhwa R., Sato F., Ohneda O. 2015. Serum-free isolation and culture system to enhance the proliferation and bone regeneration of adipose tissue-derived mesenchymal stem cells. In Vitro Cellular & Developmental Biology - Animal, 51, 5: 515-529

Sekiya I., Larson B. L., Smith J. R., Pochampally R., Cui J. G., Prockop D. J. 2002. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells, 20, 6: 530-41

Sherwood S. W., Rush D., Ellsworth J. L., Schimke R. T. 1988. Defining cellular senescence in IMR-90 cells: a flow cytometric analysis. Proceedings of the National Academy of Sciences U S A, 85, 23: 9086-9090

Shetty P., Cooper K., Viswanathan C. 2010. Comparison of proliferative and multilineage differentiation potentials of cord matrix, cord blood, and bone marrow mesenchymal stem cells. Asian Journal of Transfusion Science, 4, 14-24

Stockert J. C., Blázquez-Castro A., Cañete M., Horobin R. W., Villanueva Á. 2012, MTT assay for cell viability: Intracellular localization of the formazan product is in lipid droplets. Acta Histochemica, 114, 8: 786-796

Todaro G. J., Green H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. The Journal of Cell Biology, 17: 299-313

ThermoFisher Scientific. 2015. Adaptation of Cell Cultures to a Serum-Free Medium. http://www.thermofisher.com/si/en/home/references/protocols/cell-culture/serum-protocol/adaptation-of-cell-cultures-to-a-serum-free-medium.html (August 2015)

Ullah I., Subbarao R. B., Rho G. J. 2015. Human mesenchymal stem cells - current trends and future prospective. Bioscience Reports, 35, 2: e00191, doi: 10.1042/ BSR20150025: 18 p.

van der Valk J., Brunner D., De Smet K., Fex Svenningsen Å., Honegger P., Knudsen L. E., Lindl T., Noraberg J., Price A., Scarino M. L., Gstraunthaler G. 2010. Optimization of chemically defined cell culture media – Replacing fetal bovine serum in mammalian in vitro methods. Toxicology in Vitro, 24, 4: 1053-1063

Venes D., Taber C. W. 2009. Taber's cyclopedic medical dictionary. Philadelphia, F.A. Davis Co.: 2846 p.

Wang Y., Zhang Z., Chi Y., Zhang Q., Xu F., Yang Z. Meng L., Yang S. Yan S., Mao A., Zhang J., Yang Y., Wang S., Cui J., Liang L., Ji Y., Han Z. B., Fang X. Han Z. C. 2013. Long-term cultured mesenchymal stem cells frequently develop genomic mutations but do not undergo malignant transformation. Cell Death & Disease, 4: e950, doi: 10.1038/cddis.2013.480: 11 p.

Zisch A. H., Lutolf M. P., Ehrbar M., Raeber G. P., Rizzi S. C., Davies N., Schmokel H., Bezuidenhout D., Djonov V., Zilla P. Hubbell J. A. 2009. Cell-demanded release of VEGF from synthetic, biointeractive cell ingrowth matrices for vascularized tissue growth. The FASEB Journal, 17, 15: 2260-2262

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