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# 6

# Production of Clinical Grade Mesenchymal Stromal Cells

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## 1. Introduction

Mesenchymal stromal cells (MSC, MSCs) are cells firstly described 35 years ago in bone marrow (Friedenstein *et al*, 1976), but present basically in all adult and fetal tissues, where they reside in the vessel wall as part of the population of pericytes (Crisan et al, 2008). These rare cells ($10^{-6}$-$10^{-4}$ of nucleated cells in various tissues – Werntz, 1996) received special attention of biomedical researchers as they are easy to expand and able to differentiate in various cell and tissue types (Pittenger et al, 1999, Battula et al, 2009, and many others). Later, these cells were found to be little immunogenic and to have immunosuppressive properties, which they exert by action on T cells, B cells, NK cells and dendritic cells (Beyth et al, 2005; Corcione et al, 2006; Spaggiari et al, 2008; Spaggiari et al, 2007). Furthermore, MSCs do not necessarily need to differentiate into tissue of interest, but they can exert their therapeutic effect through secretion of various cytokines (Phinney & Prockop, 2007; Horwitz & Dominici, 2008). Use of these cells therefore appears to be a promising strategy for treatment of various disorders, including orthopedics, heart and vessel, or graft versus host disease (Shenaq et al, 2010; Mathiasen et al, 2009; Le Blanc et al, 2008).

Mesenchymal stromal cells cultivated in vitro are a mixture of cells of various clonogenic and differentiating properties, which are partly dependent on cultivation conditions, partly they are donor specific (Tsai et al, 2011; Friedl et al, 2009). There is no single marker which would distinguish MSCs from other fibroblastoid cells. Therefore, The International Society for Cellular Therapy set in 2006 minimal set of requirements which mesenchymal stromal cells should fulfill. These are: 1. adherence to plastic, 2. expression of CD73, CD90, and CD105 antigens, while being CD14, CD34, CD45, and HLA-DR negative, and 3. their ability to differentiate to osteogenic, chondrogenic and adipogenic lineage (Dominici et al, 2006). Every method used for production of MSCs must be shown to produce cells of above mentioned characteristics. There are several exception from these rules, however – for example MSCs derived from the adipose stromal vascular fraction are CD34 positive (De Ugarte et al, 2003), or can express HLA-DR in certain culture conditions (Tarte et al, 2010).

Though first clinical trial of mesenchymal stromal cells was reported as early as in 1995 (Lazarus et al, 1995), the transfer to clinics has been relatively slow and complicated by several issues, especially in the context of good manufacturing practice preparations of these cells. Among them are issues of cultivation medium, serum and supplementation used, suitable expansion systems and reproducibility of results. Mesenchymal stromal cells can be

freezed and stored (Haack-Sørensen & Kastrup, 2011), but it is not clear if autologous (customer specific) or allogeneic („off the shell or „one size fits all") products are preferable. As a result of these problems, there is still no standard or universally accepted or preferred way how to produce mesenchymal stromal cells for clinical use.

In this review, we will focus on application of good manufacturing practice standards principles to MSC production, with reference to choice of starting material, cultivation media, serum and supplements, cultivation systems, cultivation process, quality control, efficacy and safety concerns. References to universal GMP principles will be made as appropriate and selected choices of clinical grade cultivation components will be provided, as authors knowledge permits. As cellular therapy is a quickly evolving field (both from the practical and regulatory point of view), we have to state that following paragraphs will not be by any means exhaustive, and they may be also subject of changes during the time. On the other hands, some of the outlined principles may apply to other somatic cell products as well.

## 2. Mesenchymal stromal cells as advanced medicinal products

Mesenchymal stromal cells (with some exceptions mentioned below) belong to advanced therapy medicinal products (ATMPs), according the EC Regulation No 1394/2007, together with other somatic cell therapy medicinal products. According to this regulation, somatic cell therapy medicinal product means a biological medicinal product that has the following characteristics: 1. contains or consists of cells or tissues that have been subject to *substantial manipulation* so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered, or of cells or tissues *that are not intended to be used for the same essential function(s) in the recipient and the donor* (italics added by authors); 2. is presented as having properties for, or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues.

There is a significant trend to establish risk-based systems for regulation of ATMPs including mesenchymal stromal cell - based therapy products within the regulatory systems worldwide, although some countries did not follow this route so far (e.g., Australia). Numbers of governments have moved to introduce specific regulations for this sector, while others try to develop the traditional model of regulation for medicines and enable the regulatory authorities to respond to technology changes. The International Conference on Harmonization (ICH) established in 1990 in order to harmonize different regional requirements for registration of pharmaceutical drug products has no specific guidance document for cell products at the moment, although some guidance may be applicable (e.g. ICH S6, ICH Q5A-E). Clearly, there is insufficient worldwide unity of the regulatory approaches to cell based products at the moment.

Recently, the EU, USA, and Canada have implemented new systems for the regulation of ATMPs that are risk based oriented and specific for the geographic areas governed by appropriate local regulation.

The EU system formulates minimum quality and safety standards for harvesting, procurement, testing, processing, preservation, storage and distribution of human cells that need to be documented including donor selection procedures, traceability and adverse event reporting processes, GMP-based quality system, data and confidentiality protection. Within the EU, to assess the quality, safety and efficacy of ATMPs, including mesenchymal

stromal cells, Committee for Advanced Therapies (CAT) was established at the European Medicines Agency in accordance with Regulation (EC) No 1394/2007 on advanced-therapy medicinal products. The main responsibility of the CAT is to prepare a draft opinion on each ATMP application submitted to the European Medicines Agency, before the Committee for Medicinal Products for Human Use (CHMP) adopts a final opinion on the granting, variation, suspension or revocation of a marketing authorization for the medicine concerned. This decision is subsequently formalized by the decision of the European Commission that is binding in all EU member states.

The US FDA review process is conducted by the Office of Cellular, Tissue and Gene Therapies (OCTGT), Center for Biologics Evaluation and Research (CBER), under the Code of Federal Regulations Title 21 Parts 1270 and 1271. Health Canada regulates mesenchymal stromal cell derived products as medicines under Schedule D of the Food and Drugs Act. Health Canada also developed a regulatory framework under the Food and Drugs Act, "The safety of human cells, tissues and organs for transplantation regulations" (CTO Regulations 2007), which specifies requirements for the establishment of licensing and processing quality standards for cells, tissues and organs.

The Australian´s Therapeutic Goods Administration (TGA) plans to introduce new framework for human cell and tissue therapy products with a classification where mesenchymal stromal cells will fit into a Class 3 product – "A cell or tissue processed in a manner that may alter the structure and properties of the cell or tissue but does not purposefully alter the biological activity." This class of products will require: TGA Licensed Manufacturer, Relevant cGMPs, Good Tissue Practice Standards, and TGA pre-market approval.

The regulatory systems distinguish between cell products with substantial manipulation or without. Now, it is clear that in vitro cultivation represents substantial manipulation for cells isolated from human body, making from naturally occurring cells an artifact, changed by the unnatural cell culture environment. Substantial manipulation involves also cell purification or enrichment, for example their selection by monoclonal antibodies against CD34, CD49a, or CD271 antigens, as outlined below. On the other hand, the term „substantial manipulation" does not apply to simple isolation of bone marrow mononuclear cells or adipose tissue stromal vascular fraction, and also can be argued that cells isolated in this way are intended for use for similar purposes as they fulfill in the body (i.e., regeneration of damaged or aging tissues). Therefore, it can be argued that the mentioned EC Regulation does not apply to such products, though this is still under debate. This does not mean, however, that the harvesting, isolation and preparation of these otherwise unmanipulated cellular products for clinical use does not require the adherence to good manufacturing practice (GMP) principles, but regulatory requirements in these cases are similar for blood banking products and therefore these cells may be prepared in suitable transfusion or blood banking facilities. As preparation of crude cell mixtures is less difficult than preparation of better defined cell populations, these were also used in preclinical and clinical trials, sometimes with encouraging results (Hernigou et al, 2005; Chochola et al, 2008; Akita et al, 2010). There is also a frank exception from the EC Regulation No. 1394/2007: custom-made (hospital) ATMPs, which are prepared on a non-routine basis according to specific quality standards, if they are used within the same member state in a hospital under exclusive professional responsibility of a medical practitioner to comply with a medical prescription for a custom-made product for an individual patient (Sensebé, 2010).

The following text focuses mostly on further manipulated MSCs, as production of such cells are much more complex and basic principles of good manufacturing practice as well as national and international regulation requirements have to be followed.

## 3. Good manufacturing practice principles

It has to be understand that good manufacturing practice guidelines are not instructions on how to manufacture products. Rather, these are series of principles that must be fullfilled during the manufacturing process. Their goal is to obtain a final product from defined materials, by a defined, documented and traceable way, by trained operators. Currently, cell products regulated as medicines must comply with the GMP for Medicinal Products. However, the GMP for Medicinal Products is not yet fully adapted for dealing with the unique circumstances of cell based products. The main principles are as follows:

**Materials.** Raw materials have to be of a documented quality. They should be certified by their manufacturer and their batches should be registered. This does not necessarily mean that all materials have to be of clinical grade, though this is clearly an advantage. When the clinical grade material is not availlable, sufficient documentation about its production and about composition of individual batches has to be obtained to minimize the risk of its contamination by undesired elements. The documentation about used materials have to be preserved for legally determined time period.

**Manufacturing processes.** Manufacturing processes have to be clearly defined by a set of instructions known as standard operational procedures. These should be written in clear and unambiguous language and easily availlable for operators.

**Documentation.** Each part of the manufacturing process have to be documented, beginning from the storage conditions of raw materials (freezer and fridges temperatures) to the final product. These records should demonstrate that the standard operational procedures were in fact followed and that the quality of the product is as expected. Any deviations from standard operational procedures have to be documented.

**Validation.** National legislations have usually sets of recommended procedures for certain parts of the manufacturing process (e.g., the required tests for bacterial contaminations are described in pharmacopoiea). These procedures are usually designed for conventional drugs and cannot be allways used for somatic cell therapy products (e.g., sterilization, microbial tests of final product). The process called validation means the comparison of alternative procedures to the customary ones and proofs that these deviations from standard procedures bring desired outcomes.

**Standardization.** For good management of internal quality controls is a must. At present, there are also many programs of external quality controls performed by national authorities or commercial subjects. Manufacturer shall control storage areas to prevent mix-ups, deterioration, contamination, cross-contamination, and improper release or distribution of products. The storage temperature must be validated for each type of product and it is convenient to use devices with appropriate certificates.

Also set of standards have to be adopted for release of the product and these standards have to be followed and release criteria for every batch have to be documented.

Requirements for cellular products are also mentioned in International Standards for Cellular Therapy Product Collection, Processing and Administration (Fourth Edition, Version 4.1, April 2011) made by FACT-JACIE. These Standards are designed to provide

minimum guidelines for programs, facilities, and individuals performing cell transplantation and therapy or providing support services for such procedures.

**Traceability.** Records of manufacture (including distribution) that enable the complete history of a batch to be traced are retained. A system is available for recalling any batch of product from sale or supply. If undesired effect of the product occur, the causes for possible quality defects have to be investigated and appropriate measures have to be taken to exlude the defective batch from further use and to prevent recurrence of possible mistakes. Also, database of undesired drug effect should be established (pharmacovigilance).

**Training.** Operators have to be fully trained in standard operational procedures and their knowledge should be periodically examined.

GMP requirements are regulated by national and international legislatives and adherence to their principles is controlled by special agencies – in Europe, it is EMA (European Medical Agency), in the United States the FDA (Food and Drug Administration). Other countries, as Australia, Canada, Japan, Singapore or United Kingdom have highly developed GMP requirements. In other countries, especially in the developing world, the World Health Organization (WHO) version of GMP is used by pharmaceutical regulators and the pharmaceutical industry. Control of adherence to the GMP principles is performed by regular inspections by the governmental agencies.

## 4. GMP facilities

Good manufacturing practice facilities are the basic prerequisites for GMP preparation of medicinal products. They are designed to create the appropriate production environment, to prevent product contamination by raw materials and cross-contamination between batches and to ensure that standard operational procedures may be followed as intended. Again, GMP facilities for somatic cell therapy products may differ from facilities designed for manufacturing of conventional drugs.

Cleanroom desings should in general complie to International Standard ISO 14644 – Cleanrooms and associated controlled environments. ISO 14644 consists of eight parts:

- ISO 14644-1: Classification of air cleanliness
- ISO 14644-2: Specifications for testing and monitoring to prove continued compliance with ISO 14644-1
- ISO 14644-3: Test methods
- ISO 14644-4: Design, construction and start-up
- ISO 14644-5: Operation
- ISO 14644-6: Vocabulary
- ISO 14644-7: Separative devices (clean air hoods, gloveboxes, isolators and mini-environments)
- ISO 14644-8: Classification of airborne molecular contamination

It is above the scope of this chapter to run into details. In following examples, ISO 14644-1 requirements for airborne particulate cleanliness (Table 1), and scheme of contamination control concept following ISO 14644-4 (Figure 1) are shown.

The ascending requirements for cleanliness (from rooms class C to process core class A) is achieved by elaborate air conditioning systems, which ensure the highest pressure in the process core, with pressure gradient descending to peripheral parts of the facility. Air is blowed into the facility by systems of high-effective or ultrahigh-effective particle filters

(HEPA or UEPA). Between spaces of different classes of cleanliness, filters are designed for decontamination of materials as well as operating personell.
As is clear from what was written above, construction and running of GMP facility is very expensive. These expenses are naturally calculated to the final cost of the product. However, for small GMP productions, e.g. university based, or for Phase 1 clinical trials, solutions also exist (Xvivo production systems - www.biospherix.com, and others).

| ISO classification number | Maximum concentration limits (particles/m3 of air) for particles equal to or larger than the considered sizes shown below | | | | | |
|---|---|---|---|---|---|---|
| | 0,1 µm | 0,2 µm | 0,3 µm | 0,5 µm | 1 µm | 5 µm |
| ISO Class 1 | 10 | 2 | | | | |
| ISO Class 2 | 100 | 24 | 10 | 4 | | |
| ISO Class 3 | 1 000 | 237 | 102 | 35 | 8 | |
| ISO Class 4 | 10 000 | 2 370 | 1 020 | 352 | 83 | |
| ISO Class 5 | 100 000 | 23 700 | 10 200 | 3 520 | 832 | 29 |
| ISO Class 6 | 1 000 000 | 237 000 | 102 000 | 35 200 | 8 320 | 293 |
| ISO Class 7 | | | | 352 000 | 83 200 | 2 930 |
| ISO Class 8 | | | | 3 520 000 | 832 000 | 29 300 |
| ISO Class 9 | | | | 35 200 000 | 8 320 000 | 293 000 |

Table 1. Requirements for airborne particulate cleanliness

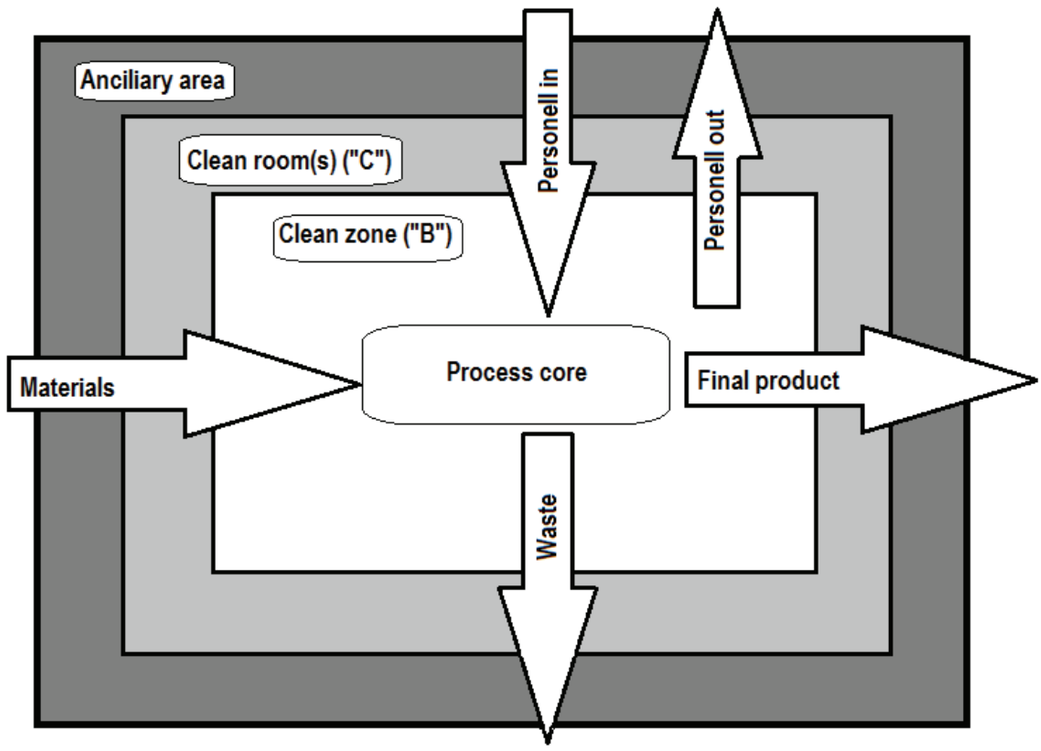


Fig. 1. Scheme of clean facility for GMP. Classification of rooms matches the requirements for airborne particulate cleanliness. For example, room of GMP class C requires cleanliness ISO Class 8 or higher

## 5. Choice of the starting material

MSCs are most easily obtained from bone marrow or adipose tissue. Other tissues of interest involve cord blood, amniotic fluid, or trophoblast.

### 5.1 Autologous or allogeneic?

Use of autologous material may be preferable for number of reasons. It eliminates or significantly reduces risks of disease transmission and overcomes the problems with a suitable donor selection. This is true even if we assume that MSCs are non-imunogenic and suitable for use across histocompatibility bariers (Niemeyer et al, 2006, Hare et al, 2009), as cells from different donors do not grow equally well. Also, there are reports that MSCs grow better in autologous plasma than in fetal calf serum (Stute et al, 2004).

On the other hand, the indisputable advantage of allogeneic cells is the possibility of their cryopreservation for later use as "off the shelf" product. This means that for acute or unpredicted indications (trauma, burns) there will be no need to wait several days or weeks untill autologous cells become availlable. Also, the quality tests, including sterility, differentiation or immunosuppressive abilities of cells, and viability of cells after thawing may be performed in full before the use of the product. Use of allogeneic, well characterized and quality-controlled cells also solves the problem of unpredicable growth of MSCs from different donors, as is autologous setting, adequate number of good quality cells may not be allways obtained. Allogeneic MSCs have been used successfully in treatment of graft versus host disease (Le Blanc et al, 2008), or myocardial infarction (Hare et al, 2009). Most donors in Le Blanc´s and all donors in Hare´s work were unrelated and HLA-mismatched individuals.

The choice of autologous versus allogeneic cells will be also guided by the wider settings of their therapeutic use. For example, in academic settings, where small-scale production is sufficient and customarized production of MSCs is feasible, autologous products might be preferrable. Also, it is possible that in certain countries it would be easier to obtain authorisation for autologous rather than for allogeneic products. However, in large-scale production setting, use of larger batches of an allogeneic product is probably inevitable.

### 5.2 Bone marrow

Bone marrow was the first source of MSCs for experimental and later for clinical use. For experimental use, it is still probably the best and most accessible, as small amounts of bone blood can be easily aspirated in local analgesia. Also, for small-scale experimental MSC cultivation, local hematology department may provide an easy access to bone blood when bone marrow samples from patients with known or suspected hematological disorder are taken for diagnostic purposes. However, one must be careful when MSCs are cultivated from hematological patients, as in certain diseases (acute and chronic leukemias, multiple myeloma, myelodysplastic syndrome) mesenchymal stromal cells may have slightly different properties from MSCs from healthy bone marrow (Garaoya et al, 2009), and may even harbor chromosomal abnormalities (Blau et al, 2007).

For larger scale and therapeutic purposes, where several hundred mililiters of bone marrow blood is necessary, bone marrow must be harvested under general anesthesia. Harvest is usually performed from posterior iliac crests similarly as for bone marrow transplantation

for hematological disorders, i.e., in collection bags of vessels with an anticoagulant, usually heparin. There are several recommendations about the volume that should be taken in one aspiration, but they are somehow contradictory. Fennema recommends to harvest at least 8 ml portions, as in lower volume the yield of nuclear cells and MSCs in unpredictable. Also, second portion of up to 10 ml may be taken from the same site without significant loss of quality due to dilution by peripheral blood (Fennema et al, 2009). On the other hand, in older work Muschler concludes that aspiration of more than 2 ml of bone blood results in smaller number of colony forming units -fibroblast (CFU-F) (Muschler et al, 1997). It may be therefore prudent for each centre to establish its own aspiration protocol, which yields the best results in their settings.

For isolation of mononuclear fraction, several methods are availlable. For smaller volumes, Ficoll centrifugation method is usually used, and GMP-compatible Ficoll is availlable. In larger volumes, bone marrow is usually processed on blood separators, which is similar to harvest of peripheral blood progenitor cells. Sometimes, the older starch or dextran sedimentation methods are still at use. In any case, closed isolation system is preferrable for mononuclear cell isolation. This may be challenging especially with small bone marrow blood volumes. Therefore, the alternative is to seed bone marrow mononuclear cells on plastic without previous enrichment, with or without preceeding red cell lysis (Tarte et al, 2010, online supplement; Horn et al, 2008; Horn et al, 2011).

### 5.3 Adipose tissue

Multipotent stromal cells in adipose tissue reside in stromal vascular fraction (SVF), which can be easily separated from fat cells after collagenase digestion (Zuk et al, 2002; Gimble & Guilak, 2003). Adipose tissue is richer source of MSCs than bone marrow, as these cells account for almost 2% of cells in SVF (Valle et al, 2009). This means that from 200-500 g of fat, 100-300x$10^6$ MSCs can be obtained, a number that might be used for treatment even without further expansion (unpublished data). Adipose tissue mesenchymal stromal cells are CD34 positive and they may differ from bone marrow stromal cells a little, but not by their differentiating potential into three main lineages (Kern et al, 2006).

Adipose tissue MSCs may be obtained after lipoexcision or lipoaspiration. If any of these procedures has a clear advantage in number or quality of cells over the other, is still a matter of debate (Torio Padron et al, 2010), but lipoaspiration is far less invasive procedure. Also, lipoaspirates are superior to excisates from the point of view of their stability, as the cell number in aspirates remains stable even after 24 hours, in contrary to rapid decrease of their yield in the excided tissue (Bieback et al, 2010). There is also uncertainity about the part of the body which is the richest in adipose tissue-derived MSCs (Fraser et al, 2007; Jurgens et al, 2008), but this does not seem to be of great clinical importance. On the other hand, it was established that higher aspiration pressures (-350 mm Hg) are preferrable to lower ones (-700 mm Hg), as they lead to higher cell yield (Mojallal et al, 2008).

The greatest disadvantage of adipose tissue in contrast to bone marrow is the necessity to digest the starting material by collagenase. From the technical point of view, besides manual method there are also automated devices which can produce SVF by good-manufacturing practice compatible method (TGI1200, Cytori Celution, Adistem et al). GMP compatible collagenase also exists, but is very expensive (Brooke et al, 2009). However, the advantages of adipose tissue seem to prevail and therefore, it may be expected that it will become more popular for MSC production than bone marrow.

### 5.4 Trophoblastic tissues

In general, trophoblastic tissues present an excellent source of cells for stem cell therapy, as they are abundant, their use is not connected with any ethical problems, and they contain developmentally young and putatively more plastic stem cells. The use of umbilical cord blood is well established in transplantation medicine (Wagner & Gluckman, 2010). Mesenchymal stromal cells can be retrieved from cord blood, Wharton jelly, and placenta (Wang, 2004; Flynn et al, 2007; Troyer & Weiss, 2008; Brooke et al, 2009). Mesenchymal stromal cells from trophoblastic tissues have been shown to have similar transcriptome, proteome, immunsuppressive and differentiation abilities as MSCs from bone marrow (Jones et al, 2007; Tsai et al, 2007; Barlow et al, 2008). Of these, umbilical cord blood MSCs were first described and probably most extensively characterized. They have higher proliferation capacity than bone marrow mesenchymal cells, but they are quite rare and even in experienced laboratories they can be successfully isolated from only about two thirds of cord blood units (Kern et al, 2006). Isolation of Wharton jelly and placental MSCs is in general similar to isolation of adipose tissue stromal cells in that digestive enzymes have to be used. However, they are more abundant and more easily isolated than MSCs from the cord blood.

One protocol for GMP isolation and expansion of placental MSCs was described recently (Brooke et al, 2009). Briefly, the placenta was aseptically collected and minced in small pieces, which were then digested by collagenase, type 1, and DNA-se I in Dulbecco´s modified Eagle´s medium, low glucose (DMEM, LG). After digestion, centrifugation tubes were pulse spun to remove large particular matter and mononuclear cells from the suspension were retrieved after centrifugation on Ficoll-Paque. Then adherent cells were isolated by cultivation on plastic for three days, in DMEM-LG with 20% FCS and 50 mg/l gentamycin. Cells were propagated for a total of five passages and cells not required for further propagation were cryopreserved after each passage.

After isolation, 74% of cells were found to be CD45+ leukocytes and 0.6% of cells were CD73+CD105+. Initial propagation in eight 175 $cm^2$ flasks yielded 40-100x$10^6$ adherent cells, from which still approximately 25% were CD45+ (P0). Percentage of CD45+ cells rose to more than 50% during first passage (P1), but fell quickly under 1% in following passages (P2-P5). In first passage, there was 40% of CD73+CD105+ cells and the percentage was above 90% in successing passages. Cell recoveries after cryopreservation were 96% from P2, 100% from P3, and 60% from P4 and P5. 120x$10^6$ MSCs from one placental unit was infused to a patient suffering with acute myeloid leukemia, who was co-transplanted with two units of umbilical cord blood (HLA-mismatched with infused MSCs). Though the hematopoietic engraftment could not be fully evaluated because of early death of the recipient, the infusion itself was reported to be uneventful.

This report shows that large numbers of MSCs can be obtained from human placenta. However, at least two passages have to be employed to deplete the final product from hematopoietic cells. The manufacturing procedure was reported to be labour-intensive and time consuming, using the open system of plastic cultivation flasks. However, this report shows that placental tissue can be used as an alternative source to bone marrow or adipose tissue for allogeneic applications, as is engraftment or treatment of graft-versus-host disease in blood progenitor cell transplantation setting.

### 5.5 Enrichment of starting material for MSCs

Mesenchymal stromal cells do not have any particular markers or antigenes to allow for an easy separation, as in case of CD34+ or CD133+ stem cells, or CD3 lymphocytes. Essentially,

two ways of enrichment of mononuclear cells for MSCs in the starting population are depletion of lineage-positive cells, or selection for MSC-containing fraction. RosetteSep© Mesenchymal Enrichment Cocktail (StemCell Technologies) contains monoclonal antibodies against hematopoietic cells (CD3, CD14, CD19, CD38, CD66b and Glycophorin A) and is mixed with bone marrow blood before separation on Ficoll gradient. Lineage positive hematopoietic cells form rosettes with erythrocytes and sediment with them. For selection of MSC-containing fraction, antibodies against CD271 (Jarocha et al, 2008; Poloni et al, 2009), or against CD105 (Jarocha et al, 2008), were used. Besides, while cited studies reported good results, Bierback reported worse results for RosetteSep© and CD271+ separation compared to plastic adherence, at least when MSCs were grown in platelet lysate (Bieback et al, 2009). Furthermore, monoclonal antibodies are expensive and they add another element into altogether complex process of GMP isolation and expansion of MSCs. If no new, revolutionary strategy for MSC enrichment will emerge, it is therefore unlikely that enrichment of starting material will become a standard procedure in GMP mesenchymal stromal cell production in the near future.

## 6. Cultivation conditions

The choice of cultivation conditions is of uttermost importance, with respect to GMP requirements. All components of the cultivation system should be fully characterized, certified or validated, and their robustness and stability of performance of the whole system have to be assured. However, in practical way, this might be quite challenging. As patented techniques and formulas are used for production of many ingredients, from surface treatment of cultivation vessels to the formulations of medium composition, both the researcher and regulatory agency have sometimes to rely on incomplete information. From this point of view, it is questionable whether the newest solutions just introduced to the market are always preferrable to older and well-tried technologies.

### 6.1 Cultivation vessels and systems

Traditionally, MSCs were cultivated in open systems. There is a plethora of companies (Corning, Nunc, TPP, to name at least few), that produce plastic flasks suitable for research-grade cultivation of MSCs. However, these do not seem to be optimal for clinical-grade production for several reasons:

1. Though these vessels are manufactured as sterile, tissue culture treated and apyrogenic, they are not certified for GMP-production.
2. They have to be opened before each manipulation. This was overcomed e.g. in RoboFlask™ produced by Corning, which have silicon rubber seal that can be repeatedly penetrated by injection needle without the need to open the vessel.
3. Classical flasks are small and difficult to manipulate. For production of clinically meaningful numbers of MSCs, tens or even more than hundred of these flasks would be needed for every single patient. This may be partially overcomed by use of larger flasks with several cultivation surfaces (e.g., CellSTACK® Culture Chambers, HYPER*Flask*®Culture Vessels, both Corning), on the other hand, the visual assessment of culture grow is very difficult under these conditions. Small bioreactors (CellCube®, Corning) offer as much as 80, 000 cm2 culture surface and accessories, as are setup kits, oxygenators, oxygen probes, etc.

4. There is a need for reseeding of MSCs after they reach critical density. Traditionally, this has been performed by trypsinization, centrifugation and reseeding of cells to new flasks. This is labour-demanding, expensive and increases the risk of microbial contamination. Possible solution of this problem may be the dynamic culture surface expansion, as described by Majd (Majd et al, 2009). The bottom of the cultivation vessel was made from high-extension silicon rubber, which could be mechanically expanded by iris-like device from the initial area of 10 $cm^2$ to area of 80 $cm^2$. In this device, cells were grown in constant densities for more than 9 weeks. Quick and hands-free harvest of adherent cells can be performed by several robotic systems, as is Tecan Freedom EVO (Tecan Group LTD).

Solutions mentioned above are mostly still suitable for small-volume production. However, larger robotic systems compatible with good manufacturing practice and good tissue practice principles, are available as well. Tecan Cellerity™ is a fully robotic modular system with several possible configurations, including HEPA filtred clean bench, robotic CO2 incubator, media refrigerator, etc. One possible configuration is shown on Figure 2. As is clear, these are already very complex and expensive solutions for large-scale commercial production. For smaller manufacturers, reasonable compromise between optimal and realistic will have to be achieved.

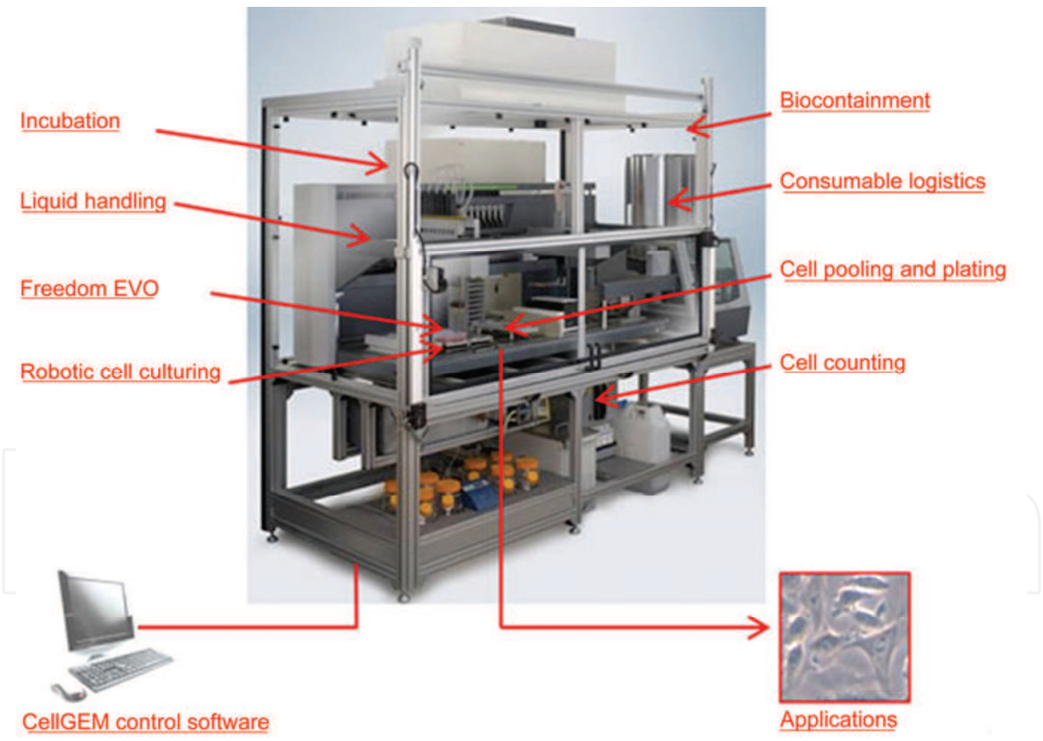


Fig. 2. Tecan Celerity configuration employing laminar box, incubator, liquid handling, cultivation media storage (down, left) and automatic processing of cell cultures

### 6.2 Choice of cultivation medium

A variety of cultivation media for mesenchymal stromal cells currently exist. Most commonly used are research-grade media DMEM (Dulbecco´s modified Eagle medium)

low-glucose, IMDM (Iscove´s modified Dulbecco´s medium) and alpha-MEM (minimal essential medium). The first of them is most commonly used and is present in EMEA approved GMP-compliant medium (Haack- Sørensen et al, 2008). It was shown that DMEM is preferrable to IMDM with the respect of preservation of MSC "stemness" (Pierri et al, 2011). However, alpha-MEM was found to better preserve osteogenic properties of MSCs than DMEM (Coelho et al, 2000), and in at least one work (Lange et al, 2007) also to lead to higher CFU-F retrieval in primary expansion (P0). Superiority of alpha-MEM over DMEM with respect to MSC expansion was found also in our own unpublished experiments.

There is a number of expansion media claimed to be GMP compliant: the LP02 basic medium (Lange et al, 2007), or the CellGro™ medium for hematopoietic stem cells (Pytlík et al, 2009). However, both of these need to be supplemented with fetal calf serum or some of its human alternatives (see below). Serum-free chemically defined media for mesenchymal stromal cells were also developed. Instead of serum they contain attachment factors for adherent cells and sometimes they have to be supplemented with recombinant cytokines. Of course, these media are all patented, thus the researcher does not know in full what is their exact composition. StemPro® is a serum-free medium pioneered by Invitrogen. While one group (Hartmann et al, 2010) were unable to cultivate MSCs in it without supplementation with 2% human serum (Hartmann et al, 2010), another group was more successfull after pre-coating of cultivation vessels with CELLStart™ xenogeneic free substrate (Invitrogen) or with human fibronectin and adding recombinant PDGF-BB, FGF-2 and TGF-beta (Chase et al, 2010). Another serum-free medium is MesenCult© ACF (StemCell Technologies) (Hartmann et al, 2010), which, however was not found comparable with DMEM and human platelet lysate in our hands (Matějková et al, unpublished data).

### 6.3 Choice of serum

First published results of clinical trials with MSCs used fetal calf serum (FCS) as a supplement to culture medium (Lazarus et al, 1995; Koç et al, 2000; LeBlanc et al, 2008). EMEA-compliant fetal calf serum does exist (Haack- Sørensen et al, 2008). Such a serum is produced e.g. by PAA Laboratories or Lonza. It origins in bovine spongiform encephalopathy-free countries (Australia, New Zealand) and is treated by irradiation to inactivate possible pathogens. However, fetal calf serum has several disadvantages. The first is great variability among batches with regard to MSC growth support, which necessitates expensive prescreening. The second is possibility of allergic reactions to xenogeneic protein. One group already reported presence of anti-fetal calf serum antibodies in blood of patients treated by MSCs expanded in FCS-containing medium (Sundin et al, 2007), and others reported anaphylatoxic reactions after administration of other cellular products prepared with FCS (Mackensen et al, 2000). Transmission of prion or viral diseases remains a theoretical possibility with fetal calf serum, too, though no zoonosis was reported in several thousands of patients treated with various cellular therapies manufactured with FCS so far.

As mentioned above, experiences with serum-free media are currently limited and reports are controversial. Therefore, before use of such media becomes widespread, human alternatives for FCS should be sought for. Autologous human plasma (AP) was reported to be at least comparable to FCS (Stute et al, 2004). However, given the amount of AP that may

be realistically obtained (200-250 ml maximum with one blood donation) and taken into account the amount of medium needed for replacements and serial passagings, AP does not seem to be a realistic option for clinical-scale MSC manufacturing. Experiences with allogeneic human serum (HS) are controversial, too. While several groups reported early senescence of MSCs grown with HS (Stute et al, 2004), others did not observe such a phenomenon (Bierback et al, 2009). In our hands, human serum (unsupplemented) performed worse than FCS and cells grown in human serum frequently underwent early adipogenic differentiation (Pytlík et al, 2009, and unpublished data). Thrombin-activated platelet releasate in plasma (t-PRP) and pooled human platelet lysate (p-HPL) are two other FCS substitutes of human origin. While both of them take advantage of release of platelet-derived cytokines and growth factors in plasma or serum, their manufacturing and subsequently their performance are substantially different. T-PRP is prepared by adding human thrombin to the platelet concentrate, with subsequent centrifugation and filtration through 0.2 μm filter (which also sterilizes the product). It is necessary to freeze t-PRP in small aliquots and thaw it just before preparation of fresh medium. The product may need aditional centrifugation to remove possibly developing clots and heparin have to be added to the complete medium to prevent gel formation.

P-HPL may be produced either from buffy coats or from expired platelet concentrates. These are briefly centrifuged at room temperature and frozen in aliquots in -30 to -80°C. The freeze-thaw cycles may be repeated several times. One team found to be advantageous to adjust the number of residual platelets in platelet-rich plasma (after centrifugation) to $1.5x10^9$/ml, as with these numbers, the performance of p-HPL was found to be optimal (Lange et al, 2007). Before use, the p-HPL should be spun at high speed (4000-8000 g) to remove the cellular debris.

In her seminal work, Bierback et al found that pHPL have better performance than tPRP, but also than HS and FBS. Besides higher yield of MSCs, use of pHPL also led to less contamination with hematopoietic cells (Bierback et al, 2009). P-HPL may be produced in most transfusion departments under GMP conditions, and its sources are not limited as are sources of autologous serum or human plasma. Even if it is not currently known which factors or cytokines in pHPL are responsible for its efficacy, it constitutes a suitable surrogate for fetal calf serum. First clinical experiences with MSCs produced in p-HPL supplemented medium were already reported (von Bonin et al, 2009).

### 6.4 Supplements

One of the first researchers who studied influence of various growth factors and other supplements on MSCs grown in serum-deprived conditions were Gronthos and Simmons (Gronthos & Simmons, 1995). They studied 25 different growth factors and found that the combination of insulin, platelet-derived growth factor BB (PDGF-BB) and epidermal growth factor (EGF), together with dexamethason and ascorbic acid, led to superior yields of MSCs over other combinations.

PDGFs were first found in platelets and they might be responsible for some of the platelet lysate activity in MSC growth. Some authors described role of PDGF during osteogenic, adipogenic and chondrogenic differentiation, however, the primary effect seems to be mitogenic. PDGF also inhibits differentiation of cells including MSCs. PDGF-BB form can activate all PDGF receptors and therefore is the best choice as a culture supplement. PDGF-BB may be obtained in GMP quality (CellGenix).

EGF has similar mode of action in MSC cultures as PDGF. It acts as a mitogen (Krampera et al, 2005), and in adition it can maintain stem cell properties of hMSCs. Indeed, Kratchmarova found that EGF and PDGF signaling leads to phosphorylation of similar set of proteins (Kratchmarova et al, 2005), with the one exception, the proteins of PI3K pathway, which is phosphorylated by PDGF only. Therefore, synergism of PDGF and EGF in Gronthos & Simmons work seems a little surprising and our experiments have shown that in certain cultivation systems, their action may be redundant (Stehlík, unpublished data).
Subsequently, other growth factors were found to be useful in MSC expansion: Fibroblast growth factor 2 (beta FGF or FGF-2) was found not only to enhance growth of CFU-F colonies, but also to preserve stem cell characteristics of MSC (Tsutsumi et al, 2001; Bianchi et al, 2003). In one work, macrophage colony-stimulating factor (M-CSF) was found also to stimulate expansion of MSCs (Jin-Xiang et al, 2004). FGF-2 factor, clinical grade, is also available from CellGenix. Transforming growth factor beta (TGF-beta) is known to induce so-called epithelial-mesenchymal transition (EMT), i.e., process that enables polarized epithelial cells to acquire a motile fibroblastoid phenotype (Wendt et al, 2009). It also induces chondrogenic differentiation of mesenchymal stromal cells. However, TGF-beta was also found to promote growth of MSCs in serum-free medium, together with PDGF-BB and FGF-2 (Chase et al, 2010).

### 6.4.1 An example of MSC cultivation with cytokine-suplemented medium

We have developed a rapid cultivation procedure of MSCs grown in CellGro™ for Hematopoietic Stem Cells clinical grade medium supplemented with 10% human serum and five Gronthos & Simmons supplements (insulin, ascorbic acid, dexamethasone, EGF, PDGF-BB), further enhanced with FGF-2 and M-CSF. This medium, though not serum-free, enabled us to expand MSCs significantly in a single step from bone marrow mononuclear cells. Yields of MSCs were consistently above $10^6$ MSCs per $10^6$ seeded bone marrow mononuclear cells after two weeks of cultivation. Furthermore, medium did not require change and also hematopoietic cells did not require removal. The only manipulation was addition of supplements three times during the two week cultivation period. MSCs cultivated in this medium had phenotype comparable with MSCs cultivated in alpha-MEM + fetal calf serum and were able to differentiate to three mesodermal lineages (Pytlík et al, 2009). During futher development, we successfully transferred this technology to RoboFLASKs™ (Corning) with silicon rubber seal, which was only three times perforated by blunted needle. Cell harvest was also successful without opening the RoboFLASK™.
This cultivation method is very simple, easily transferrable to GMP environment and enables to expand enough MSCs for clinical applications during single two-weeks expansion. After further validation, it may become a solution for MSC production by smaller companies or academic facilities.

## 6.5 Antibiotics

In preclinical research, cultivation media are often supplemented by antibiotics, usually penicilin-streptomycin combination. However, use of antibiotics, especially beta-lactams, is not advocated for clinical-scale production, as they may mask bacterial contamination. Also, they have allergogenic potential. Aminoglycoside antibiotics may be neutralized by charcoal adsorption (Kielpinski et al, 2005), or on special membrane filters (e.g., TTHVAB210 by Millipore, Steigman et al, 2008). However, the most preferrable option is not to use

antibiotics at all and to secure sterility of the product by strict adherence to principles of asepsis, rather than antisepsis.

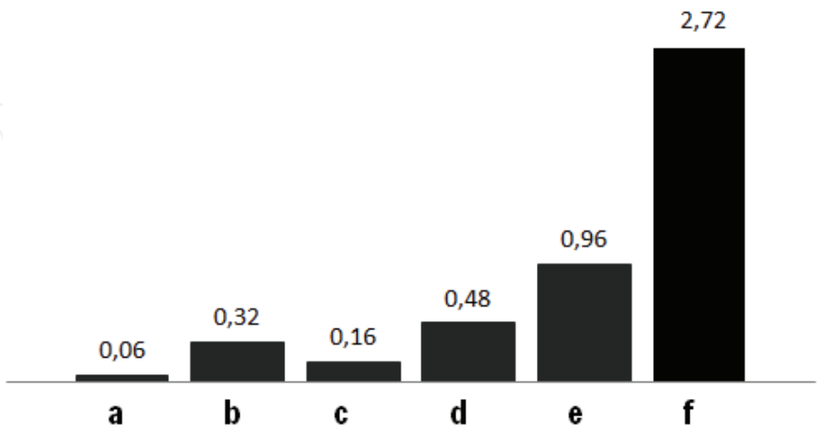


Fig. 3. Comparison of yields of MSCs grown in different media. a – alpha-MEM + FCS, b – alpha-MEM + HS, c – alpha-MEM + HS + 5 Gronthos & Simmons supplements, d – alpha-MEM + HS + 5 Gronthos & Simmons supplements + FGF-2, e – alpha-MEM + HS + 5 Gronthos & Simmons supplements + FGF-2 + M-CSF, f – CellGro™ + HS + 5 Gronthos & Simmons supplements + FGF-2 + M-CSF. Cell yields (x$10^6$) per $10^6$ seeded bone marrow mononuclear cells. Adapted from Pytlík et al, 2009

### 6.6 Harvesting of adherent cells

Harvesting of adherent cells is usually performed by EDTA-trypsin solution. Trypsin is typically porcine, and therefore not optimal for GMP production of MSCs. An alternative, TrypLE™ Select (Invitrogen) is a recombinant bacterial enzyme, produced on dedicated animal origin-free equipment, GMP compliant. It is availlable in two strenghts and the more concentrated (10x) is recommended for MSC harvest. Its performance is at least comparable to classical EDTA-trypsin and it has been already used for clinical-grade preparation of mesenchymal stromal cells (Brooke et al, 2009). Similar performance has also Sigma product from corn, TrypZean (Carvalho et al, 2011).

### 6.7 Cryopreservation of cellular products

MSCs can be cryopreserved, however, as with their expansion, no method is universally accepted and data differ significantly when recovery rates from different freezing methods and formulas are reported. As most experience in GMP cryopreservation of cellular product have been made with hematopoietic progenitor cells (HPCs), in some centres, protocols derived from HPC-freezing ones are used for MSC cryopreservation as well. Also, bags used for cryopreservation of MSCs are the same as used for HPC freezing and storage. On the other hand, other teams simply extend their experience with research-grade freezing and cryopreservation to clinics.

Most cryopreservation techniques use a mixture of cell culture media, animal sera, and dimethylsulfoxide (DMSO) as a freezing solution. DMSO has been extensively used as a cryoprotectant because of its high membrane permeability. However, despite the protection

this cryoprotectant offers, DMSO can be damaging to cells when used in high concentration, especially during the thawing procedure. Also, if not removed, it can cause adverse reactions in patients (nausea, vomiting, tachycardia, bradycardia, hypotension, etc.). Other cryoprotectants, as methylcellulose, sucrose, threhalose, glycerol, hydroxyethylstarch, polyvinylpyrrolidon, or various combinations of these were tested, however, in the end none of them has been found to be superior to DMSO. Therefore, the main issues in development of GMP freezing and cryopreservation protocols is the choice of serum (if any), and the adjustment of the DMSO concentration to lowest possible level.

Haack-Sørensen et al (Haack-Sørensen et al, 2007; Haack-Sørensen & Kastrup, 2011) advocates the use 5% concentrations of DMSO together with 95% fetal calf serum. Control-rate freezing method (freezing at rate of 1°C per minute) is employed, as this was clearly found to be better than uncontrolled freezing (Fuller & Devireddy, 2008). Cell concentration should be between 0.5-1x$10^6$/ml (Goh et al, 2007). It is essential that all procedures beginning with adding DMSO and ending with thawing, are performed at 4-8°C on ice and after thawing, the cell suspension is quickly diluted to lower the DMSO concentration. While probably not better than other protocols (the cell viability or CFU-F retrieval after thawing is not presented), this method is quite simple and may be quickly adopted for GMP conditions, if use of FCS is plausible. Autologous serum might be the best alternative to FCS, if serum is needed at all, however, its use is limited by the same problems as its use in MSC cultivation (Reuther et al, 2006). Human allogeneic serum is surely an option, too, but it still brings risk of disease transmission.

The question therefore is, if serum is needed at all for MSC cryopreservation. Other researchers found that even 2% DMSO, with culture medium (DMEM) without serum was as good as 10% DMSO with 80% human or fetal calf serum (Thirumala et al, 2010a, 2010b, 2010 c). Defined, serum-free and animal components-free freezing media, as is Cryostor™ CS 10 (StemCell Technologies, Woods et al, 2009), or Plasmalyte-A (Baxter, Steigman et al, 2008) are also availlable.

The results of freezing-thawing procedures with the respect to cell viability are controversial and difficult to compare. For similar protocols, recovery rates are as different as 50% to 90% of viable cells. Comparisons of CFU-F formation from unfrozen and frozen cells from the same passage were not reported, to our knowledge. The greatest problem with viability reports is that most researchers use only the simplest method for its evaluation, which is trypan blue exclusion. Freeze-thawing process may start early apoptosis in cryopreserved cells, while these cells still may appear as viable in the trypan blue exclusion test (Baust et al, 2002). Better tests (e.g., flowcytometric staining by $DiOC_6$ for analysis of mitochondrial transmembrane potential, together with propidiumiodine or 7-AAD to exclude dead cells) therefore should be employed. CFU-F assays, for (at least) post-hoc quality control would be also desirable, as in our hands, only half of colony forming ability could be retreieved after the freezing-thawing procerures (unpublished data). As is evident, cryopreservation of MSCs is still largerly an unresolved issue and further attempts toward its optimalization and standardization have to be performed.

## 7. Quality issues

### 7.1 Viability, clonogenicity and senescence

Viability of cells is traditionally performed by trypan blue staining in Burkers chambers. Special counting machines, which evaluate both cell concentration and tolluidin blue

permeability (Countess® from Invitrogene, Cellometer® from Nexcelcom Bioscience) are also availlable, but their running is quite expensive. Flow cytometric staining by propidium iodine or 7-AAD may be more advantageous than trypan blue, especially when combined with suitable other fluorochrome to detect still viable, but early apoptotic cells. Clonogenicity of produced cells should be compared with cells produced by standard procedure by CFU-F formation (Colter et al, 2000). This test, which involves seeding cells in densities of 1.5, 3, 5, and 10 cells/cm$^2$ in a 100 mm Petri dish, is simple, inexpensive and has been shown in our hands to be highly reproducible. Its disadvantage, however, is the lenght of this test, which lasts from 7-14 days. This may be longer than the shelf-live of the final product. However, this testing might be useful as a part of a post-release evaluation of a product quality to ensure that possible failure of the treatment procedure was not due to poor graft quality.

Senescence is an underestimated problem in MSC production. MSCs have only limited number of population doublings, known as Hayflick limit, before senescence growth arrest occur (Hayflick, 1963). In MSC, this is typically from 20 to 50 doublings, depending on cell source and culture conditions (Izadpanah et al, 2006; Suchánek et al, 2007, Cholewa et al, 2011). Senescent cells not only cease to proliferate, but their differentiation properties are also impaired and they can differentiate to osteogenic lineage only. They can display aneuploidy without transformation (Tarte et al, 2010), and exhibit certain mutations, e.g., in p53 gene. P53 mutated MSC can migrate to mammary tissue and form an inductive microenvironment for breast cancer (Houghton et al, 2010). Senescence is easy to evaluate in non-confluent cell cultures by beta-galactosidase staining (Bandyopadhyay et al, 2005), and this test has been recently adapted for flow cytometry as well (Noppe et al, 2009). Senescent MSC have typical secretome and gene expression profiles – they secrete for example interleukin-6, matrix metalloproteinases, hepatocyte growth factor, or FGF2. These molecules can reinforce the senescent arrest or stimulate growth and invasion of established cancer (Coppé et al, 2008). Senescent gene expression profile of MSC – cultivated either with fetal calf serum or human platelet lysate – includes upregulation of hyaluronan and proteoglycan link protein 1 *(HAPLN1),* keratin 18 *(KRT18),* brain-derived neurotrophic factor *(BDNF),* or renal tumor antigen *(RAGE),* while pleiotropin *(PTN)* is downregulated (Schallmoser et al, 2010). From practical point of view, beta-galactosidase testing should become a routine pre-release quality test of MSC preparations, while rt-PCR testing for selected senescence-associated genes may be a part of post-release quality surveillance (see below).

### 7.2 Product characterization

The minimal set of requirements for cells to be recognized as MSCs are set in Introduction to this chapter (Dominici et al, 2006). However, testing the full set of these requirements for every batch can be challenging, as e.g. lineage differentiation lasts several weeks, which may be longer than the expiration period of the product. On the other hand, performance of various MSC products, especially in the autologous setting, may be at least partially donor dependent (Friedl et al, 2009). Therefore, a reasonable compromise has to be achieved depending on particular situation.

Full characterization of the product has to be performed in the preclinical phase of its development. These tests should show reproducible profile of surface markers, performed by flow cytometry or immunocytochemistry. Testing for surface markers may also reveal

some impurities of the final product, especially admixture of hematopoietic cells. Release standards (percentage of cells positive for given antigen, acceptable amount of undesired cells) should be set at this point.

If the manufacturing procedure differs from accepted standard (e.g., from cultivation of cells in DMEM or alpha-MEM medium with 10% fetal calf serum), full tests for differentiation properties should be performed to prove that the final product complies with the minimal set of requirements for MSCs. This may involve comparison of cells produced by alternative procedure with cells produced by standard way. These comparisons should be not only qualitative (Oil red staining for adipogenic, von Kossa or alizarin red staining for osteogenic, and staining for collagen II for chondrogenic differentiation), but also quantitative (e.g., calcium accumulation or triglyceride synthesis). If desired effect of MSC product is immunosuppression (e.g., for treatment of graft-versus-host disease), allogeneic mixed leukocyte reactions with admixture of various ratios of MSCs should be performed (Le Blanc et al, 2003).

Preclinical characterization of the product may involve also gene expression profiling. In current literature, there is a number of papers describing gene expression profiles of MSC obtained from various tissues or cultivated by different methods (Wagner et al, 2005; Tsai et al, 2007; Secco et al, 2009), and it might not be necessary to repeat these expensive, cumbersome and poorly standardised experiments in full. If it is necessary, Wang et al. provide a detailed protocol how to perform gene expression analysis (Wang et al, 2011). In any way, qualitative or quantitative testing for expression of selected genes of interest (genes related to growth, stemness, differentiation properties or senescence) might be useful. This testing could also apply to final clinical products, either as pre-release or post-release control of quality.

For quality testing of fully developed and approved product intended to clinical use, it must be kept in mind that the donor variability might substantially influence the final quality of the batch. Full testing for clonality, differentiation, immunosuppressive properties or gene expression might not be possible because of the short shelf-life of the product. Even when there is an intention to freeze the product before use, its performance might be different before freezing and after thawing (our unpublished experiments). However, it might be advisable to perform these tests as a part of post-release testing, to ensure the released product was of sufficient quality and to be able to show that possible treatment failure was not due to poor quality of the MSC product.

## 8. Safety issues

### 8.1 Donor screening

In general, the same set of examinations as for blood banking purposes should be performed. Testing for hepatitis B, C, HIV and syphilis are mandatory. In certain areas of the world, testing for HTLV-1 and/or Chagas disease may also apply.

### 8.2 Microbial contamination

Bacterial contamination of classical pharmaceutical products is excluded by standardized tests, as set for example in European Pharmacopoiea (EP, chapter 2.6.1), or US Pharmacopoiea (USP, chapter 71). These growth promotion tests (GPT) involve two different cultivation media – Fluid thyoglicollate medium and soya-bean casein digest

medium, and two different temperatures – 22.5°C and 32.5°C – for growth of each tested sample. However, this test takes 14 days to finish and is clearly unsuitable for products with short shelf-live. There are instructions on validation of rapid microbiological tests both in USP (ch. 1223) and EP (section 5.1.6). These require for alternative microbiological testing to ensure following, compared with standard GPT:

**Specificity.** All microbial strains must be detected and confirmed. Aerobic strains must be detected in the aerobic culture bottles. Anaerobic strains must be detected in anaerobic bottles. It should be confirmed that cell cultures themselves will not generate false positive tests. Microbial strains can be bought e.g. from ATCC (American Type Culture Collection), however, the set should also include isolates from microbiologically positive samples and from environment of the facility.

**Limit of detection.** Each challenge microorganism must be detected at less than 100 CFU but greater than 0 CFU.

**Repeatability.** All replicates inoculated with challenge microorganisms are determined to be positive.

**Ruggedness.** All strains must be detected and confirmed as prepared by different analysts.

**Equivalence.** Alternative method must detect challenge organisms sooner than the compendium method.

There are several solutions for rapid microbiological testing, but all have their advantages and disadvantages. Best comparable to pharmacopoieal methods are cultivation methods based on $CO_2$ detection (BACTEC™ – Becton Dickinson, BacT/ALERT® - bioMérieux), and they have already been approved for tissue products (Kielpinski et al, 2005). These are also relatively unexpensive, easy to handle and do not require much space. Results are typically obtained in 48-72 hours. DNA detecting tests (e.g., LightCycler® Septi*Fast* Test – Roche) may be more challenging to be validated, as they may not detect all possible contaminating organisms (especially the environmental isolates) and, on the other hand, they may detect DNA from unviable organisms. Also, the number of gene copies (GC) is not easy to compare directly with the number of colony-forming units (see also 8.3). However, these tests are attractive as they can detect microorganisms in less than 24 hours. Fluorescent cytometry tests (Scan*RDI*® AES Chemunex) provide ultra-rapid detection of microorganisms (90 minutes), but are very expensive and used typically by large pharmacological companies.

It have to be stressed that validation of an alternative microbiological testing method may be very laborious and time consuming and can take several years before successfully completed. This may change, as these tests are getting more widespread. Close cooperation with the regulatory agency from the very time such a method is contemplated, is necessary in any case. For a close introduction to the rapid sterility test implementation, see Gressett (Gressett et al, 2008).

A good question is what to do when final products – especially customized ones – are eventually found to be microbiologically positive. At that time already a lot of work and money have been invested in the product, not to mention a patient who might in the meantime undergo some kind of preparative procedure for cellular treatment. This is similar to situations in hematopoietic progenitor cell transplants, where even microbiologically positive graft cannot be withdrawn and discarded, as this would mean inevitable death of the patient in many cases. Positive grafts are found in wide range of 0-43% of cases (Lowder & Whelton, 2003), but surprisingly they do not appear to present unacceptable risks. In two

large studies, (Patah, 2007; Phinney, 2007) the frequency of positive grafts was between 1-2%. While Phinney gave preemptive antibiotic treatment to the graft recipients, Phinney just observed them. The frequency of adverse events was zero in the first study and close to zero in second. Preemptive antibiotic treatment based on tested or presumed microbial sensitivity might be a reasonable strategy for transplantation of microbiologically positive products, under strictly controlled conditions.

### 8.3 Mycoplasma contamination

Mycoplasmas are microorganisms without cell wall, which may pass through sterilization with 0.2 µm filters. They have quite complex requirements for survival conditions, but cell culture media make good environment for their growth. As such, mycoplasmas present significant thread to cell and tissue cultivation. European, United States or Japanese pharmacopoieas state requirements for mycoplasma testing. Essentially, two types of tests are used: first is inoculation of cell culture samples on a solid agar or in a liquid enrichment medium, from which are mycoplasma cultures after several days transferred on agar. This test is quite sensitive (10 CFU/ml), but takes 28 days to complete. In second method, the indicator cell culture, samples are co-cultured with permissive cell lines (usually Vero cells) and then stained with fluorescent DNA-binding dyes (DAPI or Hoechst). This approach also takes time and is less sensitive than agar cultivation (100 CFU/ml).

Fortunately, there are several tests, based on nucleic acid testing (NAT), which have been already validated, though NAT is not without its problems. First, it does not distinguish dead cells from living ones. Second, the translation of gene copy numbers to colony finding units is problematic. Not only all mycoplasmas detected by NAT are not necessary live ones, but also CFU is not an equivalent to living cell – it is an expression of its ability to form typical colony. Also, cultivation methods work with larger volumes (1 to 10 ml of medium) than NAT tests (tens to hundreds µl). Therefore, enrichment of a starting material (e.g., by high-speed centrifugation) may be necessary. It has to be assured that sequences of all mycoplasmas are covered by single PCR reaction and it also has to be assured that this reaction will not amplify sequences from related microorganisms (Streptococci, Clostridia, Lactobacilli).

MycoTOOL™ (Roche Diagnostics) is a test amplifying a part of the 16S rDNA of Mycoplasmas. It was validated with the European Pharmacopoiea tests (Chapter 2.6.7.) and is able to detect Mycoplasmas with sensitivity of at least 10 CFU/ml (Deutschmann et al, 2010). A quantitative MycoSensor QPCR assay kit was developed by Stratagene and found acceptable in preclinical regulatory validation of amniotic MSC manufacturing protocol (Steigman et al, 2008). For detail description of NAT-based Mycoplasma detection techniques, problems with alternative non-microbial detection and possible other solutions, see Volokhov (Volokhov et al, 2011).

### 8.4 Endotoxin testing

Endotoxins are lipopolysaccharides from gram-negative bacteria and are the most common cause of toxic reactions resulting from contaminations with pyrogens. Reactions to endotoxin can cause serious health problems, as is diarrhea, septic shock, marrow necrosis and others (Opal & Steven, 2007). Testing for endotoxins is therefore a standard release test for cellular and gene therapy products. The acceptable level of endotoxin in these products is usually 5.0 EU/kg/dose.

Endotoxin is usually tested with the Limulus Amebocyte Lysate (LAL) method. The problem with this test is not the time (results can be usually obtained in 3-4 hours), but its sensitivity to external factors and complexity of its setting. Endosafe® PTSTM is a chromogenic LAL test that provides quantitative results in approximately 15 minutes (Gee et al, 2008). It has been already validated for testing of bone marrow mononuclear cells for cardiac regeneration (Soncin et al, 2009), and is relatively easy to use. For other applications, however, comparison with standard accepted method may still be necessary.

### 8.5 Tumorigenicity

There were several reports of spontaneous transformation of human MSC in cultures (Rubio et al, 2005; Wang et al, 2005; Rosland et al, 2009). Most, if not all, these results reflect cross-contamination of mesenchymal cultures with exogenous tumor cell lines (Torsvik et al, 2010), which hardly can be a concern in a well-conducted GMP facility. However, transformation of MSC was observed after prolonged cultivation in human telomerase immortalized cells (Serakinci et al, 2004). This should not again cause concern in production of non-manipulated MSC, however, it shows to potential danger in case MSC were genetically manipulated. Furthermore, these immortalized transformed MSCs lost the $p16^{ink4a}$ gene, which was shown to occur occasionally even in non-immortalized MSC cultures (Shibata et al, 2007). In conclusion, risk of spontaneous malignant transformation of human MSC products does not seem to be very high. The question of routine cytogenetic testing of MSC product has to take in account the fact of low sensitivity of classical cytogenetic examination which may easily miss potentially dangerous but still very small clone and certainly will miss most losses of heterozygozity or similar small genetic changes. Also, cytogenetic testing can lead to false-positive results, as it was shown that aneuploidy might in fact be quite common in MSC undergoing senescence, but not transformation (Tarte et al, 2010).

### 8.6 Clinical safety and surveillance principles

As the experience with somatic cell therapy is still limited, there are no universally applicable principles of clinical safety monitoring. Until sufficient information will be availlable, all recipients of somatic cell therapy, including the treatment with MSC, should be followed indefinitely (for a lifetime), and monitored for possible adverse effects of treatment. Adverse events should be collected in context of the clinical trials in the premarketing phase, and according to general pharmacovigilance principles in the postmarketing phase.

Possible acute complications connected with mesenchymal stromal cell treatment, as perceived from preclinical evaluation, are infusion related complications, immunological reactions (more probable with use of xenogeneic proteins during MSC production and/or after repeated use), and local reactions (with local application). Significant number of MSCs, especially from the Stro-$1^-$ fraction, engrafts in lungs (Devine et al, 2003; Bensidhoum et al, 2004), and lungs are the first organ attended by intravenously administered MSCs. Therefore, the possibility of MSC induced lung injury have to be taken seriously. In experimental animals, administration of large numbers of MSCs may cause stroke or even death. Therefore, it is desirable that all systemically treated patients would be closely monitored during the infusion and some time thereafter.

MSCs are little immunogenic and immune reactions caused by their administration therefore should not be problem. However, when cultivated in xenogeneic protein-

containing systems, they may internalize and present these proteins to recipient. This problem may become significant especially if repeated administrations of MSCs are planned (e.g., for graft versus host disease treatment), as first exposure of xenogeneic protein may cause priming of the recipient immune system and subsequent administrations may trigger an allergic reaction.

In the long-term follow-up, three issues seem to be particularly important: "maldifferentiation" of MSCs, tumor propagation, and disease transmission.

"Maldifferentiation" of MSCs refer to differentiation in a tissue type not desired in the particular organ. It was shown that MSCs, in contrast to hematopoietic progenitor cells, produce calcifications after local injection to an infarcted heart (Breitbach et al, 2007). In another model of glomerular injury, MSCs prevented progressive renal failure when administered intraarterially to rats, but degraded in kidney to fat cells, surounded by fibrotic tissue (Kunter et al, 2007). To our knowledge, nothing similar was observed after intravenous infusion or in humans, but similar undesired effects of human MSCs cannot be excluded.

For tumor formation, patients should be followed indefinitely. As shown above, the risks of spontaneous transformation of human MSCs are probably very small, however, there are concerns that MSCs may support tumor growth by a variety of mechanisms, involving immunosuppression, transformation of MSCs to CAFs (cancer associated fibroblasts), or tumor vasculature support (Momin et al, 2010; Klopp et al, 2011). Human MSCs have been shown to promote tumor development in several animal models (Zhu et al, 2006; Karnoub et al, 2007). In clinical practice, rather than facilitating growth of previously undiagnosed tumors, MSCs may promote tumor growth when applied to patients with established cancer, for example in hematopoietic cell transplantation setting. There is one report showing that patients who had cotransfused MSCs together with hematopoietic progenitor cells, had less graft versus host disease but more leukemia relapses (Ning et al, 2008). On the other hand, there are also reports that unmanipulated MSCs may also suppress tumor growth (Khakoo et al, 2006; Qiao et al, 2008). Large series of patients, optimally in randomized clinical trials, need to be followed for the frequency of various types of spontaneous tumors before the tumorigenicity of human MSCs may be excluded. The question of tumorigenesis will undoubtebly become even more significant if genetically engineered MSCs will be used for treatment of cancer or metabolic diseases, however, this is beyond the scope of this chapter (reviewed in Aboody et al, 2008 and Momin et al, 2010).

To our knowledge, disease transmission was not reported yet after mesenchymal stem cell therapy. Usual infection surveillance should be sufficient. Infectious origin of any febrile reaction during and after MSC application should be excluded and MSC recipients should be tested for hepatitis or HIV transmission in a fixed time after cellular therapy. If blood-transmitted infection is confirmed in recipient, donor of MSC should be investigated as well, in case of allogeneic therapy. If fetal calf serum is used for MSC expansion, it must be from bovine spongiform encephalopathy-free area, as noted above.

## 9. Conclusion

Mesenchymal stromal cell therapy offers solutions for a number of currently unmet clinical needs in modern medicine. These solutions might be less than optimal in certain cases, or may not fulfill the expectations at all. Mesenchymal stromal cell therapy may well provide only temporary clinical solutions, before better understanding of underlying principles of

diseases and their treatment become availlable and better treatment approaches (e.g., targeted delivery systems for cytokines and gene products, small molecules, etc.) will be developed. At this time, however, it seems that somatic cell therapy is worth exploring, despite the new challenges connected with it.

Because of the complex regulatory requirements, cell therapy will probably be very expensive and during this time, when its safety and efficacy are being tested, it will be difficult to find reimbursement of expenses connected with its development. An inequality in access to new treatments may result on one hand and difficulties with accrual of patients to clinical trials on the other. Therefore, it is crucial that all involved in mesenchymal stromal cell treatment, including funding institutions, regulatory institutions, academic facilities and private subjects, would cooperate closely together, on national or international platforms. On these platforms, fabrication of GMP-compatible facilities and development of GMP prepared products for cellular therapy will undoubtedly prove to be crucial in transferring the experimental knowledge into clinical practice. Falling behind the international level of knowledge and experience may have very undesired effects on health care in underdeveloped countries or regions. The purpose of this chapter was to provide at least partial solutions to challenges in this exciting new area of medicine.

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**Regenerative Medicine and Tissue Engineering - Cells and Biomaterials**

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Tissue Engineering may offer new treatment alternatives for organ replacement or repair deteriorated organs. Among the clinical applications of Tissue Engineering are the production of artificial skin for burn patients, tissue engineered trachea, cartilage for knee-replacement procedures, urinary bladder replacement, urethra substitutes and cellular therapies for the treatment of urinary incontinence. The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues reconstructed from readily available biopsy material induce only minimal or no immunogenicity when reimplanted in the patient. This book is aimed at anyone interested in the application of Tissue Engineering in different organ systems. It offers insights into a wide variety of strategies applying the principles of Tissue Engineering to tissue and organ regeneration.



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