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Mesenchymal Stem Cells: Isolation, Characterization and Potential Clinical Applications

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Abstract of the dissertation

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Chairperson of the Commission for Dissertation Defences
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Summary

Stem cells offer a promising avenue to therapy for a wide range of human diseases. However, for this potential to be realized, a consistent and plentiful supply of well-characterized stem cells is essential. To date, there has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells (MSCs).
Current obstacles to the large-scale manufacture of MSCs suitable for therapeutic use in humans include: a lack of standards for the characterization, isolation or identification of MSCs; the absence of standard protocols for differentiation of MSCs to various lineages; a lack of specificity for surface markers used for MSC characterization ; the absence of standardized cryopreservation protocols; the requirement of current production methods to use animal-derived serum resulting in major contamination implications.

Following my first experiments with low fetal bovine serum containing media and experimental work with dental pulp stem cells I was invited to be a part of the collaborative 7FP EU project - PurStem. As a team leader I was personally responsible for the work package focused on serum-free isolation, growth and differentiation of MSCs. Objectives, standard operating protocols and results summarized in this thesis are in relation to this project.

PurStem project set out to identify the MSC “receptome” and use this repertoire of growth factor receptors to develop novel serum-free media suitable for large-scale MSC production. Furthermore, the PurStem project aimed to create novel antibody reagents for specific MSC characterization and contribute to Good Manufacturing Practice (GMP) standards to enable rapid progression to production of serum-free MSC for clinical, therapeutic applications.

Goals of this thesis and PurStem project were achieved by: developing and validating a collaborative, standardized procedure for the isolation, culture and cryopreservation of MSCs that produced consistent cell types even when grown in different laboratories; using the wealth of information obtained from characterisation of the MSC “receptome” to develop low and serum-free culture conditions where MSCs can survive and proliferate, thereby reducing or eliminating potential contamination issues associated with current serum-based culture methods.

These results will contribute to the optimization of GMP manufacturing and banking of cells for use in clinical trials initially and ultimately as a commercial product. Furthermore, this thesis and related PurStem project have also advanced our basic understanding of MSC biology by defining the surface “receptome”, setting the stage for the development of next generation therapies which will exploit the self-repair potential of adult stem cells or stem cell targeting.

Stem cell therapeutics are expected to lead to the treatment of diseases with no current effective treatment options, as well as contribute to tissue engineering of new tissues or organs for replacement purposes. The findings of this work and PurStem project will enable the translation of promising stem cell therapies, initially in the area of osteoarthritis and autoimmune diseases. These novel regenerative therapies will reduce the economic and social costs of disease to the European Community. The primary social benefits from this work and PurStem will accrue from improvements in treatment options for a range of diseases. These improved treatments will improve patient quality of life and reduce the economic burden associated with chronic diseases.

Souhrn

Mezenchymové kmenové buňky: Izolace, charakteristika a potenciální klinické aplikace

Kmenové buňky jsou vybaveny vlastnostmi, které z nich dělají atraktivní “kandidáty” pro léčbu dosud neléčitelných lidských onemocnění. Aby mohl být tento potenciál kmenových buněk realizován, konzistentní kvalita a dostatečné množství dobře charakterizovaných kmenových buněk je zásadní. Přestože potenciál kmenových buněk je v současné době již dobře doceněn a celá řada vědců se snaží nalézt způsob, jak kmenové buňky využít pro léčbu, doposud stále neexistuje standardní protokol, podle něhož lze kmenové buňky izolovat a charakterizovat z dospělých tkání. Každá laboratoř používá své vlastní protokoly pro izolaci kmenových buněk, stejně jako kritéria toho, co kmenová buňka je a co není.

Mezenchymové kmenové buňky (MKB), jímž je věnována tato práce, se poměrně snadno izolují, neboť zdroj pro jejich izolaci je dobře dostupný, buňky v kultuře obvykle dobře proliferují, ale výsledná populace je značně heterogenní. Vykazují schopnost diferencovat se v buňky kostí (osteocyty), chrupavek (chondrocyty), tukové tkáně (adipocyty) i šlach. Zároveň se vyznačují pozoruhodnou plasticitou – jsou schopny produkovat takové rozmanité typy buněk, jako jsou buňky nervové, srdeční, jaterní, endotelové a další.

Mezi překážky omezující nasazení MKB pro terapeutické použití u člověka patří: nedostatek standardů pro izolaci, charakterizaci a identifikaci MKB, neexistence standardních protokolů pro diferenciaci MKB; nedostatek specifických povrchových znaků používaných pro charakterizaci MKB; absence standardizovaných protokolů kryokonzervace a též závislost současných výrobních postupů na použití zvířecího séra.

Po mých prvních experimentech s kultivačními médii s nízkým obsahem bovinního séra a experimentální práci s kmenovými buňkami zubní pulpy jsem byl přizván ke spolupráci na projektu 7. RP EU - PurStem. Jako vedoucí týmu jsem byl osobně zodpovědný za pracovní balíček zaměřený na bezsérové izolace, růst a diferenciace MKB. Cíle této práce, standardní operační protokoly a prezentovaná data jsou výsledkem mé práce pro projekt PurStem.

Překládaná disertační práce a projekt PurStem mají za cíl vyvinout standardní protokol pro izolaci a kultivaci kmenových buněk se snahou o produkci uniformní populace kmenových buněk použitelných kteroukoli laboratoří. Pokud se podaří zajistit dostatečný přísun dobře charakterizovaných kmenových buněk, značně se usnadní úloha vědcům, jejichž produkty musí být standardně opakovány a stejně tak lékařům, kteří potřebují vyvíjet léčebné postupy s předpověditelnými a garantovanými vlastnostmi.

Tato práce a projekt PurStem pokládají základy právě tomuto přístupu tím, že vytváří shodné linie kmenových buněk pro široké potřeby dalšího výzkumu a buněčné terapie. Primární sociální výhody z této práce a projektu PurStem vyplývají ze zlepšování možností léčby řady chronických (např. autoimunitních) onemocnění. Zdokonalení léčby zlepší kvalitu života pacientů a pomůže snížit ekonomickou zátěž spojenou s léčbou chronických onemocnění.

Background

Regenerative medicine

Regenerative medicine represents a new paradigm in human healthcare with the potential to resolve unmet medical needs by addressing the underlying causes of disease. The emerging field of regenerative medicine is unique in its aim to augment, repair, replace or regenerate organs and tissue that have been damaged by disease, injury or even the natural aging process. This rapidly evolving, interdisciplinary field is transforming healthcare by translating fundamental science into a variety of regenerative technologies including biologics, chemical compounds, materials and devices. It differs from other fields of medicine in the array of disciplines it brings together and in its ability to create or harness the body's innate healing capacity. Currently, the vast majority of treatments for chronic and/or life-threatening diseases are palliative. Others delay disease progression and the onset of complications associated with the underlying illness. Very few therapies in use today are capable of curing or significantly changing the course of disease. The result is a healthcare system burdened by costly treatments for an aging, increasingly ailing population, with few solutions for containing rising costs. The best way to significantly improve the economics of our current healthcare system is to develop more effective treatments for the most burdensome diseases and conditions - diabetes, neurodegenerative disorders, autoimmune disease, stroke and cardiovascular diseases, for example - to facilitate longer, healthier and more productive lives.

Personally, I am in the field of regenerative medicine for 10 years, and during that time stem cell therapy became a commercial and medical reality. Even though the majority of people perceive regenerative medicine as something of the future, it is actually here and now. A significant number of regenerative medicine products are already commercially and clinically successful. In addition to over 60,000 stem cell transplants annually performed worldwide for the treatment of oncology and blood-based disorders, it is estimated that in 2012 cell therapy products distributed by biotherapeutic companies generated over $900 million with 160,000 patients receiving treatments. It is widely believed that these numbers are easily doubled when including non-cell-based regenerative medicine products such as scaffolds and other materials.

Analysts suggest there are at least 2,500 ongoing regenerative medicine clinical trials involving tens of thousands of patients for a myriad of clinical indications. An estimated 15 percent of this is industry-sponsored, and the remainder is being sponsored by leading academic centers around the world. Regenerative medicines encompass an array of technologies and therapeutic approaches including cell-based therapies, small molecules and biologics as well as synthetic and bio-based materials designed to augment, repair, replace or regenerate organs and tissues, thereby targeting the root cause of disease. Arguably the most prominent segment of the regenerative medicine industry, the cell therapy sector, is currently engaged in over 1,900 clinical trials around the world (www.clinicaltrials.gov).

Living cells, a pillar of the field, are incorporated into regenerative medicines to achieve a variety of positive effects: - to replace damaged or diseased cells and/or tissue; - to stimulate an endogenous response that promotes the body's own healing such as an immune response or regeneration in diseased tissue; - to deliver genetic or molecular therapies to targets.

Serum free culture of adult human bone marrow derived mesenchymal stem cells

The use of MSCs in the clinic will most likely involve more than the minimal manipulation of harvesting and infusion. Expansion and differentiation steps in MSC culture involve the use of bovine serum-containing media. Bovine serum is the most widely used growth supplement for in vitro culture because of its high levels of growth-stimulatory factors and low levels of growth-inhibitory factors. However, use of animal derived products greatly enhances the risk of prion, viral or zoonose contamination. By eliminating our reliance on animal or human based products, the risk of infection is eliminated (Mannello et al., 2007, Halme et al., 2006).

A greater risk than prion or viral infection through fetal bovine serum is the risk of immune reaction. Proteins from serum attach to cells cultured in its presence, acting as antigenic substrates for immune reactions (Tuschong et al., 2002). Selvaggi et al. (1997) showed that human lymphocytes cultured in media containing fetal calf serum resulted in immunogenic reactions when used therapeutically, resulting in an arthus-like reaction. Patients were sensitized to fetal calf proteins carried on the donor cells despite repeated washing of the cells before infusion. Additionally, the detection of newly formed antibodies to fetal calf serum indicated that immune complex formation followed cell transplantation. More recently Chachques et al. (2004) demonstrated that autologous myoblasts cultured in the presence of autologous human serum and transplanted into infarcted hearts decreases the risk of arrhythmia that was identified in previous studies using bovine serum.

An alternative to bovine serum albumin is autologous or allogeneic human serum. Autologous serum has proven to be as effective as bovine serum regarding the isolation and expansion of human MSCs (Stute et al., 2004). Additionally, autologous human serum has been shown to maintain higher cell motility compared to bovine derived serum (Kobayashi et al., 2005) and has eliminated the immune reaction observed with bovine serum cultured cells (Selvaggi et al., 1997 and Chachques et al., 2004). Culturing MSCs in allogeneic human serum results in growth arrest and cell death (Shahdadfar et al., 2005). Microarray experiments demonstrated several regulated genes comparing MSCs cultured in autologous serum as compared to fetal bovine serum. Interestingly, several of these regulated transcripts involve cell cycle and differentiation genes including anti-apoptotic genes and cell cycle genes (Shahdadfar et al., 2005). Alternatively, fresh or frozen human plasma and platelets have been shown to be even more effective than bovine derived serum in maintaining human MSCs proliferation (Muller et al., 2006). Pytlik et al. (2009) described a simple, yet efficient method of one-step cultivation of large numbers of human multipotent mesenchymal stromal cells. Key components of this method were the co-cultivation of adherent and non-adherent bone marrow cells, the use of special medium supplemented with human pooled serum and the use of additional growth factors and cytokines. However, the use of human serum poses many of the same problems as fetal bovine serum such as variability in composition between donors and the collection of serum from patients with pathology, eventually to be treated with cultured MSCs, making the use of human serum an unattractive option.

The need for a rationally developed serum free medium for the expansion and differentiation of human MSCs is clear for quality control of experiments between laboratories as well as for the culture of cells intended for clinical use. Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating (Pochampally et al., 2004). These cells contained longer telomeres than

control cells and expressed genes associated with embryonic cells such as octomer-binding transcription factor 4. A new compliment of medium, growth factors, cytokines, etc must therefore be developed specifically to compliment the display of receptors expressed on MSC cell surface ensuring uniform expansion of the MSCs population.

Serum free differentiation of adult human bone marrow derived mesenchymal stem cells

Differentiation of MSCs into osteochondral lineages in serum-free media relies upon the addition of cytokines and/or growth factors (Kuznetsov et al., 1997; Gronthos et al., 1995). Several cytokines and growth factors that promote chondrogenesis have been identified (Heng et al., 2004). For example, various isoforms of the TGF-beta superfamily (Chimal-Monroy et al., 1997), fibroblast growth factor 2 (Quarto et al., 1997) and insulin-like growth factor 1 (Fortier et al., 2002). Furthermore, several non-proteinaceous chemical factors can promote chondrogenesis, such as ascorbic acid (Farquharison et al., 1998), prostaglandin E2 (Miyamoto et al., 2003) and concanavalin A (Mikhailov et al., 1988). The challenge is to find the subtle combination of factors that will induce differentiation down the expected lineage. This is especially difficult in osteochondral differentiation as these tissues (and the supportive growth factors and cytokines) are closely intertwined, dose dependent and temporally dependent. Further, depending upon the dosage and timing of exposure, these supplements will have pleiotropic effects on MSCs.

Serum-free media for the expansion and differentiation of non-human cells, such as embryonic chick mesenchymal cells (Quarto et al., 1997) and rat MSCs (Lennon et al., 1995) have been published using a combination of supplements such as insulin, dexamethasone, FGF-2, PDGF-bb and EDGF were used to maintain proliferation and differentiation. Significantly less success has been achieved with human cells.

Conflicting results regarding the use of human derived medium supplements have been published. Lin et al. (2005) demonstrated the multipotentiality of human MSCs is maintained when cultured in media containing human plasma. Yamamoto et al. (2003) published similar results that human serum contains factors that not only enable MSC expansion and differentiation, but augments differentiation down the osteogenic and adipogenic lineages (Stute et al., 2004, Oreffo et al., 1997). Alternatively, Shahdadfar et al. (2005) observed that human MSCs grown in autologous human serum proliferated faster than cells cultured in fetal bovine serum; however, they differentiated more slowly than MSCs expanded in fetal bovine serum. Using a microarray, Shahdadfar et al. (2005) demonstrated the differences in gene expression between human and bovine serum cultured cells. MSCs cultured in fetal bovine serum expressed genes indicating they were taken farther along a differentiation pathway as compared to MSCs cultured in human serum. But again, the use of human serum poses many of the same problems as fetal bovine serum such as variability in composition between donors and the collection of serum from patients with pathology, eventually to be treated with cultured MSCs, making the use of human serum an unattractive option. In the end, a serum free medium containing a specific complement of growth factors (at the appropriate times) must be developed to enable MSC differentiation in a GMP setting for clinical application.

Objectives

From my early scientific steps I tried to constantly challenge conventional wisdom, to take the contrarian route and to create novel solutions. Following my first experiments with low-FCS containing media and experimental work with dental pulp stem cells I was invited to be a part of the collaborative project - PurStem. As a team leader I was personally responsible for the work package focused on serum-free isolation, growth and differentiation of MSCs. The objectives summarized in this chapter are in relation to this project work package.

Stem cells offer a promising avenue to therapy for a wide range of human diseases. However, for this potential to be realized, a consistent and plentiful supply of well-characterised stem cells is essential. To date, there has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells. The current state-of-the-art has several weaknesses, in that, there are no standards for the characterisation, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSC characterisation lack specificity and cryopreservation protocols are not standardised. Critically, current production methods for MSC require the use of animal products with major contaminant implications.

The objectives of this thesis were to:

- Examine existing methods and approaches to the preparation of MSCs
- Identify current best practice
- Standardise the technology by means of a unified operating protocol
- Develop new methods of culture and new media formulations by identifying the repertoire of growth factor receptors that exists on the surface of the cell
- Use this information to develop new media using a combinatorial approach to the selection of growth factor supplements to modulate and optimise the growth kinetics and differentiation of the cells
- Utilise recombinant human growth factors and so benefit from freedom of reliance on serum
- Generate a validated standard operating procedure for serum-free MSCs culture

Material and methods

For this study, bone marrow was obtained from 8 consecutive patients (healthy donors) undergoing total hip replacement where 10-15ml of BM were aspirated. Moreover 18 reference DPSCs lines from impacted third molars obtained from healthy donors were isolated. Donors or their legitimate representative were supposed to subscribe informed consent according to guidelines of Ethical committee of the Medical Faculty in Hradec Králové.

For the validation purposes and for creation of collaborative standardized procedures, 4 bone marrow samples were bought and distributed (AllCells, CA).

Processing of BM was performed as described in following Standard Operating Protocol, processing of dental pulp was performed as described previusly by Suchánek et al., 2009 and a pre-screen of factor combinations implemented using passaged MSCs grown in serum or reduced serum (Soukup et al., 2006, Karbanova et al., 2011) and the directly isolated in vivo MSC that had not been exposed to serum.

Protocols, presented in this disertation thesis, served as a uniform method of MSC isolation, culture and cryostorage procedures in each PurStem partner laboratory, resulting in comparability in culture protocols, differentiation protocols and cell characterization by differentiation and FACS. Outlined here are the protocols agreed upon by PurStem partners in the beginning of the PurStem project.

A) MSCs isolation protocol

B) MSCs subculture protocol

C) MSCs cryopreservation protocol and Preparation of cryopreservation medium

D) Osteogenic differentiation of hMSCs

E) Chondrogenic differentiation of hMSCs

F) Adipogenic differentiation of hMSCs

To evaluate CFU-F frequency (CFU-F assay), 100 µl volume of the marrow suspension were plated in 75cm^2 Petri dishes (TPP, Switzerland). The medium was changed after 72 hours from the first plating and then twice a week. After 2 weeks of primary culture, cells were washed with D-PBS, fixed with 3.7% formaldehyde in D-PBS, stained with 1% methylene blue in borate buffer (10 mM, pH 8.8) for 30 minutes, then washed with distilled water and the colonies were counted. All determinations were performed in duplicate and expressed as mean values. CFU-F frequency was used to calculate the population doublings of first-confluence cultures.

Within P0, P1 and P3 DNA analysis and phenotype were assessed using propidium iodide staining and flow cytometry (Cell Lab Quanta, Beckman Coulter, USA). For phenotypic analysis, cells were detached and stained sequentially with immunofluorescent primary antibodies.

Percentage of positive cells was determined as percentage of cells with higher fluorescence intensity than the upper 0.5% isotype immunoglobulin control. Classification criteria: <10% no expression, 11-40% low expression, 41-70% moderate expression and >71% high expression.

For karyotyping cells (subcultured at a 1:3 dilution, both early passages and after reaching Hayflick´s limit) were after 24 hours cultivation subjected to a 4-hour Demecolcemid (Sigma-Aldrich, USA) incubation followed by trypsin-EDTA detachment and lysis with hypotonic KCl and fixation in acid/alcohol. Metaphases were analyzed after GTG banding using software Ikaros v5.0 (MetaSystems, USA).

For detection of cytoskeletal proteins, cells were fixed with methanol at -20°C for 2 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For other intracellular molecules, cells were fixed with 4% paraformaldehyde at 20°C for 10 minutes and permeabilized with 0.1% Triton X-100 for 10 minutes. For cell surface antigens, cells were fixed with 4% paraformaldehyde at 20°C for 10 minutes. Blocking and diluent solution consisted of phosphate-buffer saline (D-PBS), 1% BSA, and 1% serum (Sigma-Aldrich, USA) from the same species as was the primary antibody. Slides were blocked for 30 minutes, incubated sequentially for 30 minutes each with primary antibodies and fluorescein- or phycoerythrin-coupled anti-mouse or anti-rabbit IgG antibody. Between each step, slides were washed with PBS containing 0.3% BSA.

The core outputs of this thesis are also standardized procedures. Subsequent protocols serve as uniform methods of MSC serum-free isolation, culture and cryostorage.

A) MSCs Serum Free Isolation Protocol

B) MSCs Serum Free Sub Culture Protocol

C) MSCs Serum Free Cryopreservation Protocol

D) MSCs Serum Free Differentiation Assays

E) xCELLigence MSCs profiling

Results

Clinical application of MSCs will require expansion and differentiation of primary MSCs in order to obtain sufficient therapeutic cell numbers as it is not possible to get enough MSCs for clinical use during a single expansion. Usually, the cells have to be passaged 1-2 times in media that contains animal-derived reagents, which increases the risk of contamination of the cell culture and also increases the time from the bone marrow harvest to the final production of the cellular treatment to 4-6 weeks. Currently, there are commercially available serum-free media. Mesencult-ACF from Stem Cell Technologies and Thera Peak from Lonza were tested during the PurStem project. Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating. A new complement of medium, growth factors, cytokines, etc. must therefore be developed specifically ensure consistent MSC cell surface receptor expression yielding uniform expansion of the MSC population in culture.

Comparison of PurStem media and commercial serum-free media

Colony forming assays and expansion cultures were set up in standardized PurStem (PS) media, which contains serum, low serum media (2% fetal bovine serum (FBS) and two commercially available serum-free media (Mesencult-ACF and Thera Peak). The cellular morphologies of the cultures were compared after 14 and 30 days.

MSC colonies were successfully cultured in commercially available serum-free media (Mesencult-ACF, Thera Peak) over the short term (14 days). After 14 days, the colonies observed in the standardized PurStem media were large, with densely packed cells, while those from the Mesencult-ACF media were small, containing fewer and more loosely packed cells. After 30 days, the cells in serum free media failed to proliferate and morphological changes were observed while the cells in PurStem media were confluent. In Therapeak media, spheroids were visible following the first passage. Despite the same initial seed concentration, the Mesencult-ACF cultures contained only 25% of the number of cells of the PurStem media cultures (Figure 1). Although our results demonstrated that it is possible to culture MSCs in commercially available serum-free media, these media were not suitable for sustaining MSCs in long term culture. Therefore, improved serum-free media conditions are required to culture MSCs for use in clinical therapy.

Effect of Growth Factor Combinations and ECM Molecules on MSC Viability and Proliferation

In the beginning of the project PurStem team leaders compiled a list of relevant factors with the potential enhance MSCs cell attachment and proliferation, including mitogenic factors and ECM molecules, for screening. A serum-free media from the literature developed for human articular chondrocytes, containing a cocktail of recombinant growth factors to drive cells towards osteogenic or chondrogenic differentiation was also tested. MSCs cells cultured under these serum-free conditions were elongated and fibroblast-like, similar to those cultured in PS media, and retained the ability to undergo osteogenic differentiation.

MSCs attachment after 24 h was examined using a range of ECM molecules, including vitronectin, fibronectin, laminin and hyaluronic acid. For MSCs isolated in the presence of serum, vitronectin

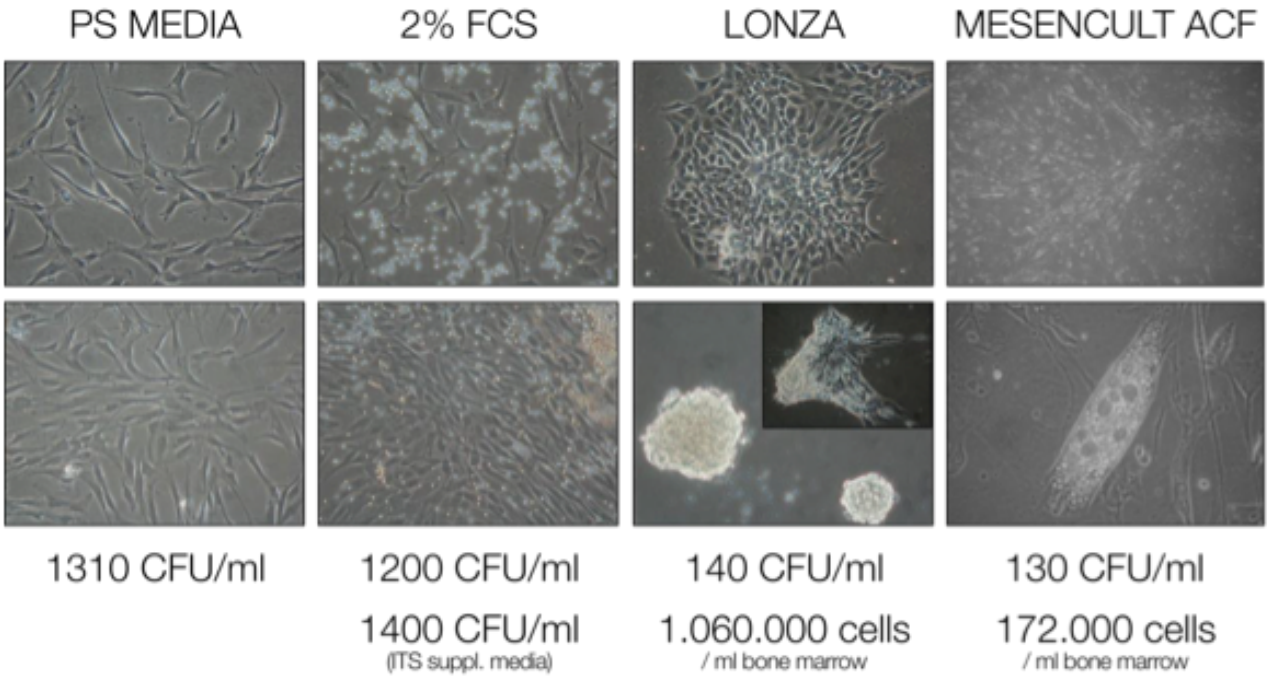

Figure 1. MSCs after 30 days in culture in PS (serum-containing), low serum (2% FCS), Lonza Thera Peak (serum-free) and Mesencult-ACF (serum-free) media.

seemed to result in the best initial cell attachment without altering cell morphology. However, after 6 days, MSC proliferation was greatest on fibronectin coated plates. Further study showed that fibronectin promotes MSCs attachment by phosphorylation of protein FAK after 90 minutes.

MSCs proliferation in the presence of growth factors on fibronectin coated plates in serum free media was examined. The growth factors examined included PDGF-AA, bFGF, FGF4, IGF2, EGF and GM-CSF. Initial results suggested that bFGF and FGF4 play a role in proliferation. Further optimization of the serum-free media formulation was conducted, and western blot analysis revealed that the presence of EGF in serum-free media promoted MSC attachment, but did not promote cell proliferation. There was a statistically significant increase in cell proliferation for media with PDGF-AA/FGF2 or FGF4/FGF2 or FGF4 compared to serum free media. There was no statistically significant difference between MSCs cultured in FGF2/FGF4 and FGF4 alone.

Using colony forming assays and xCELLigence technology which allows monitoring of cellular events in real time, several other combinations of growth factors/supplements were tested and high throughput analysis of proliferation and viability was performed. Basic combinations of growth factors/supplements with serum-free media were tested using high throughput analysis of proliferation, viability assessment and phenotyping. Cells cultivated in high concentrations of PDGF-BB were more numerous but less stable during long term cultivation. Moreover CD146 expression decreased as the amount of PDGF-BB used was increased. A combination of EGF (10 ng/ml) and ITS (10 µl/ml) with the addition of HSA (Human serum albumin) (0.125%) yielded an optimum basal serum-free media composition.

Surface markers of MSCs cultured in serum-free conditions

To assess the effect of the supplements, CD146 and CD271 expression levels on the cells were examined at frequent time points. Cells grown under standardized PurStem conditions (10% serum) had a standard MSC phenotype (Figure 2). Cells grown in other media conditions had an MSC phenotype (expressing CD73, CD90, etc. and lacking expression of lineage markers) but did differ in the expression levels of some markers. The most prominent difference was a decrease in expression of CD105 and CD146 for the three media tested and an increased expression of CD271 for Medium A when compared to cells grown in standardized Purstem media (Figure 3).

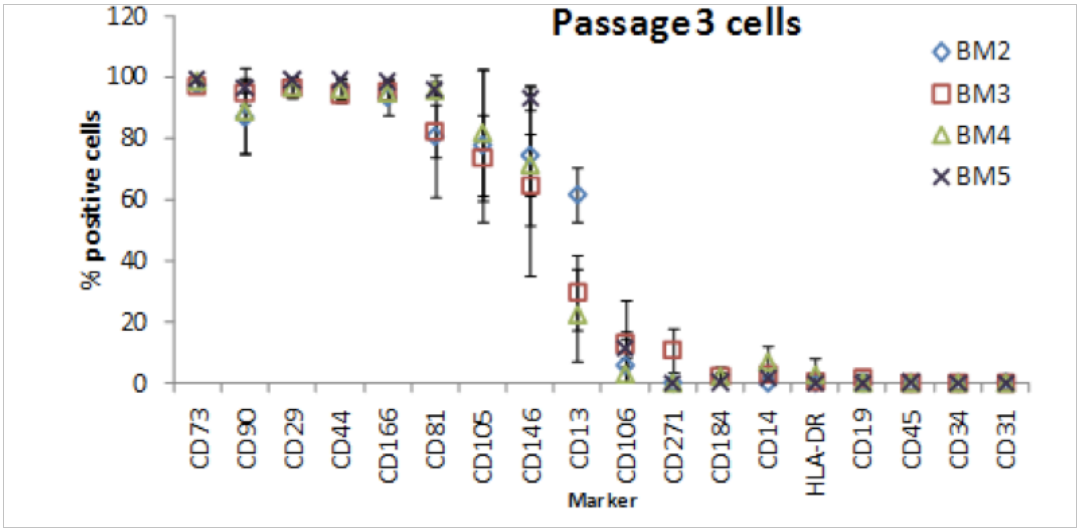

Figure 2. Phenotype of cultured MSCs (passage 3) from all donors cultured under standardized PurStem conditions in the project partner laboratories. Data points show the average value for each donor across the 4 partner laboratories. Error bars show the standard deviation of the values between different centers.

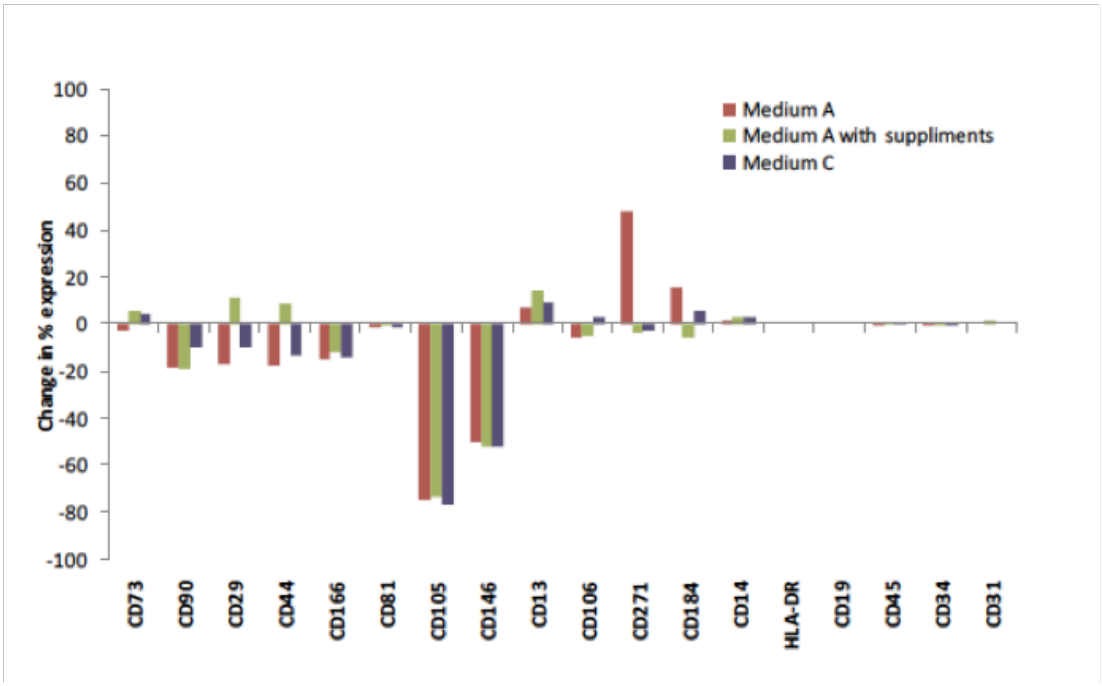

Figure 3. Difference in expression of cell surface markers of MSCs cultured in different media relative to MSC cultured under standardized PurStem conditions. Phenotypes of cells at Passage 2 were compared (Medium A (1% FCS, EGF, ITS), Medium A with supplements - IGF1 +FGF4 +Activin A and Medium C (Medium A with lower concentration of Ascorbic acid).

MSCs were also cultivated in special culture dishes (PureCoat Amine and Carboxyl, BD) to determine if cells could be cultured in serum-free media, supplemented with EGF, without plate coating to meet GMP requirements. PureCoat Amine dishes were suitable for serum-free culture, while PureCoat Carboxyl was unsuitable (Figure 4).

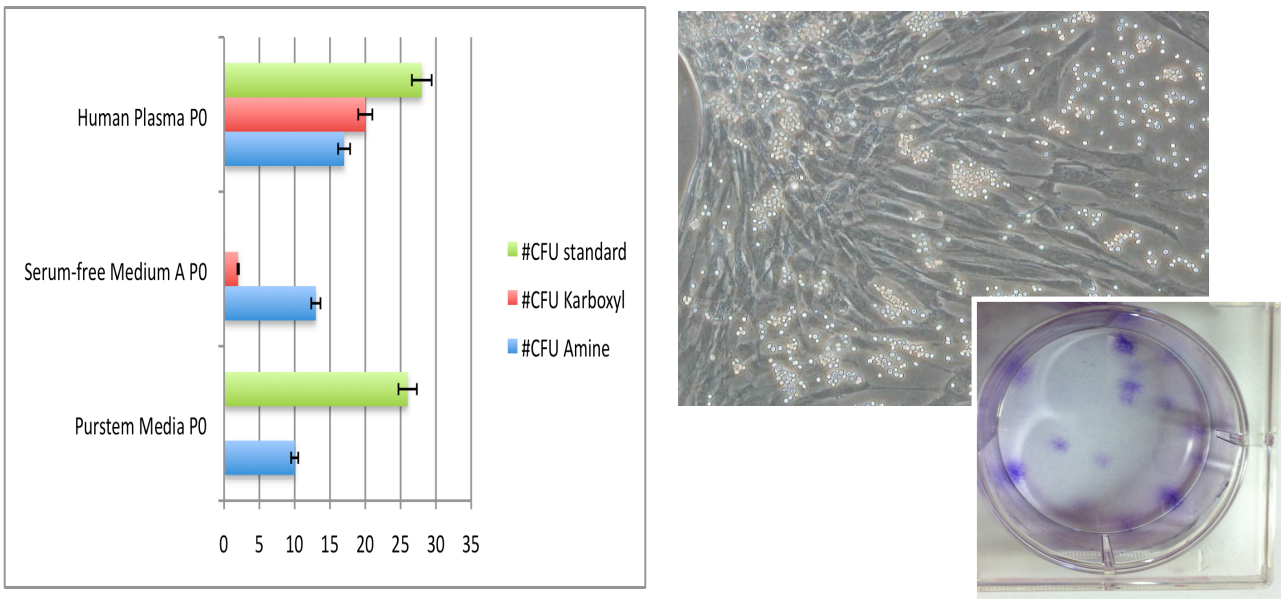

Figure 4. PureCoat Amine surface has been tested as beneficial for serum-free cultivation. No. of CFUs, crystal violet stained colonies (serum-free medium) and phase contrast microscopy of serum-free sample are presented. PureCoat Carboxyl surface is not suitable for both serum-free media and FCS containing media. Human plasma containing samples successfully proliferated in all culture vessels.

Differentiation potential of MSCs cultured in serum-free conditions

Moreover, the potential whether the MSCs grown under the optimized PurStem serum-free conditions differentiated towards mesenchymal lineages was examined.

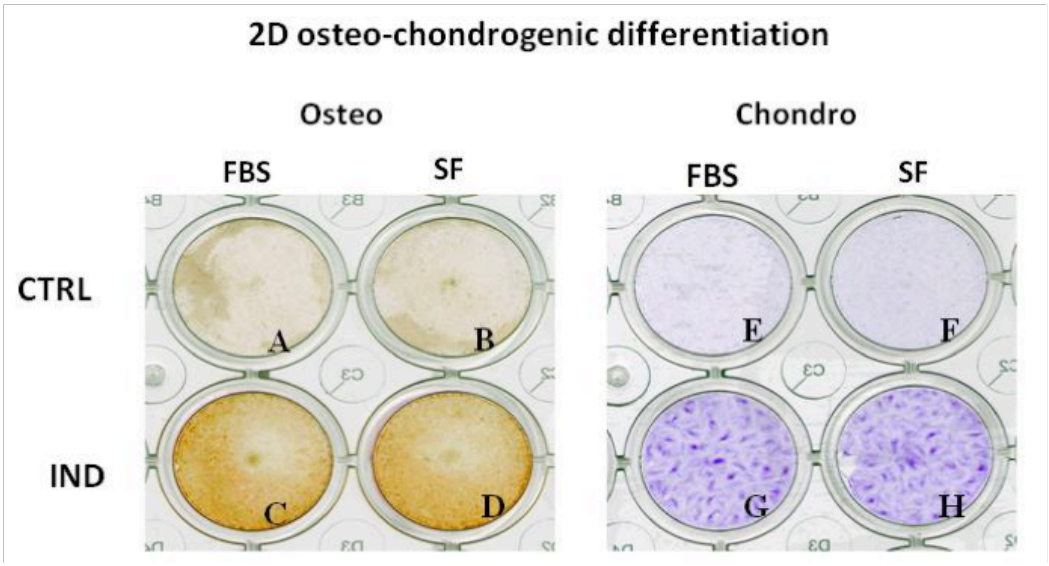

Figure 5. MSCs differentiation was induced with an osteogenic medium for 3 weeks in 10% FBS medium (C) or serum-free medium (D) or with a chondrogenic medium for 2 weeks in 10% FBS medium (G) or serum-free medium (H). Control cells were maintained in 10% FBS medium (A, E) or serum-free medium (B, F).

The serum-free medium contained a cocktail of recombinant growth factors specifically used to drive cells towards osteogenic or chondrogenic differentiation, in association with other components involved in normal cell homeostasis and metabolism. The osteo- and chondrogenic potential of serum free expanded cells was compared with the potential of cells grown in serum containing media (Figure 5). Results indicated that MSCs cultured under the PurStem serum-free conditions show an elongated and fibroblast-like phenotype. Nonetheless, they maintain the ability to undergo osteogenic and chondrogenic differentiation.

Serum-free cryopreservation

In classic serum-containing freezing media, serum exerts a protective function for the cells. BIOFREEZE (Biochrom AG, Germany) is a commercially available media free of animal-derived substances that is also free of genetically modified organisms. MSCs (passage 1) cells cultivated in serum-free media supplemented with EGF were cryopreserved using the standardised PurStem cryopreservation procedure and BIOFREEZE for comparison. There was no significant difference between the number of cells frozen and number of thawed viable cells (Figure 6). The number of recovered cells at passage 2 and viability of the samples immediately after thawing was significantly better for the standardised PurStem procedure than the BIOFREEZE procedure. However, after one consecutive passage (Passage 2), the viability of both protocols was comparable.

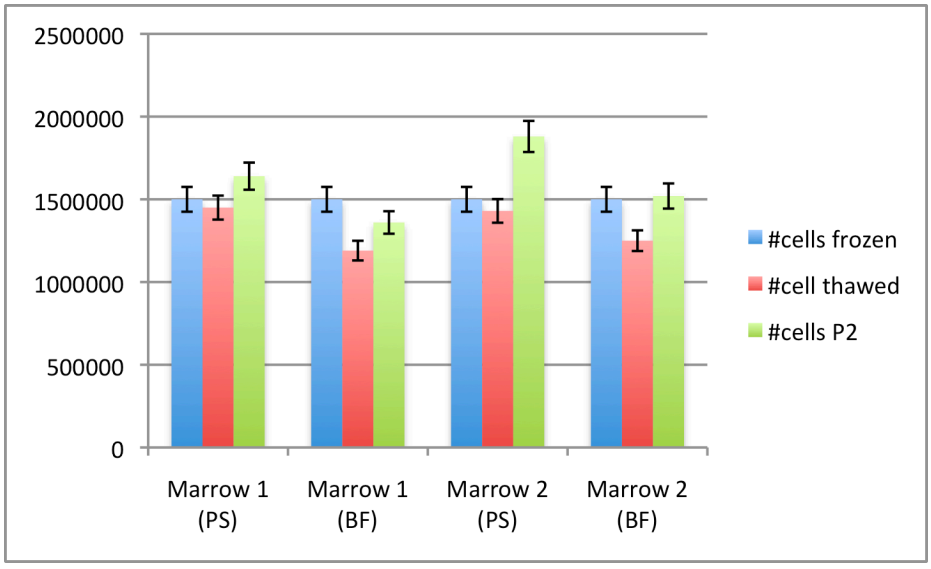

Figure 6. Cell No. after thawing and at P2. Standard PurStem (PS) cryopreservation protocol vs BIOFREEZE serum free cryopreservation media (BF).

Discussion

There has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells (MSCs). There is a strong possibility that this limited ability to produce stem cells will result in delays to the translation of new therapies to the clinic. The current state of the art has several weaknesses, in that, there are no standards for characterization, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSCs characterization lack specificity and cryopreservation protocols are not standardized. Critically, current production methods for MSCs require the use of animal products with major contaminant implications. One of the goals of this thesis and PurStem project in parallel was to identify GMP-compliant raw materials, develop an Standard operating protocol for small scale serum-free media production, and validate analytical assays for use as Quality Control release assays for the production of GMP-compliant cells for clinical applications.

What are the requirements for GMP production of therapeutic MSCs?

PurStem initiative aimed to prepare for the advanced future manufacturing demand for MSCs in support of the industrialization of stem cell technology. To enable MSCs to be used in the clinic in a safe and efficient manner, all cell based medicinal products must comply with EU safety requirements and legislation. GMP requires that raw materials and other consumables used in the production and quality control analysis of products are of GMP grade to ensure the consistency of release results and patient safety. Standard operating procedures (SOPs) and validation of quality control release assays are also essential for GMP compliance with EU regulations.

In order to advance the bioprocessing of MSCs for clinical applications, initial in vivo studies were conducted using the MSCs isolated and cultured using the PurStem unified protocols. Furthermore, a list of the GMP compliant raw materials was identified and an SOP for the small-scale production of serum free media was written.

To comply with the GMP requirements we strived to cultivate MSCs in coating free environment. In 4 bone marrow samples freshly isolated MSCs were separated using Ficoll density gradient centrifugation and CD271+ immunomagnetic separation, then analyzed by flow cytometry and seeded in serum free media supplemented with EGF in special culture dishes (PureCoat Amine and Carboxyl, BD), then compared with directly plated MSCs. Following 14 days in culture, colonies were stained and duplicated samples were frozen for further qPCR analysis of telomere length. Directly plated MSCs were seeded in three different media (PS standard medium with 10% FCS, Serum-free medium supplemented with EGF and Human plasma+pHPL containing medium) and 3 different surfaces were tested to optimize serum-free cultivation protocol.

Why are serum-free culture conditions needed?

Clinical application of MSCs will require expansion and differentiation of primary MSCs in order to obtain sufficient therapeutic cell numbers as it is not possible to get enough MSC for the clinical use during a single expansion. Usually, the cells have to be passaged 1-2 times, which increases the

risk of infectious contamination of the cell culture and also increases the time from the bone marrow harvest to the final production of the cellular treatment to 4-6 weeks.
Traditionally, MSC culture has been performed using bovine serum containing media. Bovine serum is the most widely used growth supplement for in vitro culture because of it possesses high levels of growth-stimulatory factors and low levels of growth-inhibitory factors. However, there are a number of risks associated with using animal derived products for the culture of therapeutic cells, including prion, viral or zoonose contamination (Mannello et al., 2007, Halme et al., 2006) and patient immune reaction (Tuschong et al., 2002, Selvaggi et al., 1997). In addition, there is a need for serum-free culture conditions for quality control of experiments between laboratories, as the variability of bovine serum can affect the reproducibility of results.

What are the current alternatives to animal-derived serum?

Human serum - An alternative to bovine serum albumin is autologous or allogeneic human serum. Autologous serum has proven to be as effective as bovine serum for the isolation and expansion of human MSCs (Stute et al., 2004), maintains higher cell motility compared to bovine derived serum (Kobayashi et al., 2005) and has eliminated the immune reaction observed with bovine serum cultured cells (Selvaggi et al., 1997 and Chachques et al., 2004). However, culturing MSCs in allogeneic human serum also results in growth arrest and cell death (Shahdadfar et al., 2005). Alternatively, fresh or frozen human plasma and platelets have been shown to be even more effective than bovine derived serum in maintaining human MSC proliferation (Muller et al., 2006). However, the use of human serum possess many of the same problems as bovine serum, such as variability in composition between donors and the collection of serum from patients with pathology, making the use of human serum an unattractive alternative to animal-derived serum.

Serum-free media - Unfortunately, the use of current serum-free conditions in culturing human MSCs selects for a subpopulation of cells that can survive serum deprivation and continue proliferating (Pochampally et al., 2004). A new complement of medium, growth factors, cytokines, etc. have to be developed specifically ensure consistent MSC cell surface receptor expression yielding uniform expansion of the MSC population in culture. Currently, there are commercially available serum-free media. Mesencult-ACF from Stem Cell Technologies and Thera Peak from Lonza were tested during the study with unsatisfactory results.

PurStem approach - PurStem project set out to identify the MSC “receptome” and use this repertoire of growth factor receptors to develop novel serum-free media suitable for large-scale MSC production. Goals of this thesis and PurStem project were achieved by: developing and validating a collaborative, standardized procedure for the isolation, culture and cryopreservation of MSCs that produced consistent cell types even when grown in different laboratories; using the wealth of information obtained from characterisation of the MSC “receptome” to develop low and serum-free culture conditions where MSCs can survive and proliferate, thereby reducing or eliminating potential contamination issues associated with current serum-based culture methods.

Can human MSC differentiation be ensured in serum-free media?

During passaging, MSCs tend to lose their ability to differentiate into specialized tissues (Sugiura et al., 2004). As early as in 1995, Gronthos and Simmons (1995) have explored the effect of 25

recombinant growth factors on the growth of marrow stromal cells. The best results were obtained with the combination of ascorbic acid, dexamethasone, platelet-derived growth factor BB (PDGF-BB) and epidermal growth factor (EGF). Jing-Xiang et al. (2004) have found that the 5 recombinant human monocyte colony stimulating factor (rh M-CSF) increases the number of CFU-F by 25% and the total number of MSC eight- to tenfold. Tsutsumi et al. (2001) have shown that mesenchymal stem cells expanded with fibroblast growth factor 2 (FGF-2) retain better differentiation ability when compared to MSCs expanded without this factor. These works have shown that the use of certain supplements or growth factors, most of which can be produced by recombinant technology, can lead both to a higher yield of mesenchymal stem cells and to the preservation of their ability to differentiate into specialized tissues.

In our hands, the osteo- and chondrogenic potential of serum free expanded cells was compared with the potential of cells grown in serum containing media. MSCs cultured under the PurStem serum-free conditions show an elongated and fibroblast-like phenotype. Nonetheless, they maintain the ability to undergo osteogenic and chondrogenic differentiation.

Significant/Novel Results

1) Standardized serum-free protocols were generated for MSCs isolation, sub-culture, cryopreservation, differentiation assays and xCELLigence profiling.
2) MSCs cultured under PurStem serum-free conditions maintain their osteogenic and chondrogenic differentiation potential.
3) Serum-free culture methods are GMP compliant and suitable for the culture of stem cells for use in clinical applications.

Conclusions

Stem cells offer a promising avenue to therapy for a wide range of human diseases. However, for this potential to be realized, a consistent and plentiful supply of well-characterized stem cells is essential. To date, there has been relatively little progress in the development of new culture technologies for the large-scale manufacture of mesenchymal stem cells (MSCs). The current state-of-the-art has several weaknesses, in that, there are no standards for the characterization, isolation or identification of MSCs from any tissue, nor are there standard protocols for differentiation of MSCs to various lineages. Additionally, surface markers used for MSC characterization lack specificity and cryopreservation protocols are not standardized. Critically, current production methods for MSC require the use of animal products with major contaminant implications. Therefore, in an effort to overcome these issues, this thesis and PurStem project in parallel seek to identify the MSC "receptome" and use this repertoire of growth factor receptors to develop novel serum-free media for MSC production.

The objectives were to:

- Examine existing methods and approaches to the preparation of MSCs
- Identify current best practice
- Standardize the technology by means of a unified operating protocol.
- Develop new methods of culture and new media formulations by identifying the repertoire of growth factor receptors that exists on the surface of the cell.
- Use this information to develop new media using a combinatorial approach to the selection of growth factor supplements to modulate and optimize the growth kinetics and differentiation of the cells.
- Utilize recombinant human growth factors and so benefit from freedom of reliance on serum.
- Identify new reagents from this effort that will be used to characterize the cells.

As a first step to achieving these goals, all partners agreed on current best practice MSC methods. Consequently, a collaborative standardized procedure for the isolation, culture and cryopreservation of MSCs was developed and validated in each partner laboratory. Using FACS analysis, preliminary work on identifying the "in vivo" MSC transcriptome and changes due in vitro culture commenced. In parallel, the repertoire of growth factors expressed on the surface of the MSC was identified. Furthermore, a list of novel antibodies for new surface antigens was characterised using a novel combination of transcriptional and biochemical approaches. With respect to the serum-free media formulation, preliminary studies examined MSCs viability in low serum conditions containing a number of growth factors and serum free conditions using growth factors in combination with various attachment factors. In order to advance the bioprocessing of MSCs for clinical applications, initial in vivo studies were conducted using the MSCs isolated and cultured using the PurStem unified protocols. Furthermore, a list of the GMP compliant raw materials was identified and an SOP for the small-scale production of serum free media was written.

A number of key results were achieved over the course of the project. Using the unified protocols for the isolation and characterisation of MSC, initial results revealed that there was variability between partners at the start, but these variations reduced over time. Cumulative population doublings throughout the culture process from primary to the end of passage 2 culture indicated that the culture process was relatively reproducible between partner institutions. Moreover, using FACS analysis, it was revealed that the standardised PurStem culture conditions produced cells with a consistent cell surface phenotype even when grown in different laboratories. Using FACS and real time PCR, the repertoire of growth factor receptors and active transcription factors in the "in vivo" MSC was characterised; initial results revealed that cultured MSCs were a heterogeneous population of undifferentiated and more-committed, less proliferative cells, which evolve during passaging towards cellular senescence.

A wealth of information was generated by the surface receptome analysis, where a list of growth factor receptors expressed on the surface of the MSC was identified. The receptome was used to develop the serum free media. In a further effort to optimize the serum-free media formulation, a range of growth factor combinations were examined, and it was found that MSCs can attach, survive and proliferate in low serum and serum free conditions.

In the end, a rigorous protocol for the isolation and culture of MSCs has been developed and validated. The repertoire of identified growth factors was used to:

- Narrow down the growth factor combinations for the serum free media formulation,
- Provide information on new reagents that can strengthen the release criteria for MSCs,
- Help identify new methods for directly isolating MSCs from tissue based on immunoselection rather than adherence
- Help enable new methods for endogenous manipulation of MSCs.

The ability to produce GMP-grade human MSCs has advanced the state of the art in MSCs standards of preparation and release criteria. As a part of the PurStem project we have generated new standard operating procedures for the isolation and growth of MSCs, as well as new criteria that will be used to define the MSC for current applications in tissue engineering. These will contribute to the optimization of GMP manufacturing and banking of cells for use in clinical trials initially and ultimately as a commercial product.

Our effort has contributed not only to optimal stem cell manufacturing processes for Regenerative Medicine applications in the short-term, but has also advanced our basic understanding of MSC biology by defining the surface receptome. This database will improve our knowledge of the pathways that control mobilization and growth of the "in vivo" stem cell in response to injury, setting the stage for the development of next generation therapies which will exploit the self-repair potential of adult stem cells or stem cell targeting.

Since the onset of the PurStem project, a greater awareness of the research required to advance the large-scale manufacture of stem cells for clinical applications is becoming more apparent. In an effort to highlight the research currently underway in Europe, the PurStem website was created and has been constantly updated over the course of the project. Furthermore, PurStem has made an imprint on the world stage, with several conference presentations in the United States, United Kingdom, Ireland and the Czech Republic.

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List of published articles and delivered scientific lectures

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Patents:

- Způsob pěstování lidských mezenchymových kmenových buněk, zejména pro léčbu nehojících se fraktur, a bioreaktor k provádění tohoto způsobu (PV/49/2006; P1057CZ00)

Bibliographic data	**Web of Science**	**SCOPUS**
No. of published items	22	17
No. of published items 2006-2013	21	17
Sum of the times cited	53	73
H- index	4	5

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- MOLINSKY, J. et al. Pattern of angiogenesis-related gene expression during in vivo growth of mantle cell lymphoma cell line JEKO-1. *Haematologica,* 2008, 93(s1), 98.
- SOUKUP, T. et al. Kmenové buňky zubní pulpy a periodoncia. *Chem. Listy,* 2008, 102, 393-394. **(IF=0,683)**
- SOUKUP, T. et al. Dental pulp stem cells and their characterization. *Acta Medica (Hradec Králové),* 2008, 51(1), 88.
- KARBANOVÁ, J. et al. Neural cell differentiation of dental pulp stem cells. *Acta Medica (Hradec Králové),* 2008, 51(1), 74.
- PYTLIK, R. et al. Spontaneous in vitro transformation of human lymph node and bone marrow stromal cells. *Experimental hematology,* 2006, 34(9), 78-79. **(IF=3,147)**
- MOKRÝ, J. et al. Kmenové buňky z pohledu imunologie. *Alergie,* 2006, 8(s2), 44-45.
- KIDERYOVA, L. at al. Spontaneous transformation of lymph node and bone marrow stromal cells from cancer patients. *Haematologica/The hematology journal,* 2006, 91(s1), 310.
- **SOUKUP, T.** et al. Biological properties and flow cytometric analysis of human dental pulp stem cells. *Cytotherapy*, 2006, 8(s2), 3. **(IF=3,553)**
- KARBANOVA, J. et al. Dental pulp stem cells – a source of cells for tissue engineering. *Cytotherapy,* 2006, 8(s2), 3. **(IF=3,553)**
- MOKRY, J. et al. Nestin – a useful marker in regenerative medicine. *Cytotherapy,* 2006, 8(s2), 12. **(IF=3,553)**

Scientific lectures:

(invited speaker and/or international conferences)

- **SOUKUP, T.** et al. Sledování biomarkerů kmenových buněk zubní pulpy pomocí průtokové cytometrie. DNA analýza 2010, Praha, 2010.
- **SOUKUP, T.** xCELLigence system and real time analysis of stem cells. ACEA**,** San Diego, 2010. **(invited speaker)**
- **SOUKUP, T.** et al. Vliv metotrexátu uvolněného in vitro z kostního cementu na proliferaci a buněčný cyklus mezenchymových kmenových buněk. DNA analýza VI., Praha, 2009.
- **SOUKUP, T.** et al. Kmenové buňky zubní pulpy a periodoncia. X. Brněnské dentální a implantologické dny, Brno, 2008.
- **SOUKUP, T.** et al. Radiosenzitivity of human dental pulp stem cells. Morphology 2008, Olomouc, 2008.
- **SOUKUP, T.** et al. Kmenové buňky zubní pulpy a periodoncia. Konference mladých chemiků, biochemiků a biologů, Milovy, 2008.

(vítězná přednáška v oboru biochemie a molekulární biologie – cenu udělila firma Sigma-Aldrich, Česká společnost chemická a Česká společnost pro biochemii a molekulární biologii)

- **SOUKUP, T.** et al. Vliv ionizujícího záření ^{60}Co na kmenové buňky zubní pulpy. Konference DNA analýza V., Praha, 2008.
- **SOUKUP, T.** et al. Potenciál kmenových buněk zubní pulpy a periodoncia v regenerativní medicíně. Implantologický seminář, Praha, 2008.
- **SOUKUP, T.** Mesenchymal Stem Cells – Facts and Mysteries. The University of Hong Kong, HKG SAR, 2007. **(invited speaker)**
- **SOUKUP, T.** Kmenové buňky zubní pulpy: Změny fenotypu ovlivněné kultivačními podmínkami. Mesenchymal Day, Liběchov, 2007. **(invited speaker)**
- **SOUKUP, T.** et al. Dental pulp stem cells: Phenotypic changes according to culture conditions. International Conference Analytical Cytometry IV., Brno, 2007.
- **SOUKUP, T.** et al. Stanovení základních biologických charakteristik a fenotypizace kmenových buněk v laboratoři tkáňových kultur. Konference DNA analýza IV, Praha, 2007.
- **SOUKUP, T.** et al. Mezenchymové kmenové buňky. zvaná přednáška v rámci projektu ESF „Zvýšení kvalifikace a flexibility absolventů doktorského studijního programu na LF UP“, Olomouc, 2007.
- **SOUKUP, T.** et al. Mezenchymové kmenové buňky u nemocných s osteoporózou. XVI. Ortopedické sympozium s mezinárodní účastí, Hradec Králové, 2007.
- **SOUKUP, T.** et al. Mezenchymové kmenové buňky u nemocných s osteoporózou. 2. morfologický postgraduální kurz, Hradec Králové, 2006.
- **SOUKUP, T.** et al. Spontaneous transformation of stromal cells from cancer patients. 3rd International Medical Postgraduate Conference „New frontiers in the research of PhD students“, Hradec Králové, 2006.
- **SOUKUP, T.** et al. In vitro and in vivo characteristics of spontaneously transformed human cells. Morphology 2006, Prague, 2006.
- **SOUKUP, T.** et al. Biological properties and flow cytometric analysis of human dental pulp stem cells. 2nd International Conference “Strategies in Tissue Engineering”, Würzburg, 2006. **(presentation O-1)**
- **SOUKUP, T.** Antigenic profile of mesenchymal stem cells isolated from the human dental pulp. 2nd Medical Postgraduate Conference „New frontiers in the research of PhD students“, Hradec Králové, 2005.
- **SOUKUP, T.** et al. Antigenic profile of mesenchymal stem cells isolated from the human dental pulp. I. Fakultní konference studentů doktorského studia, Hradec Králové, 2005. **(winner of the basic science section)**
- **SOUKUP, T.** et al. Isolation and cultivation of dental pulp stem cells. 43rd Congress of the Czech Anatomical Society and 42nd Lojda Symposium, Brno, 2005.

- **SOUKUP, T.** et al. Mesenchymal progenitor cells from vertebral body bone marrow. 43rd Congress of the Czech Anatomical Society and 42nd Lojda Symposium, Brno, 2005.
- **SOUKUP, T.** et al. Izolace, ex vivo expanze, diferenciace a fenotypizace mezenchymových progenitorových buněk získaných aspirací kostní dřeně obratlového těla. Analytická cytometrie III, Červenohorské sedlo, 2005.
- **SOUKUP, T.**, MOKRÝ, J. Bone Marrow-Derived Mesenchymal Progenitor Cells: Their Isolation, Characteristics and Potential Clinical Applications. Ms Chen Yang Foo Oi Telemedicine Ctr, Hong Kong, 2004.
- **SOUKUP, T.** et al. Degenerace žlutého vazu: Stanovení elastin/collagen fibres ratio. Onemocnění páteře, Ústí nad Labem, 2003. **(awarded lecture - cena Zdravotnických novin)**
- **SOUKUP, T.** et al. Ligamentum Flavum: Research Summary 1999-2003. Research Meeting - Schulthess Clinic, Zürich, 2003.
- **SOUKUP, T.** et al. Degenerative changes of ligamentum flavum: Histopathological study, 46th Congress of the Czech and Slovak Revmatologists with International Participation, Liberec, 2002.
- **SOUKUP, T.** et al. Interactive atlas of human embryology. Trends and perspectives of contemporary morphology, 40th Congress of the Czech Anatomical Society with International Participation, Plzeň, 2002.
- **SOUKUP, T.**, TUREK, Z. Role of the Ligamentum Flavum in Spinal Surgery: Histopathological Study. Prix de Medicine 2002, Laboratoires Fournier - Ambassade de France, Prague, 2002.
- **SOUKUP, T.**, HRADIL, J. Nový injikovatelný bioaktivní kostní cement pro páteřní chirurgii: Úprava metodiky biomechanického testování. SVK VFÚ, Brno, 2002.

Poster presentations:

(first author or presenting author)

- **SOUKUP, T.** Activation of p53, Cell Cycle Arrest and Senescence Following Irradiation of Human Mesenchymal Stem Cells – Single Cell Analysis. Single Cell Analysis Summit, San Diego, 2010.
- **SOUKUP, T.** PurStem – Revolutionising the Large-scale Production of High Quality Adult Stem Cells. Stem Cells World Congress 2010 – Embassy Suites, South San Francisco, 2010.
- **SOUKUP, T.** et al. Influence of ITS supplemented media on human dental pulp stem cells in vitro. World Stem Cell Summit 2009, Baltimore, 2009.
- **SOUKUP, T.** et al. Purification and ex vivo expansion of mesenchymal progenitor cells isolated from human vertebral body bone marrow. 2nd World Congress on Regenerative Medicine, Leipzig, 2005.
- SUCHOMEL, P., **SOUKUP, T.** Pyogenic Osteomyelitis of the Odontoid Process: Single Stage Decompression and Fusion, WORLD SPINE II, Chicago, 2003.
- **SOUKUP, T.** et al. Myoskeletální medicína: interaktivní výuková pomůcka pro studenty LF, 3. pracovní seminář České kineziologické společnosti na téma Kineziologie nohy a plantografie, Olomouc, 2003.
- **SOUKUP, T.** et al. Gait Analysis and a Model for 3D Analysis of the Spine: Present State and Future of Motion Analysis, 8th Congress of the Czech Society of Myoskeletal Medicine with International Participation, Liberec, 2002.

Grant projects participation:

- 7FP EU project – PURSTEM (11/2008 – 2011) - team leader

- GA ČR No. 304/09/1568 (2009 – 2011)

- VZ LF No. MSM 0021620820

- VZ FN No. MZO 00179906

- IGA MH NR/9182-3 (2007-2009)

- Nadace Tomáše Bati 2008 – „Srovnání fenotypu kmenových buněk zubní pulpy a periodoncia“

- Nadace Tomáše Bati 2007 – „Biologické charakteristiky kmenových buněk v lab. tkáňových kultur“

- Rozvojový projekt: Vybudování edukačního odborného morfologického centra LF UK v HK pro podporu rozvoje nových akreditovaných studijních programů (MŠMT 2006 č. 635-4c)

- Interní grant ROCHE pro postgraduální studenty LF v Hradci Králové (2005/2006)

- Grant FRVŠ 2529/G3/02 – Embryologie v 3D (2002)