Methods in Molecular Biology 1283

Springer Protocols

Kursad Turksen *Editor*

Stem Cells and Good Manufacturing Practices

Methods, Protocols, and Regulations

Humana Press

METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

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ISSN 1064-3745 ISSN 1940-6029 (electronic)
Methods in Molecular Biology
ISBN 978-1-4939-2434-9 ISBN 978-1-4939-2435-6 (eBook)
DOI 10.1007/978-1-4939-2435-6

Library of Congress Control Number: 2015931543

Springer New York Heidelberg Dordrecht London

Printed on acid-free paper

Humana Press is a brand of Springer
Springer Science+Business Media LLC New York is part of Springer Science+Business Media (www.springer.com)

Preface

To actualize the potential of stem cells for regenerative therapies requires that the cells be handled using Good Manufacturing Practices. There are increased efforts to prepare stem cell populations under such conditions. With this in mind, it seemed timely to collect a series of protocols describing the kinds of infrastructures, training, and standard operating procedures currently available. I am very grateful to the authors who contributed in this volume, which I think will be invaluable to both basic and clinical practitioners in stem cell biology.

I would like to thank Dr. John Walker, Editor in Chief of the *Methods in Molecular Biology* series, for his support for this idea; Patrick Marton, Executive Editor, Springer Protocols, for his continuous support and encouragement; and David Casey, Editor, Springer US, for tirelessly answering my endless questions to get this volume to the print stage.

Ottawa, ON, Canada ***Kursad Turksen***

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Methods in Molecular Biology (2015) 1283: 1–11
DOI 10.1007/7651_2014_175

Published online: 24 December 2014

Therapeutic Mesenchymal Stromal Cells: Where We Are Headed

Patrick J. Hanley

Abstract

With a range of therapeutic uses, from diabetes and Crohn's disease to wound repair, interest in the function, characterization, and expansion of mesenchymal stromal cells (MSCs) is growing rapidly. When considering the therapeutic use of MSCs, one must take into account a multitude of options including the ideal source of MSCs, the ideal donor, and the best means of expansion. Here we discuss different sources of MSCs, including cord blood, bone marrow, and adipose tissue, the option of using autologous and allogeneic donors, and finally we discuss GMP-applicable expansion protocols aimed at expanding MSCs for clinical use.

Keywords: Mesenchymal stromal cells (MSC), Cell therapy, Clinical trials

1 Introduction

Mesenchymal stromal cells (MSCs) were first described as adherent cells with a fibroblast morphology (1). Since those initial observations, our understanding of MSCs has increased greatly. These cells are adherent, nonhematopoietic cells that contribute to the structure and architecture of bone marrow and other tissues in which they reside, such as adipose tissue (2–4). They are characterized by their tri-lineage potential or their ability to undergo adipogenesis, chondrogenesis, or osteogenesis given the appropriate stimuli (5). Phenotypically, the cells express the markers CD73, CD90, and CD105 but do not express CD34, CD45, CD19, CD14, or HLA-DR (6). Additional markers have also been used to identify MSCs, such as STRO-1 (7, 8), CD106 (9), CD166 (10), CD29 (5), CD44 (5), MSCA-1 (11, 12), CD271 (13), and others (10) with varying degrees of success. A limitation to using only one phenotypic marker to identify an entire population of cells is that the marker may be indicative of a population of cells skewed towards a specific lineage. Moreover, the international society of cellular therapy (ISCT) issued a position paper on mesenchymal stromal cells and have defined them as >95 % adherent cells expressing CD73, CD90, CD105 but expressing <2 % of CD45, CD34,

CD14 or CD11b, CD79α or CD19, and HLA-DR. The cells also must differentiate as described above into the three lineages in vitro (6, 14).

The functional properties of MSCs are what make them such an appealing modality. MSCs release a host of soluble factors that interact with various factions of the immune response. They suppress T cells (15) and NK cells (16) directly by releasing Indoleamine 2,3-dioxygenase (IDO), which depletes tryptophan, a necessary amino acid for T- and NK-cell proliferation (17, 18). B cells stimulated with pokeweed antigen are also suppressed by MSCs via Prostaglandin E2 (PGE2), which leads to a decrease in their proliferation and prevents their differentiation to plasma cells (19). MSCs are able to orchestrate the immune system via dendritic cells (DCs) (20) as well by releasing PGE2, M-CSF, IL-6, IL-10, and transforming growth factor-beta (TGF-β), which prevent the differentiation of monocytes into immature DCs (18). The maturation of immature DCs is also inhibited by TGF-beta and PGE-2. This not only impairs the activation of T cells but also enhances the differentiation and function of regulatory T cells (T regs) (21), which perpetuate the immunosuppressive environment. Some reports claim that MSCs also activate T regs in vivo and this interaction with T regs is responsible for most of the immunosuppression attributed to MSCs (21, 22). Most reports suggest that this MSC:Treg axis is contact independent and attributed to the soluble factors listed above (15, 23–25).

2 Clinical Efficacy of MSCs

The first successful infusion of MSCs for accelerating hematological recovery was reported in 1995 by Lazarus et al. This group showed the feasibility of collection and ex vivo expansion of MSCs present in small bone marrow aspirates, and infusion of these cells was not associated with significant adverse events or GvHD (26). In a multicenter phase 1/phase 2 study, recipients of an allogeneic T cell-depleted stem cell transplant also received HLA-disparate ex vivo-expanded MSCs from a relative. All recipients showed sustained engraftment in a group that has a 20 % failure rate in historical controls (27). Beyond their role in promoting engraftment, the immunosuppressive properties of MSCs have also been tested by investigators looking to control inflammation or autoimmunity (28).

One of the most studied areas of the therapeutic potential of MSCs is in graft versus host disease (GvHD) after allogeneic stem cell transplant, where immune cells—primarily T cells—transferred with the graft attack organs of the host. Le Blanc et al. were the first to utilize MSCs as a therapy for GvHD; ex vivo-expanded haploidentical MSCs were infused intravenously to a 9-year-old patient

with Grade IV GvHD of the gut and liver. After 5 days the patient had a notable decrease in bilirubin levels and was able to resume oral food intake after 2 weeks (29). A larger study phase 2 from this group was published in 2008 using MSCs derived from HLA-identical sibling donors, haploidentical donors, and third-party HLA-mismatched donors. The median dose was 1.4×10^6 MSC/kg. Of 55 patients, 30 had a complete response and 9 showed improvement; those with a complete response had a lower incidence of transplant-related mortality after 1 year and a higher overall 2-year survival rate (30). A number of early-phase clinical trials using MSCs have since been initiated, many with promising results, including one study by Yin et al. where MSCs were not only used as a therapy but were used to identify biological markers that correlated with clinical responses (31).

However, trials with MSCs have not been limited to early-phase clinical studies. Recently, Osiris published their phase 3 trial using third-party MSCs in patients with steroid refractory GvHD. In this study, 2 × 106/kg MSCs were given twice weekly for 4 weeks. Although the overall response rate for Prochymal was not significantly different from the placebo, there was a significant improvement in gut and liver GvHD (32), and this product has subsequently been approved in Canada and New Zealand as a treatment for pediatric acute GvHD. Moreover, the controversial results of the Osiris trial indicate that there is an urgent need to identify the optimal MSC product—including the optimal dosing, method of manufacture, product characteristics, and whether the product is best used fresh or previously cryopreserved (33).

3 Requirements for Manufacturing MSCs for Clinical Trials

Successful manufacturing of MSCs for clinical use is not limited to the expansion of the MSCs themselves but also includes other core components of Good Manufacturing Practices (GMP) and Good Tissue Practices (GTP), which ensure that products are manufactured according to a controlled and auditable process and limit the risk of pathogenic transmission (34, 35). Core components such as storage and distribution can easily be overlooked but are important aspects of the manufacturing process. For instance, one decision investigators must make is whether to cryopreserve the final product or to use them fresh. For allogeneic banks, cryopreserved cells offer the convenience of having a fully characterized product available before the product is infused to the patient. Sterility tests, potency assays, and purity and identity tests can all be performed in advance and reviewed by quality assurance before it is released for use (36). Even so, reports suggest that many cell therapy products are adversely affected by cryopreservation and subsequent thawing (37). Indeed, Francois et al. demonstrated that cryopreserved

MSCs upregulate heat shock proteins and lack suppressive function upon thawing of cryopreserved MSCs (37). When cells are used fresh, characterization assays must be performed prior to administration, which also has limitations. Firstly, the product being characterized may not be identical to the infused protocol. Secondly, the product will likely be released while sterility testing is still pending, which increases the risk that a contaminated product will be administered to a patient. Rapid microbiological tests are available but can sometimes result in false negatives (38, 39).

Regardless of the storage conditions, the MSC product will need to be distributed or, at the very least, transported to the local infusion site. For local infusions, transporting fresh or cryopreserved products is relatively straightforward. However, shipping cryopreserved products to other institution typically requires the use of a temperature-controlled liquid nitrogen dry shipper with a validated courier service. Temperature tracking is also important and should be recorded, minimally, at the start of shipment and once it is received; this can easily be tracked digitally using a digital temperature probe. For fresh products, MSCs pose more of a challenge because they are adherent cells. For this reason, MSCs can be shipped in flasks—which requires that the receiving institution is capable of the end processing required prior to infusion and is trained in the process—or they can be stored in gas-permeable bags that limit the adherence of cells. The optimal conditions for shipment—including the bags, vials, or vessels used, the temperature during shipment, and the method of harvest—should all be validated.

4 Current Methods of MSC Expansion

Current manufacturing platforms for expanding MSCs consist of basic cell culture techniques that have been available for decades. Traditionally, MSCs are expanded using standard T-flasks (40). The source of MSCs—bone marrow, adipose tissue, cord blood, placenta, or other sources—is plated on the tissue culture-treated polystyrene surface, typically at a density of 1,000–500,000 cells/cm^2. Cells are left to attach for 2–5 days, at which point cells not adhering to the plastic are aspirated and the flask is fed with fresh medium. Once the cells reach 70–80 % confluence, the cells must be passaged by aspirating the medium, washing the cells with PBS, adding a dissociation reagent such as TrypLE select, and harvesting the cells in MSC medium. Cells are then replated based on a set seeding density or they are passaged 1–4 into new flasks (36). This routine is repeated until the desired cell dose has been reached. While this method is simple, does not require specialized equipment, and uses supplies that are relatively inexpensive, this approach does require multiple CO_2 incubators, sufficient staff to harvest

hundreds of flasks, and has an increased risk of contamination due to the extensive manipulation required with hundreds of flasks.

Another common approach for expanding large numbers of MSCs is the use of cell stacks or cell factories of varying sizes. As the name suggests, these cell stacks are stacks of individual flasks with part of the cell stack dedicated to media or reagent exchange. Commercial companies also offer these cell stacks with tubing accessories to make the manufacturing process functionally closed, hence limiting the likelihood of contamination. Manufacturing in cell stacks is similar to that in individual T-flasks but requires additional technical expertise. Nonetheless, cell stacks offer an alternative to individual flask-based expansion and require less space while reducing the labor involved.

Additional manufacturing paradigms include a combination of flasks and cell stacks; initial plating of MSCs occurs in multiple flasks, with each passage then proceeding to cell stacks of increasing size. Using this technique, Sabatino et al. were able to plate eight 10-layer cell stacks at passage 3 to recover 1–4.76 billion MSCs from a single donor (41). Other centers requiring large numbers of MSCs plate 12–25 mL of bone marrow into multiple 10-layer cell stacks at initiation. Feeding cells weekly, they are able to recover 2–5 billion MSCs after 1 month without needing to passage the MSCs (42, 43).

5 Next-Generation MSC Expansion

The aforementioned methods were used in a phase 3 trial by Osiris, but the questionable success of this trial forced those interested in the translation of MSCs to rethink their approach to MSCs, including the optimal means of expansion (33). To this end, the increasing demand for MSCs clinically, along with commercial interest from companies with significant capital and GMP experience, has pushed the development of next-generation MSC expansion platforms. One of the biggest advancements was the introduction of stand-alone, functionally closed expansion systems (44). Unlike suspension cells that can grow in large cell culture bags or vessels, MSCs are adherent and hence their growth potential is greatly limited by the surface area. Next-generation systems have overcome this limitation using a variety of technologies. One system, the Wave by GE Healthcare, utilizes the same cell culture bag platform that they use for suspension cells combined with microbeads (45). One commercially available microbead product is the Cytodex microcarriers made of cross-linked dextran. These microcarriers are ~175–180 μm in diameter and support the attachment and expansion of MSCs. Agitation or stirring of the cells is required to ensure that the microbeads and cells remain in suspension (46).

Media and gas can be added to the system as with suspension cells, and at harvest, a dissociation reagent is added to the system to remove the cells from the beads. This technique has many advantages, including growth in several dimensions and at high densities. Even so, one limitation of this approach is that it is difficult to detach cells from the microcarriers, and the rigorous dissociation required can cause decreased viability of the cells. PBS Biotech also offers a Vertical-Wheel Bioreactor System that uses microcarriers kept in suspension by the vertically oriented wheel to grow MSCs to large numbers. The system comes in different sizes to accommodate varying demands. Similarly, the Xpansion system by Pall is a 2-dimensional bioreactor designed to mimic flask-based expansion but in a condensed, stacked system with 10- and 50-stack systems with up to 115,000 cm^2. The Xpansion system controls pH and oxygen by allowing diffusion through the thin silicon tubing in the central column with media circulation controlled by a central pump. One advantage of using the Xpansion system is that the first three layers of the stack can be observed using an LED microscope (Ovizio, Belgium) (http://www.atmi.com/lifesciences/products/bioreactors/xpansion.html, accessed on 11/07/2014). While the above-mentioned bioreactor systems offer novel ways of manufacturing MSCs in a manner more applicable to GMP regulations, how these systems compare to traditional flask- or cell stack-based expansion platforms remains to be tested.

Another large-scale expansion system that has been tested by academic labs is the Quantum Cell Expansion System by Terumo, BCT. Like some of the systems mentioned above, the Quantum's hollow-fiber bioreactor maximizes the surface area without creating a large footprint. This system is a semiautomated, functionally closed, temperature-controlled stand-alone system that allows media to be perfused through the bioreactor chamber and into a waste bag at a user-defined rate that can be changed to account for cell growth and metabolism of media. In fact, because the cells cannot be visualized, lactate production by the cells and glucose consumption are used as surrogates of growth. In this system, 10–20 million cells at passage 1 can be expanded to 500–1,000 million cells in 6 days (47–49). By using a functionally closed system, the number of open events is reduced by 99.9 % and over the course of an early-phase clinical trial, the number of open events (manufacturing events where the product is exposed to the environment) would be reduced by as much as 50,000 events (47). Nevertheless, the Quantum and its disposable bioreactor set are only available in one size (2.1 m^2), so companies or cell therapy labs looking to increase productivity would need to scale out instead of scale up as with other bioreactor systems. Additionally, residual ethylene oxide used to sterilize the expansion sets could lead to chromosomal instability; however, Jones et al. have recently shown that MSCs expanded in the Quantum are genetically stable (50).

6 Additional Considerations for Future Clinical Trials Utilizing MSCs

6.1 Source of MSCs

Most clinical trials to date have used bone marrow (BM)-derived MSCs (BM-MSCs). Once the bone marrow is harvested from an eligible donor, the bone marrow mononuclear cells (BMMC) are either isolated using a manual BMMC separation technique with a carbohydrate gradient and centrifugation or seeded directly onto the culture vessel. MSC expansion from BM is considered to be relatively straightforward and does not require expensive immunomagnetic selection techniques. However, due to the infrequent nature of the BM-MSCs, multiple passages and numerous cell doublings are typically required in order to reach clinical cell doses. Additionally, when creating a third-party MSC bank, donors can be screened for ideal characteristics such as young age (where the frequency of MSCs is 10–100× higher) to identify the best donors (51, 52). In some cases, multiple donors can be selected and tested for MSC expansion and function prior to initiating the MSC bank.

Another popular source of MSCs is adipose tissue. MSCs are 2,500 times more abundant in adipose tissue (53), and it has been reported that adipose-derived MSCs have superior suppressive capabilities to BM-MSCs (54). Furthermore, although adipose-derived MSCs have distinctive advantages, going from the liposuction product to a source of homogenous MSCs is more laborious than BM-MSCs, as cellular debris and extracellular matrices must be removed followed by enzymatic treatment of the tissue/adipose MSCs (55).

With over 400,000 cord blood (56) units available in cord blood banks worldwide, expanding MSCs from cord blood has also generated interest in the field of cellular therapy. While more challenging than BM-MSCs or adipose-derived MSCs, cord blood-derived MSCs have been used in around 37 clinical trials (44). Of the three sources of MSCs mentioned here, CB has the lowest frequency of MSCs at around 0.00003 % of all cells in the cord blood unit (57). Despite the infrequent nature of CB MSCs, some reports suggest that CB-derived MSCs have excellent proliferation potential that can yield as many as 1×10^{15} cells (58). Even so, multiple reports have suggested that MSCs were only successfully expanded in 20–63 % of CB units tested. Other reports have suggested more reproducible expansion methods by selecting various cell populations, but these selections often favor the differential of one or more of the chondrogenic, osteogenic, or adipogenic lineages. Indeed, in some cases, even without selection, CB-derived MSCs showed no adipogenic differentiation (59). Nevertheless, clinical studies have shown the feasibility of this approach as well as their potential clinical benefit.

6.2 Autologous vs. Allogeneic Third Party

Another consideration when designing trials using MSCs is whether to use autologous MSCs or allogeneic, third-party MSCs that are stored as a cell bank. Autologous MSCs should, in theory, last longer since they would not be rejected by the host nor elicit HLA-specific antibodies. However, this approach is not ideal for elderly donors or donors who are not suitable for bone marrow collection. An additional drawback is that it takes at least 3–4 weeks to expand enough cells for clinical use. Moreover, autologous MSCs are most likely to benefit chronic conditions where patients need long-lasting treatment strategies and are on current treatment strategies so they can afford to wait until the MSCs have been expanded.

Allogeneic MSCs have many benefits as well. Although they are more likely to be rejected or elicit a rejecting antibody response, they are a cheaper, more universal option since a bank of them can be created. And since there is a pre-made bank, these cells can be infused urgently. Unlike autologous MSCs, a number of BM donors can be screened to ensure that only MSCs that rapidly proliferate and are highly functional are used. Banked cells will have been previously characterized and released which allows for rapid treatment. Still, limiting the number of donors should be considered carefully, as repeated cell doublings, which would be necessary to create a massive cell bank, can limit the stemness and function of the final MSC product (60).

7 Summary

MSCs have shown promise as a therapy for GvHD and an array of other diseases. While late-phase clinical trials were not an overwhelming success, they did show the potential impact MSCs could have on inflammatory diseases while also indicating that additional iterations with MSCs will be needed for the biologic to reach its potential. Increasing interest and investment in the field of cell therapy will continue to make processes more streamlined, will reduce the complexity of MSC expansion, and should help elucidate the optimal manufacturing method, media, donor source, and potency assay to correlate clinical efficacy with the cell product (61).

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Methods in Molecular Biology (2015) 1283: 13–21
DOI 10.1007/7651_2014_118

Published online: 24 December 2014

The Suspension Culture of Undifferentiated Human Pluripotent Stem Cells Using Spinner Flasks

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Abstract

Suspension cell culture systems with superior scalability, controllability, and monitoring options are an attractive alternative to static adherent culture methods for expansion and production of human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent cells (iPSCs). In this chapter, we describe a scalable suspension culture system using serum-free, feeder-free, matrix-free, and defined culture conditions with spinner flasks for hPSC maintenance and expansion. This suspension culture system provides an efficient and GMP-compatible process for large-scale manufacture of hPSCs.

Keywords: Human embryonic stem cells, Human induced pluripotent stem cells, Suspension culture, Spinner flasks

1 Introduction

Human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), have enormous proliferation capacity and the potential to differentiate into all cell lineages of the three germ layers. With these characteristics, hPSCs have become attractive as cell therapies in regenerative medicine and for use in drug screening. As hPSCs, notably hESCs, move toward preclinical and clinical studies, a robust scalable culture system for cell manufacture is essential to provide sufficient quality cells to seed hPSC differentiation processes at clinical scale.

Conventionally, hPSCs are propagated in adherent cell culture systems, using feeder cells or suitable matrices that support cell attachment and growth. In the last decade, developments in defined matrices, medium, and reagents for adherent hPSC culture systems to meet clinical quality have resulted in significant progress in establishing GMP-compliant culture systems (1–7). Nevertheless, handling large numbers of adherent culture flasks, roller bottles or multi-stacked "factories" entailing vessel coating, cell passaging, and harvesting for large-scale production remains challenging and has inherent practical scalability limitations.

Large-scale processes with adherent cultures are labor-intensive and time-consuming, potentially affecting the reproducibility and quality of cell cultures. Particularly, hPSC adherent cultures are usually passaged or harvested in the form of small cell clusters (8–10). These passage processes suffer from unreliable cell quantification and poor controllability of cluster size, and result in variability among cell culture vessels and cryopreserved vials of banked products. For scale-up production of hPSCs, a more controllable and scalable process of manufacturing is desirable.

A suspension cell culture system is an attractive platform for manufacture of cell products. It has several advantages including scalability, monitoring, controllability, and facile cell feeding and harvesting options. A suspension culture system adapted to a bioreactor is also more amenable to automation, which provides even greater process monitoring and system control capability. We and several groups have successfully demonstrated that hPSCs can be maintained in cell aggregates and serially passaged in suspension cultures (11–19). An essential step for culturing hPSCs in suspension has been to grow cells in the form of cell aggregates of controlled size by addition of a ROCK inhibitor, such as Y-27632, which enhances the formation of cell aggregates and survival of dissociated hESCs in suspension (20). However, our previous report showed that while hESC growth and pluripotency marker expression are maintained in aggregates up to 200–300 μm in diameter, growth and pluripotency decrease beyond this range (19), presumably due to a reduction in nutrient and metabolite penetration into the center of large cell aggregates. Therefore, monitoring and control of cell aggregate size, managed by periodic passage of the cultures, is critical to obtain efficient continued expansion and high quality of suspension cultures.

To develop a scalable and reproducible hPSC suspension culture system compliant with GMP regulations, the selection of starting cell cultures, medium, culture vessels, and control of cell aggregate sizes need to be taken into consideration. While adherent cultures on feeder cells or undefined matrices such as Matrigel with various medium conditions have been used as starting cell sources for suspension adaptation, a starting culture with a defined matrix and a medium suitable for use in both adherent and suspension cultures provides a more reliable transition from adherent to suspension culture. Several common hPSC media, such as feeder cell-conditioned based-medium, KSR-based media, mTeSR™, and StemPro™ hESC SFM, have been used alone or modified for use in reported hPSC suspension culture systems. To achieve predictable cell culture quality and GMP compliance, serum-free and defined medium, preferably delivered by qualified vendors, are favored for reproducible cell manufacturing. For scale-up production, it is critical to select a proper type of culture vessel which can provide better scalability. Various sizes and geometries of culture

vessels, including plates, Petri dishes, Erlenmeyer flasks, and spinner flasks, have been used to carry hPSC suspension cultures in agitation or static state. Among these culture vessels, spinner flasks provide superior scalability and are more suitable for automation.

Here we describe a scalable and GMP-compliant method to grow and expand hESC and hiPSC in the form of cell aggregates in suspension (Fig. 1) using spinner flasks (Fig. 2) under serum-free, feeder-free, matrix-free, and defined culture conditions. This method has been demonstrated to support long-term serial passage of hESC cells with consistent and predictable performance equal or superior to the growth of the same lines in optimized adherent systems, such as on Matrigel or mouse embryo fibroblasts (MEF). Cells generated from these suspension cultures can be stably cryopreserved in serum-free and defined medium conditions with high cell viability at thaw. This process has been applied to pilot scale GMP-compliant production of hPSCs and shown efficient for the production of >500 vial master and working cell banks.

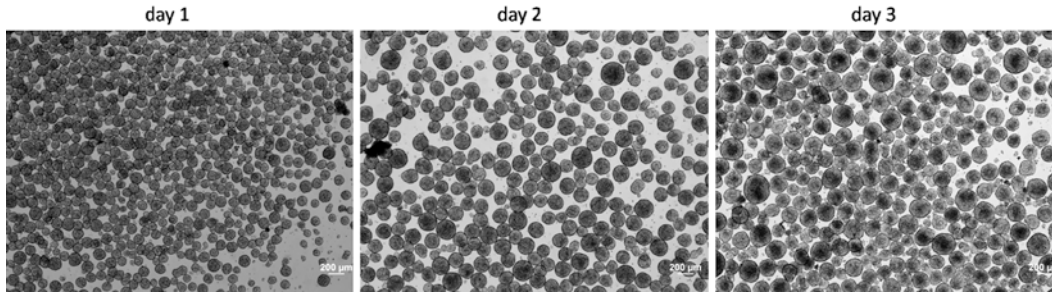

Fig. 1 Cell aggregates of H7 line formed on day 1, 2, and 3 suspension cell culture

Fig. 2 The hESC suspension cultures setup with disposable 125-mL spinner flasks on a slow-speed magnetic stirrer in a incubator

2 Materials

1. Recombinant Human Fibroblast Growth Factor-basic (bFGF) (Life Technologies): 10 μg/mL bFGF stock solution, dissolve 1 mg lyophilized bFGF in 100 mL filtered PBS (containing calcium and magnesium) with 0.1 % BSA. Aliquot and store at −20 °C.
2. Y27632 (EMD Millipore): 5 mM Y27632, dissolve 10 mg of Y27632 in 5.91 mL sterile distilled water to a 5 mM stock solution. Aliquot, protect from light, and store at −20 °C.
3. StemPro hESC SFM kit (Life Technologies): The StemPro hESC SFM kit contains three components, DMEM/F-12 with Glutamax (store at 2–8 °C), Bovine Serum Albumin (BSA) 25 % (store at 2–8 °C), and StemPro hESC Supplement (store at −20 °C). All of the components should be protected from light. Supplement is supplied as a frozen vial. Thaw supplement prior to use, aliquot, and store at −20 °C. Avoid multiple freeze-and-thaw cycles.

   Preparation of StemPro hESC SFM: Thaw supplement at 4 °C overnight. To prepare 500 mL of StemPro medium, mix 454 mL DMEM/F-12 with Glutamax with 10 mL StemPro supplement, 36 mL 25 % of BSA, 2 mL 10 μg/mL of bFGF (**Note** 1). Compounded StemPro hESC SFM medium is stable for 7 days when stored at 2–8 °C with protection from light. Add β-Mercaptoethanol to 0.1 mM final concentration before use.
4. 55 mM β-Mercaptoethanol (Life Technologies).
5. DMEM/F-12 (Life Technologies).
6. PBS without Ca^{2+} Mg^{2+} (CELLgro).
7. PBS with Ca^{2+} Mg^{2+} (Irvine Scientific).
8. Accutase (EMD Millipore): Thaw Accutase at 4 °C, aliquot, and store at −20 °C. Accutase can be stored for up to 2 months after thawing at 4 °C.
9. Stemsol (Protide Pharmaceutical).
10. Cryopreservation Medium: 10 % DMSO (Stemsol) and 90 % StemPro medium supplemented with 10 μM Y27632, and filtered through a 0.2 μm filtering system with PES membrane. Make freeze medium fresh just before cell cryopreservation and keep on ice.
11. Cimarec Biosystem Slow-Speed Stirrer (Thermo Scientific).
12. Cimarec Biosystem 40B Controller (Thermo Scientific).
13. 125 mL Disposable Spinner Flasks (Corning).

3 Methods

3.1 Selection of Starting Adherent Cultures

hESCs and hiPSCs cultured on feeder cells with Knock-out Serum Replacement (KSR) medium plus bFGF, on Matrigel with mTeSR or E8 medium, and on CELLstart with StemPro hESC SFM have been directly used as starting cells for adaptation to our suspension culture system. Several hPSC lines, HES-2, H1, H7, H9, H14, and a hiPSC line, have been adapted from different adherent culture systems to the suspension culture using the method. As StemPro hESC SFM is the medium for our suspension culture system, an adherent culture with this medium may provide a better transition to the suspension culture.

3.2 Adaptation of Adherent to Suspension Cultures

1. Expand hPSC adherent cultures to at least 1.5–1.8 $\times$ 10^7 cells for a 60 mL suspension culture in a 125-mL spinner flask. The working volume for the spinner flask is 40–100 mL.
2. Pretreatment of the adherent culture with Y-27632: Dilute Y-27632 with proper volume of culture medium and add it into the adherent culture at final concentration of 10 μM. Incubate the culture with Y-27632 at 37 °C for 1 h prior to cell dissociation. For adherent culture using a 6-well plate, 0.5 mL culture medium mixed with 7 μL of Y-27632 stock solution is added for a well of 3 mL culture.
3. After incubation, remove medium and wash wells with 1–2 mL of pre-warmed PBS without Ca^{++}/Mg^{++}.
4. Dissociate cells with 1 mL of Accutase solution per well. Incubate at 37 °C for 1–5 min or until colonies loosen up. Gently pipette cells to dissociate them into single cells (**Note 2**).
5. Collect dissociated cells into conical tubes and use 3–4 mL of DMEM/F12 to wash 1 mL of Accutase.
6. Centrifuge cells at RCF 300 $\times$ *g* for 5 min.
7. Remove supernatant and perform a second wash.
8. Resuspend pellet in a proper volume of StemPro medium containing 10 μm Y-27632 to make cell counts.
9. Add cell into StemPro medium containing 10 μM Y-27632 to make 60 mL suspension culture with cell density 2.5–3 $\times$ 10^5 cells/mL in a 125-mL spinner flask.
10. Place flask(s) on magnetic stirrer at stirring rate 70 rpm and culture cells at 37 °C with 5 % CO_2.
11. Change medium everyday by replacing 50 % of spent medium with equal volume of fresh medium without Y-27632. Take out 50 % of spent medium and spin down cell aggregates. Remove supernatant and resuspend cell aggregates in equal volume of fresh medium. Add the cell suspension back to the

spinner flask. Alternatively, if the aggregates are big, let cell aggregates precipitate for 5–10 min and replace 50 % of spent medium with fresh medium.

12. Passage cells every 3–4 days (**Notes 3–5**).

3.3 Subsequent Passaging of Suspension Culture

1. Harvest cells aggregates from spinner flasks, spin down cell aggregates at RCF 200 × *g* for 5 min, and aspirate supernatant.
2. Gently resuspend and wash cell aggregates using PBS without Ca^{++}/Mg^{++}. Spin down cell aggregates at RCF 200 × *g* for 5 min and carefully aspirate supernatant.
3. Add pre-warmed Accutase (~1 mL per 20 mL of culture) and mix gently.
4. Incubate in a water bath at 37 °C for 3–5 min with gentle shaking.
5. Gently pipette cell aggregates with a glass pipette or p-1000 to dissociate them into single cells (**Note 2**).
6. Wash the dissociated cells with DMEM/F12 (Accutase: DMEM/F12 ~1:10 dilution).
7. Spin down cells at RCF 300 × *g* for 5–10 min and aspirate supernatant.
8. Resuspend cell pellet in an appropriate volume of StemPro medium containing 10 μM Y-27632 to make cell counts.
9. Seed single cells at ~2.5–3 × 10^5 cells/mL (**Note 6**) with StemPro medium containing 10 μM Y-27632 into spinner flasks.
10. Place spinner flasks on magnetic stirrer set to a speed of 70 rpm (**Notes 6–8**) and culture cells at 37 °C with 5 % CO_2.
11. Change medium everyday with the procedure described previously in step 11 of "*Adaptation of adherent to suspension cultures.*"
12. Passage cells every 3–4 days (**Notes 3–5**).

3.4 Cryopreservation of hESCs from Suspension Cultures

1. Turn on the controlled rate freezer and precool the chamber to 4 °C approximately 30 min before use.
2. Program the controlled rate freezer with the setup in Table 1.
3. Centrifuge dissociated single cell suspension at RCF 300 × *g* for 5 min to obtain cell pellet and remove supernatant.
4. Gently resuspend cells in StemPro and perform a cell count.
5. Adjust cell concentration to 8 × 10^6 cells/mL with cold StemPro medium.
6. Mix cell suspension in StemPro with equal volume of 2× cold freeze medium to make cell density 4 × 10^6 cells/mL for vialing.

Table 1
Program setup of the controlled rate freezer for cryopreservation of hPSCs generated from suspension cultures

Step	Rate (°C/min)	End temperature (°C)
1	−1	−4
2	−25	−40
3	10	−12
4	−1	−40
5	−10	−90

7. Aliquot 1 mL cell suspension in each cryovial. Mix remaining cell suspension frequently during vialing to ensure even suspension.
8. Keep vialed cells on ice.
9. Upon completion of vialing, immediately transfer vials to the precooled controlled rate freezer and start the program.
10. After the program is finished, transfer the frozen vials into liquid nitrogen for cryopreservation.

4 Notes

1. The original StemPro hESC SFM contains 8 ng/mL bFGF. Increasing the concentration to 40 ng/mL in suspension culture promotes cell yield, undifferentiation rates, and culture consistency.
2. Proper dissociation of cell aggregates into single cells is a critical step for success of the suspension culture. Overtreatment of cells with enzyme, or excessive trituration during cell dissociation, will cause cell differentiation and low cell expansion rate.
3. An observed fold expansion of <2 after 3–4 days may be expected for the first 1–2 passages in suspension when seeding from an adherent culture or after thawing a previously adapted suspension line.
4. A stable fold expansion rate, high expression of pluripotency markers, and compact sphere morphology are usually signs of good adaptation. For a successful suspension adaptation, the anticipated expansion ranges between two and fivefold, and most typically three to fourfold, every 3–4 days post-passage. Pluripotency markers, such as Tra-1-60, Oct-4, and SSEA-4, are expected to be over 90 %.
5. The optimal time interval for cell passage can be established by daily evaluation of the average cell aggregate size, pluripotency

marker expression, and fold cell expansion. Once parameters are established for a specific cell line, visual observation of aggregate size at day 3 or 4 is usually sufficient to confirm that a culture is ready to be passaged. Aggregate size can affect the efficiency of nutrient and metabolite penetration in cell aggregates, and thus affect cell growth and cell quality. A decline in the expression of pluripotency markers or fold expansion indicates a need to passage the cultures. Aggregate sizes in the range of 200–300 μm, typically occurring between days 3–4, are common for the hESC and iPSC lines we have tested.

6. Some adherent PSC lines may take several passages to adapt to suspension culture. For difficult adaptation, increasing cell seeding density or reduced stir rates may provide a better transition.
7. The sensitivity of different cell lines to shear stress varies and has to be optimized for each line. Optimization of stirring rates may be necessary if aggregate fusion, significant amounts of cell debris, or failure of aggregates to increase in size are observed.
8. The suspension culture system has been successfully applied to disposable 125-mL, 500-mL, and 1-L spinner flasks (Corning). Optimized stirring rates used for 500-mL and 1-L spinner flasks are typically around 70, 65, and 50 rpm, respectively, but may vary ±5 rpm for different lines.

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Methods in Molecular Biology (2015) 1283: 23–29
DOI 10.1007/7651_2014_106

Published online: 10 August 2014

Scalable Expansion of Human-Induced Pluripotent Stem Cells in Xeno-Free Microcarriers

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Abstract

The expansion of human-induced pluripotent stem cells (hiPSCs) is commonly performed using feeder layers of mouse embryonic fibroblasts or in feeder-free conditions in two-dimensional culture platforms, which are associated with low production yields and lack of process control. Robust large-scale production of these cells under defined conditions has been one of the major challenges to fulfil the large cell number requirement for drug screening applications, toxicology assays, disease modeling and potential cellular therapies. Microcarrier-based systems, in particular, are a promising culture format since they provide a high surface-to-volume ratio and allow the scale-up of the process to stirred suspension bioreactors. In this context, this chapter describes a detailed methodology for the scalable expansion of hiPSCs in spinner flasks and using xeno-free microcarriers to allow further translation to Good Manufacturing Practice (GMP) conditions.

Keywords: Human-induced pluripotent stem cells, Microcarriers, Spinner flask, Xeno-free

1 Introduction

Human-induced pluripotent stem cells (hiPSCs) present a great therapeutic potential in the field of regenerative medicine, due to their inherent capacity to self-renewal and to differentiate into the multiple cell types that arise from the three embryonic germ layers. These cells are emerging as a potential alternative to human embryonic stem cells (ESCs), since they avoid the ethical concerns associated to the manipulation and destruction of human embryos and allow overcoming immune rejection issues, when performing transplantation of patient-specific hiPSCs in personalized medicine settings (1, 2). Importantly, patient-specific hiPSC may also be used in several in vitro biomedical applications such as drug screening (3) and disease modeling (4).

One major limitation associated to the production and clinical application of hiPSCs in cellular therapy is the development of standardized and scalable culture processes to produce a clinically relevant quantity of cells, while maintaining their pluripotent state,

differentiation potential, and their genetic stability. This is not possible when using the traditional two dimensional cell culture platforms (5) since these culture systems present low cell yields and lack of control, which leads to variability on product quantity and quality.

In order to overcome these issues, focus is being given to hPSC expansion on microcarrier-based suspension cultures since these microparticles support the growth of anchorage-dependent cells, providing high surface area to volume ratios (6, 7). Moreover, when combined with stirred-tank bioreactors, microcarrier-based systems can provide a solution for bioprocess scale-up (8) since stirring and control characteristics of this system reduce the heterogeneity of the culture environment, minimizing gradients of dissolved oxygen and metabolite concentrations, while avoiding problems of undesired spontaneous differentiation and genetic instability. Moreover, and envisaging further bioprocess translation to GMP conditions, a great effort has also been made towards the translation of these scalable culture systems to chemically-defined and xeno-free conditions (9).

In this chapter, a microcarrier-based methodology for the scalable expansion of hiPSCs under chemically defined conditions is described. A single-cell strategy for hiPSC inoculation is used in order to allow a more homogeneous distribution of the cells throughout the microcarriers. In the first place it is described a methodology for screening of different microcarriers under static culture, in order to select the most suitable for translation to dynamic conditions, by evaluating their ability to promote hiPSC adhesion, proliferation, and pluripotency characteristics. Afterwards, it is described a methodology for microcarrier-based expansion of hiPSCs under dynamic culture conditions in a 15 mL-laboratory-scale stirred spinner flask. The protocol described was developed based on the use of synthetic microcarriers, consisting of a peptide-acrylate surface functionalized with synthetic peptides and designed for long-term support of hPSC self-renewal under xeno-free conditions (10, 11). Importantly, this protocol is scalable, allowing further translation to a fully controlled large-scale stirred tank bioreactor, in order to provide a highly reproducible and robust process for the large-scale, clinical-grade production of hiPSCs.

2 Materials

2.1 Microcarriers

Xeno-free microcarriers (Synthemax II surface, Corning) were used to support hiPSC expansion in suspension both under static conditions and in the spinner flask. Matrigel-coated Plastic microcarriers (Solohill Engineering, Inc.) were used as a positive control for initial microcarrier evaluation under static conditions (*see* **Note 1**).

2.2 Disposables and Equipments

1. Sterile low-attachment 24-well plates (Corning Inc.).
2. Spinner flask (Wheaton, U.S. Patent 3572651).
3. Magnetic stirrer platform (Thermo Scientific, Variomag Biosystem Direct).
4. Fully humidified cell culture incubator with Temperature and CO_2 control (Memmert, Inco2).
5. Water bath (Memmert, WB22).
6. Cell culture centrifuge (Hermle, Z 400 K).
7. ThermoMixer (Eppendorf, Comfort).
8. Micropipette (1–10, 10–100, 100–1,000 μL).
9. Pipettor.
10. Laminar flow hood.

2.3 Culture Media and Solutions

1. mTeSR1 culture medium for hPSC maintenance (Stem Cell Technologies): Aseptically add the 5× Supplement to the basal medium for a total volume of 500 mL (*see* **Note 2**).
2. KO-DMEM/SR medium: Prepare and sterile-filter 392 mL of Knockout-DMEM, 100 mL of Knockout-Serum Replacement, 5 mL of MEM nonessential amino acids, 2.5 mL of L-Glutamine, 0.5 mL of β–Mercaptoethanol (0.1 M) and 5 mL of Penicillin/Streptomycin (All from Invitrogen). Keep KO-DMEM/SR medium at 4 °C (*see* **Note 3**).
3. Phosphate buffered saline (PBS) solution: Dissolve (1×) PBS powder (Life Technologies) in 1 L of distilled water and filter the solution using a sterile 0.22 μm filter and store at room temperature.
4. Matrigel (BD Biosciences): Thaw Matrigel aliquot on ice at 4 °C overnight (*see* **Note 4**). Dilute Matrigel (1:30) in cold DMEM-F12 (Invitrogen) and incubate the sterile microcarriers for 2 h at room temperature (50 μL of diluted Matrigel per cm^2 of growth area).
5. Accutase (Sigma) (*see* **Note 5**).
6. ROCK inhibitor (Y27632, StemGent): For a stock solution of 10 mM, dilute 2 mg of Y27632 in 624 μL of DMSO (Sigma). Store aliquots at −20 °C. Use a working concentration of 10 μM.
7. Ethanol 70 % (v/v).
8. SIGMACOTE® (Sigma), solution of chlorinated organopolysiloxane in heptane. Store at 2–8 °C.
9. Trypan blue stain solution 0.4 % (Gibco).

3 Methods

3.1 Screening of Xeno-Free Microcarriers Under Static Culture in Low-Attachment 24-Well Plates

3.1.1 Preparation of Microcarriers

1. Weigh to a falcon tube the amount of microcarriers needed to guarantee 3 cm^2 of microcarrier surface area per well.
2. For sterilization, add 1 mL of ethanol 70 % for 10 mg of microcarriers and incubate for 60 min with rocking agitation.
3. Wait until the microcarriers settle down and remove the ethanol. Wash three times with sterile PBS (3 mL each time).
4. Incubate microcarriers with mTeSR1 culture medium for at least 30 min, at 37 °C and 5 % CO_2.

3.1.2 Single-Cell Inoculation

1. Start the protocol when hiPSCs are 80 % confluent in a Matrigel-coated plate.
2. Remove the exhausted medium from the wells and add 1 mL/well of prewarmed KO-DMEM/SR medium containing 10 μM of ROCK inhibitor (ROCKi).
3. Incubate cells at 37 °C and 5 % CO_2 for 60 min.
4. Remove the medium and wash the wells with PBS (1 mL/well).
5. Add 1 mL/well of accutase and incubate at 37 °C for 5–10 min (*see* **Note 6**).
6. Add 1 mL/well of prewarmed KO-DMEM/SR medium (with ROCKi) and flush the cells. Transfer cell suspension to a falcon tube. Add more 1 mL/well of medium, flush, and transfer cells to the same falcon tube (*see* **Note 7**).
7. Centrifuge for 5 min at 208 × *g*.
8. Remove supernatant and ressuspend the pellet in 1 mL of mTeSR1 medium containing 10 μM of ROCKi, with a 1,000 μL pipette.
9. Count viable and dead cells in a hemocytometer under the optical microscope using the trypan blue dye exclusion test.
10. Prepare cell suspension in mTeSR1 medium (250 μL per well) supplemented with ROCKi for inoculating at the desired cell density (25,000–50,000 cells/cm^2).

3.1.3 Inoculation of Microcarriers Under Static Conditions in 24-Well Plates

1. After performing the procedure described in Section 3.1.1 wait until the microcarriers settle down and remove the incubation medium.
2. Add the volume needed (250 μL per well) of mTeSR1 medium supplemented with ROCKi (10 μM), in order to have a final microcarrier concentration of 12 cm^2/mL.
3. Distribute 250 μL per well of microcarrier solution in sterile low-attachment 24-well plates (*see* **Note 8**).
4. Distribute 250 μL of resuspended hiPSCs per each well of the 24-well plate (*see* **Note 8**).

3.1.4 Culture Conditions

1. Place the 24-well plates in the incubator at 37 °C and 5 % CO_2.
2. After 24 h, change 400 μL/well of the exhausted medium, without removing any microcarriers, by 400 μL/well of prewarmed mTeSR1 medium without ROCKi.
3. Change medium every day until the end of the culture period.

3.2 Dynamic Culture in Spinner Flask

3.2.1 Preparation of the Spinner Flask

1. Clean and dry the internal glass surface of the spinner flask before the siliconization.
2. To siliconize the flask, take 25 mL of SIGMACOTE® (undiluted) and cover the internal glass surface of the spinner flask (*see* **Note 9**).
3. Allow the treated glass surface to air dry in a hood (overnight). No heating is required.
4. Rinse the siliconized flask with distilled water to remove the HCl by-products before use.
5. Fill 1/4 of the spinner flask with distilled water and autoclave at 121 °C for 20 min, for sterilization. The side arm caps must be kept loose.
6. After sterilization, close the side arm caps and leave it inside the hood.
7. When spinner flask reaches the room temperature, remove the water and wash the flask with KO-DMEM/SR medium.

3.2.2 Preparation of Microcarriers for hiPSC Culture

Proceed as in Section 3.1.1, however guarantee a total surface area of 100 cm^2/spinner (working volume of 15 mL).

3.2.3 Single-Cell Inoculation

Proceed as in Section 3.1.2.

3.2.4 Inoculation of Microcarriers for Spinner Flask Culture

1. Place the microcarrier solution (100 cm^2 of microcarriers in 3 mL of mTeSR1 medium supplemented with ROCKi) inside the spinner flask.
2. Add the resupended hiPSC suspension (2.5–5 × 10^6 cells in 3 mL of mTeSR1 medium supplemented with ROCKi) to the microcarriers inside the spinner flask.

3.2.5 Culture Conditions

1. Close the spinner flask and place it inside the incubator at 37 °C and 5 % CO_2 in static conditions. The side arm caps must be kept loose.
2. After 24 h, remove 3 mL of the exhausted medium, without taking any microcarriers, and add 12 mL of prewarmed mTeSR1 medium without ROCKi (to a final working volume of 15 mL).

3. Leave the spinner flask overnight at an intermittent agitation of 40 rpm for 3 min every 2 h.
4. Start the continuous agitation at 40 rpm afterwards.
5. Replace 50 % of the exhausted medium by prewarmed mTeSR1 medium every day.

3.3 Collect Cells at the End of the Culture

1. Remove the exhausted medium from the spinner flask and incubate cell confluent microcarriers with KO-DMEM/SR medium supplemented with ROCKi (10 μM) at 37 °C for 60 min.
2. Wash cell confluent microcarriers with sterile PBS.
3. Incubate cell confluent microcarriers with 5 mL of accutase in a ThermoMixer for 30 min at 37 °C, using 750 rpm of mixing speed.
4. Dilute with 5 mL of KO-DMEM/SR medium supplemented with ROCKi.
5. Dissociate cells from microcarriers by pipetting up and down with a 1,000 μL pipette.
6. Sterile filter the mixture through a 40 μm mesh to remove microcarriers.
7. Centrifuge the cells for 5 min at 1,000 rpm.
8. Remove supernatant and resuspend the cell pellet in 1 mL of mTeSR1 medium containing 10 μM of ROCKi, with a 1,000 μL pipette.
9. Count viable and dead cells in a hemocytometer under optical microscope using trypan blue dye exclusion test.
10. Collected hiPSCs should be then evaluated in terms of their pluripotency state, by analyzing the expression of pluripotent markers by immunocytochemistry and flow cytometry, and genetic stability by karyotype analysis. Cell differentiation capacity should also be evaluated in vitro via embryoid body formation and spontaneous differentiation.

4 Notes

1. Plastic microcarrier coating with Matrigel was performed during 2 h of incubation at room temperature using 0.25 mL of Matrigel solution (1:30), supplemented with ROCKi (10 μM), per cm^2 of microcarrier superficial area.
2. Aliquots of mTeSR1 medium stored at −20 °C are stable for 6 months. Thaw the complete mTeSR1 medium at 4 °C for use (stable for 2 weeks).

3. Protect KO-DMEM/SR medium from light, since KO-SR is light sensitive.
4. Since above 10 °C Matrigel starts to form a gel, it is necessary to work on ice when handling this solution.
5. After thawing, accutase may be stored up to 2 months at 4 °C.
6. Tap the cell culture plate gently and wait until when cell-colony contours become loose.
7. To flush the cells from the culture plate, use a 1,000 μL pipette.
8. Move the plate sideways several times to ensure an even distribution.
9. The reaction of the glass with SIGMACOTE® is almost instantaneous. Excess solution can be removed for reuse.

Acknowledgments

This work was funded by Fundação para a Ciência e a Tecnologia, Portugal (Project PTDC/EQU-ERQ/105277/2008). Sara M. Badenes, Tiago G. Fernandes, and Carlos A.V. Rodrigues were supported by fellowships from Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BPD/74449/2010, SFRH/BPD/86316/2012 and SFRH/BPD/82056/2011, respectively).

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Methods in Molecular Biology (2015) 1283: 31–42
DOI 10.1007/7651_2014_124

Published online: 11 October 2014

Derivation of GMP-Compliant Integration-Free hiPSCs Using Modified mRNAs

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Abstract

The clinical use of human induced pluripotent stem cells (hiPSCs) and the development of patients-specific gene and cell therapies rely on the development of fast, reliable, and integration-free methods of derivation of pluripotent stem cells from somatic tissues. Here we describe an integration-free protocol for the rapid derivation of hiPSCs from dermal fibroblasts using modified mRNAs. This method is inexpensive, highly efficient, and makes use of reagents that are xeno-free and chemically defined and can therefore be adopted by any Good Manufacturing Practice (GMP) facility.

Keywords: Xeno-free hiPSCs, GMP, Clinical use, Modified mRNA, Regenerative medicine, Cell therapy, Gene therapy

1 Introduction

Derivation of human induced pluripotent stem cells (hiPSCs) from virtually any adult tissue provides numerous opportunities for development of therapeutic strategies in regenerative medicine, cell therapies, and drug discovery applications. Substantial progress has been made in the derivation and optimization of hiPSCs since its technology has first been described in 2007 (1). Moreover and in contrast to human embryonic stem cell (hESC) lines, hiPSCs provide an unlimited cell source without the destruction of human embryos, thus presenting a promising, unlimited, and alternative supply of undifferentiated pluripotent cells. Despite this, the promise of hiPSCs in regenerative medicine relies on overcoming several hurdles. In particular, clinical application of hiPSCs derivatives necessitates a protocol of derivation with minimal risk of integration of exogenous DNA as random integration can lead to insertional mutagenesis with unpredictable effects on the quality of the cells and the potential safety after transplantation (2). Although non-integrative DNA-based methods of inducing pluripotency including episomal vectors (3) and minicircles (4) have been developed, it is difficult to exclude the possibility of integration of very small fragments of DNA. Other proposed non-integrative methods

like protein-based reprogramming (5) or Sendai virus (6) are very inefficient (protein based) or require extensive passaging to remove residual viral expression (Sendai virus).

More importantly, the clinical application of hiPSCs requires that their production has to meet GMP-compatible standards but also that the derivation of specified cell derivatives (used for transplantation) from pluripotent stem cells is performed under GMP conditions. Previous regulatory oversight suggests that two methods may be acceptable for this purpose: (1) Derivation of cells and cell products under GMP requirements and (2) Conversion of cells or cell products derived under research-grade conditions to GMP quality standards (http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070273.pdf). Here, we developed a standardized platform of human iPSC derivation by making use only of chemically defined matrices and animal-free reagents that are already in use in GMP facilities. This protocol is integration-free, fast, efficient, and easily adoptable by any GMP facility.

2 Materials

Prepare all solutions using ultrapure RNase/DNase-free water and analytical grade reagents. All reagents and equipment are handled under sterile and GMP-compliant conditions.

2.1 Culture of Human Fibroblasts

- CELLstart (Life Technologies).
- TrypLE Express (Life Technologies).
- Serum-free Fibroblasts Medium (e.g., FibroLife from Life Technologies).
- DBPS with calcium and magnesium (Life Technologies).

2.2 Derivation of Induced Pluripotent Stem Cells

- Modified mRNAs encoding for OCT4, SOX2, KLF4, cMYC, LIN28A, and GFP (100 ng/μl) (TriLink)

  Preparation of mRNA cocktail and single-use aliquots

  – Thaw individual mRNAs on ice (**Note 1**). Combine mRNA factors into a 1.5 ml RNase-free microcentrifuge tube on ice as follows: 385 μl OCT4 mRNA + 119 μl SOX2 mRNA + 156 μl KLF4 mRNA + 148 μl cMYC mRNA + 83 μl LIN28A mRNA + 110 μl nGFP mRNA. Mix well and aliquot 50 μl of the mRNA cocktail into 20 individual sterile, 1.5 ml RNase-free microcentrifuge tubes. Store aliquots at −70 °C (**Note 2**).
- Opti-MEM basal medium (Life Technologies).
- RNAiMAX (Life Technologies).

- Pluriton Basal Medium (Stemgent).

  Preparation of aliquots

  – Thaw Pluriton Basal Medium overnight at 4 °C. Pipet 40 ml aliquots of Pluriton Basal Medium into seven 50 ml conical tubes (280 ml total). Store aliquots at −20 °C (**Note 3**).
- Pluriton Supplement (Stemgent)

  Preparation of single-use aliquots

  – Thaw 200 μl of Pluriton Supplement on ice. Pipet 4 μl of Pluriton Supplement into 50 sterile, low protein-binding microcentrifuge tubes and store at −70 °C (**Note 4**).
- B18R interferon inhibitor (eBioscience)

  Preparation of single-use aliquots

  – Thaw 40 μg of B18R protein (0.5 mg/ml stock concentration, 80 μl total volume) on ice. Pipet 4 μl of B18R protein into 20 sterile, low protein-binding microcentrifuge tubes and store at −70 °C (**Note 4**).
- CELLstart (Life Technologies).
- StainAlive DyLight 488 Mouse anti-Human TRA-1-60 (Stemgent).
- StainAlive DyLight 488 Mouse anti-Human TRA-1-81 (Stemgent).

2.3 Culture and Cryopreservation of Induced Pluripotent Stem Cells

- TeSR2 Basal Medium (Stem Cell Technologies).
- TeSR2 5X Supplement (Stem Cell Technologies).
- TeSR2 250X Supplement (Stem Cell Technologies).

  Preparation of Complete TeSR2 medium

  – Thaw TeSR2 5X Supplement and TeSR2 250X Supplement at room temperature (15–25 °C) (**Note 5**). Add 100 ml of thawed TeSR2 5X Supplement and 2 ml of thawed TeSR2 250× Supplement to 400 ml TeSR2 Basal Medium. Mix well and store at 4 °C (**Note 6**).
- Nutristem (Stemgent).
- CELLstart (Life Technologies).
- Accutase (Innovative Cell Technologies).
- Bambanker (Wako).
- Liquid nitrogen.
- Cryovials.

2.4 Characterization of GMP-Compliant hiPSCs

- Gene expression analysis of pluripotency markers.
- Immunocytochemistry of pluripotency markers.
- EB formation and spontaneous in vitro differentiation.

- Gene expression analysis and immunocytochemistry of differentiation markers.
- Teratoma formation.

2.5 Equipment

- Pipette-aid.
- Serological pipettes (2, 5, and 10 ml).
- 2, 10, 200, and 1,000 μl pipettors.
- 6-well tissue culture plates.
- 100 mm dishes.
- 15 ml and 50 ml conical centrifuge tubes.
- Syringe filters (0.22 μm).
- Triple gas tissue culture incubator set at 37 °C, 5 % CO_2, 5 % O_2.
- Tissue culture incubator set at 37 °C, 5 % CO_2.
- Tissue culture centrifuge.
- Phase-contrast microscope.
- Fluorescent microscope.
- Stereo microscope.
- Dissection scope.
- Hemocytometer.
- Coverslips.
- 70 % Ethanol.
- Cell scrapers.
- Low protein-binding microcentrifuge tubes.
- RNase-free aerosol-barrier tips.
- Cell lifters.
- Needles (20 Gauge).
- Glass pipettes.
- Vertical laminar flow hood certified for Level II handling of biological materials and GMP compliant.
- Vortexer.
- Table centrifuge.

3 Methods

Carry out all procedures in a sterile tissue culture hood under GMP-compliant conditions unless indicated otherwise. This protocol describes mRNA-based reprogramming of fibroblast cells that are cultured in 4 wells of 6-well tissue culture plate (Fig. 1). Three wells are used to reprogram target fibroblasts at different

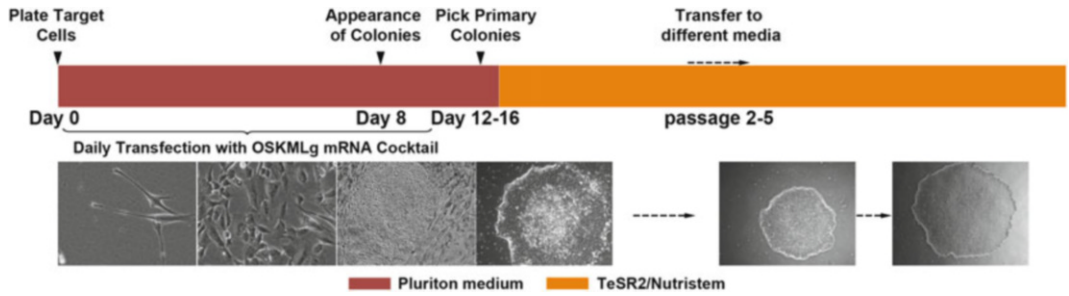

Fig. 1 Overview of feeder-free derivation of mRNA-induced pluripotent stem cells under GMP-compliant conditions. Morphology tracking of reprogrammed human fibroblasts during the course of reprogramming and passaging. Fibroblasts show early epithelioid morphology and small cluster formation that lead into small hES cell-like colonies. Small colonies grow in size and become mature iPSC colonies

starting densities while the remaining well serves as positive control and is used to reprogram BJ fibroblasts. Reprogramming experiments are performed in a triple gas tissue culture incubator set at 37 °C, 5 % CO_2, 5 % O_2 as it has been shown to increase reprogramming efficiencies (7).

3.1 Coating 6-Well Tissue Culture Plate

1. Dilute CELLstart 1:50 in DPBS.
2. Add 750 µl diluted CELLstart to 4 wells of a 6-well tissue culture plate (final volume is 0.078 ml/cm^2).
3. Incubate in the triple gas incubator for 2 h (**Note 7**).
4. Aspirate CELLstart. The plate is now ready for plating of cells.

3.2 Culturing and Plating of Target Fibroblasts

1. Aspirate culture medium from fibroblasts (**Note 8**).
2. Add 0.05 % Trypsin/EDTA to the cells.
3. Rinse twice with DPBS
4. Incubate the cells for 3–5 min in the triple gas incubator.
5. Add 2× (volume) fibroblast culture medium to the cells to dilute the Trypsin/EDTA.
6. Transfer the harvested cell solution into a 15 ml conical tube with a serological pipette.
7. Centrifuge the cells for 4 min at 200 × *g*.
8. Remove the supernatant and resuspend the cell pellet in 5 ml of fibroblast culture medium.
9. Count the cells with the hemocytometer and calculate the plating cell density (**Note 9**).
10. Plate the cells in three independent wells of a CELLstart-coated 6-well tissue culture plate at densities of 1×10^4, 2.5×10^4, and 5×10^4 in 2 ml culture media. Plate BJ fibroblasts at a density of 1×10^4 into the fourth well of the CELLstart coated 6-well tissue culture plate.
11. Incubate the cells overnight in the triple gas incubator (**Note 10**).

3.3 Transfect Cells and Start Reprogramming

1. Add 10 ml of Pluriton Medium to a sterile 100 mm dish (**Note 11**).
2. Incubate Medium for 2 h in triple gas incubator (**Note 12**).
3. Just prior to use, thaw one aliquot of Pluriton Supplement and B18R protein on ice (**Note 13**).
4. Add 4 μl of each aliquot to the equilibrated Pluriton Medium to generate Complete Pluriton Medium.
5. Aspirate cell medium from all 4 wells of 6-well tissue culture plate and add 2 ml of Complete Pluriton Medium (**Note 14**).
6. Incubate the cells at least 2 h in the trigas incubator prior to transfection (**Note 15**).
7. Thaw one 50 μl aliquot of mRNA cocktail on ice.
8. Add 200 μl of basal Opti-MEM to the tube containing the mRNA cocktail and gently mix up and down 4–5 times (=*tube 1*) (**Note 16**).
9. In a second sterile, RNase-free 1.5 ml microcentrifuge tube, add 225 μl of Opti-MEM (=*tube 2*).
10. Add 25 μl of RNAiMAX to *tube 2* directly into the Opti-MEM.
11. Using a 1,000 μl RNase-free, aerosol-barrier pipet tip, pipet gently but thoroughly to mix and immediately transfer diluted RNAiMAX to *tube 1* containing the diluted mRNA cocktail.
12. Briefly vortex the mRNA transfection complex and spin down with a table centrifuge.
13. Incubate the mRNA transfection complex at room temperature for 15 min for mRNA-lipids complex formation (**Note 17**).
14. During the incubation time, equilibrate fresh Pluriton Medium to be used after transfection as follows: add 10 ml of Pluriton Medium to a sterile 100 mm dish and put into trigas incubator for equilibration.
15. Remove equilibrated cells in 6-well tissue culture plate from trigas incubator and in drop-wise fashion, add 120 μl of the mRNA transfection complex to each of the 4 wells (**Note 18**).
16. Gently rock the 6-well tissue culture plate from side to side and front to back to distribute mRNA transfection complex.
17. Put back into the trigas incubator and incubate for at least 3 h (**Note 19**).
18. After 3 h of incubation, thaw one aliquot of Pluriton Supplement and B18R protein on ice.
19. Take out the 100 mm dish with pre-equilibrated Pluriton medium (see step 14) and add 4 μl of both aliquots to generate Complete Pluriton Medium.

20. Take out transfected cells (see step 17), aspirate cell medium from all 4 wells of 6-well tissue plate and add 2 ml of fresh Complete Pluriton Medium.
21. Incubate cells overnight in trigas incubator.
22. Repeat transfection procedure, medium equilibration, and medium change for 12–18 consecutive days as described in steps 7–21 (**Note 20**).

3.4 Assess Cell Proliferation Rate and Density of Target Cells

1. One of three wells should have the optimal cell density throughout the reprogramming experiment (see Fig. 2 for optimal cell density). Monitor cell death and proliferation rate every other day under a phase contrast microscope.
2. In the first week of reprogramming, positive GFP signal helps to assess transfection efficiency (should be between 80 and 90 %) (**Note 21**).
3. From the second week onwards, positive GFP signal aids in identifying early colony formation (Fig. 2) (tightly packed colonies are more difficult to transfect, thus appear as dark round "holes" under a fluorescent microscope) (**Note 22**).
4. Balance between cell death and cell proliferation of target cells should be approximately the same compared to the positive control (fourth well with BJ fibroblasts) (**Note 23**).

3.5 Identify Emerging Colonies

1. Around day 8 the first colonies may appear and will grow in size. Between day 12 and 16 the iPSC colonies should emerge and be clearly visible.
2. Primary colonies can be identified with live staining of TRA-1-60 or TRA-1-81 (**Note 24**) as described below.
3. Add 4 ml of Pluriton Medium to a 15 ml conical centrifuge tube.
4. Add 40 µl of either StainAlive DyLight 488 TRA-1-60 or TRA-1-81 antibody to the conical tube (**Note 25**).
5. Aspirate medium from all 4 wells of 6-well tissue culture plate containing cells.
6. Add 1 ml of diluted antibody to each of the 4 wells.
7. Incubate cells in Triple gas incubator for 30 min.

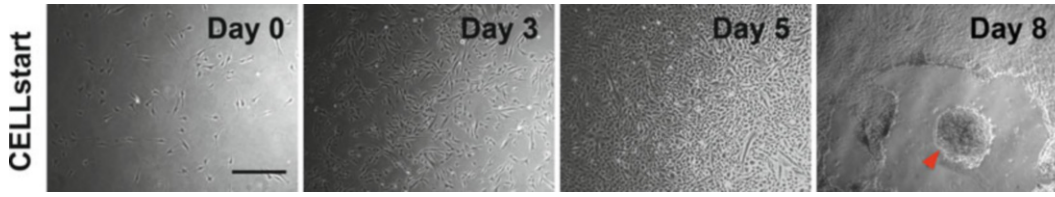

Fig. 2 Derivation of iPSCs on GMP compatible CELLstart matrix. Red arrow indicated emerging iPSC colony

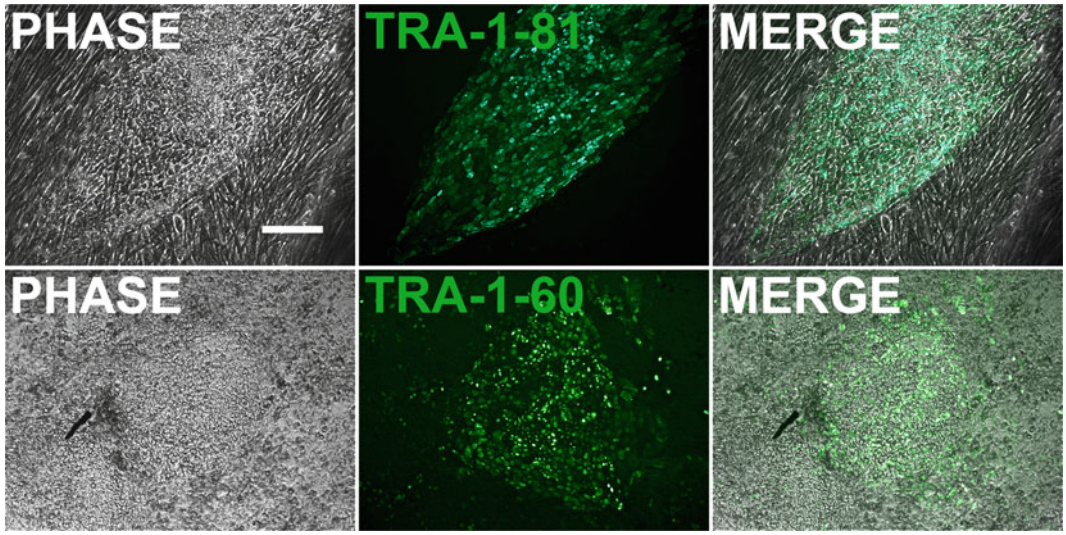

Fig. 3 TRA-1-60 and TRA-1-81 live immunostaining during reprogramming used for colony identification. Scale bar = 150 μm

8. Aspirate Pluriton Medium containing the antibody from each well and wash with Pluriton Medium twice.
9. Add 2 ml of Complete Pluriton Medium (with B18R and Pluriton Supplement) to each of the 4 wells.
10. Examine colony formation under a fluorescent microscope with the appropriate wavelength (Fig. 3) (**Note 26**).
11. Mark primary colonies for manual dissection.

3.6 Manual Dissection of Primary Colonies

1. Dilute CELLstart 1:50 in DPBS.
2. Add 250 μl diluted CELLstart to 8 wells of a 12-well tissue culture plate (final volume is 0.078 ml/cm^2).
3. Incubate in a cell culture incubator (37 °C and 5 % CO_2) for 2 h (**Note 7**).
4. Aspirate CELLstart. The plate is now ready for plating of cells.
5. Add 10 ml of Pluriton Medium to a sterile 100 mm dish.
6. Incubate Medium for 1 h in cell culture incubator.
7. Thaw one aliquot of Pluriton Supplement and B18R protein on ice.
8. Add 4 μl of both aliquots to the pre-equilibrated Pluriton Medium to generate Complete Pluriton Medium.
9. Add 1 ml of Complete Pluriton Medium to 8 wells of a 12-well tissue culture plate coated with CELLstart.
10. Return 12-well tissue culture plate to cell culture incubator.
11. Take out 6-well tissue culture containing primary iPSC colonies.

12. Using a stereo microscope, locate iPSC colonies based on morphology and based on positive TRA-1-60 or TRA-1-81 signal (see Section 3.5).
13. Using a glass picking tool, gently separate the colonies from the surrounding fibroblasts.
14. Using the same tool, gently cut each colony into 6–8 pieces (**Note 27**).
15. Using either the same tool or a cell lifter, gently detach the colony pieces from the bottom of the tissue culture plate.
16. Using a pipet with a sterile 20 μl pipet tip, transfer the detached colony pieces into an individual well of a 12-well tissue culture plate filled with freshly prepared Complete Pluriton Medium (see step 10).
17. Repeat picking and transferring of primary colonies until 8 individual colonies are picked (**Note 28**).
18. Return both plates (6-well tissue culture plate with primary iPSC colonies and 12-well tissue culture plate with freshly picked colonies) to cell culture incubator. Shake 12-well tissue culture plate back and forth and left and right a few times inside the incubator to distribute colony pieces.
19. Let pieces attach overnight.
20. Change culture medium daily with Pluriton Medium containing Pluriton Supplement (**Note 29**).
21. When cells are 80–90 % confluent, manually passage into a fresh cell culture plate by repeating steps 1–19.
22. Cells are now ready to be transitioned to a new cell culture medium environment.

3.7 Transition of Culture Medium

1. After manual passaging of iPSC colonies to passage 2, gradually transition culture medium from Pluriton Medium containing Pluriton Supplement to a 1:1 mix of Nutristem and Complete TeSR2 Medium.
2. With each daily media change, convert culture from Pluriton Medium to Complete TeSR2/Nutristem as follows: 1:0, 0.8:0.2, 0.5:0.5, 0.2:0.8, 0:1 (ratio of volumes of culture media) (**Note 30**).
3. Cells are now fully converted to GMP-compliant conditions.

3.8 Characterization of GMP-Compliant iPSCs

Cells can now be characterized for their pluripotent character by applying several assays including gene expression analysis of pluripotency markers, immunocytochemistry of pluripotency markers, embryoid body (EB) formation and spontaneous in vitro differentiation, gene expression analysis and immunocytochemistry of differentiation markers, and teratoma formation.

4 Notes

1. Keep mRNAs on ice at all times and avoid repeated opening and closing of vials containing mRNAs. Use RNaseZAP (Life Technologies) or similar RNase contamination solution to wipe down surface of work area. Modified mRNAs should be made in a GMP-compliant environment and in vitro transcribed as previously described (8).
2. Aliquots can be stored up to 3 months. Refreezing aliquots is not recommended. Aliquots are prepared to reprogram 4 wells at a time. Each aliquot will be used for each day of transfection. The molar stoichiometry of all mRNA molecules is 3:1:1:1:1:1 for OCT4, SOX2, KLF4, cMYC, LIN28A, nGFP. nGFP is used to track transfection efficiency over the course of reprogramming and aids to identify early forming colonies.
3. The remaining 220 ml of Pluriton Basal Medium can be stored at 4 °C for use during the first week of reprogramming.
4. Aliquots can be stored up to 3 months. Refreezing aliquots is not recommended. Aliquots are prepared to reprogram 4 wells at a time. Each aliquot will be used for each day of transfection.
5. TeSR2 5× Supplement and 250× Supplement can be dispensed into working aliquots and stored at −20 °C. Frozen aliquots can be stored up to 6 months. Thawed aliquots should be used within 1 day to prepare Complete TeSR2 media. Refreezing aliquots is not recommended.
6. Complete TeSR2 is stable up to 2 weeks or when frozen at −20 °C for up to 6 months. Frozen aliquots of Complete TeSR2 can be thawed at room temperature (15–25 °C) or overnight at 4 °C.
7. Optimal results are achieved when coated 6-well tissue culture plates are used the same day or 1 day after. If precoating the day before, the plates must be stored at 4 °C and wrapped with Parafilm to avoid drying.
8. It is recommended to have target fibroblast in culture prior passaging. Target cells can be directly plated from frozen stocks, though attaching efficiencies are generally lower. It is crucial that the passage number of target cells to be reprogrammed is between 1 and 5.
9. Calculated cell density is crucial for the success of reprogramming. It is recommended to count twice and calculate the average of both results. Also, the initial cell density to be used for counting should not exceed 5×10^5 cells/ml.

10. Rock 6-well tissue culture plate in the Trigas incubator back and forth and left and right to evenly distribute the target fibroblast cells.
11. Due to a high risk of contamination we recommend keeping a full stack of 100 mm dishes at a designated and sterile area to be used only for the reprogramming experiment.
12. Pluriton Medium has to be equilibrated at low O_2 tensions and to 37 °C prior use. Conical tubes for equilibration are not recommended since the smaller surface increases incubation time for complete equilibration.
13. We recommend keeping all aliquots (mRNA cocktail, B18R protein, Pluriton Supplement) at one designated area at −20 °C. All aliquots will be used on a daily basis.
14. Incubation of small volumes of culture medium is not recommended due to evaporation. A total of 8 ml (4 × 2 ml) is needed. The remaining volume can be discarded.
15. Cells have to be pretreated with the B18R protein (200 ng/ml) as it is required prior the first transfection on Day 0 to presuppress the cells' interferon response. Subsequent culture and transfections on the following days will be carried out in Complete Pluriton Medium that is already supplemented with the B18R protein.
16. Incubate Opti-MEM at room temperature prior to use.
17. It is essential that the tube containing the mRNA transfection complex be not disturbed during incubation.
18. The addition of the whole mRNA transfection should be applied dropwise to guarantee an even distribution of the complex and avoid citotoxicity.
19. Shake 6-well tissue culture plate again back and forth and left and right as the mRNA transfection complex may have concentrated towards the middle of each well. A minimum of 3 h incubation is recommended. Do not incubate for more than 4 h.
20. It is important to transfect the cells at the same time each day (±2 h) in order to maintain sufficient levels of mRNA transcripts to allow for continual expression of the mRNA factors. The transfection procedure must be repeated each day exactly as done on Day 0.
21. Cell cultures should be not more than 70 % confluent in the first week as overgrown cultures significantly decrease transfection efficiencies, thus reducing the amount of mRNA entering the cells.
22. Although we do not recommend passaging cells during a reprogramming experiment, they can be passaged if they are close to 100 % confluence by day 6 or day 7.

23. BJ fibroblast cells (positive control) should not have reached 100 % confluence by day 6 or 7 and therefore do not need to be split. If they have reached confluence than adjust your cell counting prior to the transfection accordingly. Non-reprogrammed fibroblast that grows around early emerging colonies can be removed to allow for colony growth (see Fig. 2).
24. Cell cultures are very confluent and should appear overgrown. It is sometimes difficult to identify derived colonies from fibroblast.
25. This dilution will yield 5 μg/ml solution of StainAlive antibody. If desired one can down to 2.5 μg/ml.
26. Avoid cell cultures to be kept outside of the incubator for more than 15 min.
27. Optimal sizes of clumps should be empirically determined. Avoid single cells passaging for the first 10 passages.
28. For each individual picked colony use a separate glass picking tool and cell lifter to avoid cross contamination between cell clones.
29. Addition of B18R is not necessary since mRNA transfections have stopped.
30. Cells have to be closely monitored throughout the transitioning process. Allow for modifications (e.g., change ratio every other day or increase ratio steps), as each cell line behaves differently. Also, consider extending transition time up to 4 passages if needed.

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Methods in Molecular Biology (2015) 1283: 43–52
DOI 10.1007/7651_2014_123

Published online: 11 October 2014

Labeling Pluripotent Stem Cell-Derived Neural Progenitors with Iron Oxide Particles for Magnetic Resonance Imaging

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Abstract

Due to the unlimited proliferation capacity and the unique differentiation ability of pluripotent stem cells (PSCs), including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), large numbers of PSC-derived cell products are in demand for applications in drug screening, disease modeling, and especially cell therapy. In stem cell-based therapy, tracking transplanted cells with magnetic resonance imaging (MRI) has emerged as a powerful technique to reveal cell survival and distribution. This chapter illustrated the basic steps of labeling PSC-derived neural progenitors (NPs) with micron-sized particles of iron oxide (MPIO, 0.86 μm) for MRI analysis. The protocol described PSC expansion and differentiation into NPs, and the labeling of the derived cells either after replating on adherent surface or in suspension. The labeled cells can be analyzed using in vitro MRI analysis. The methods presented here can be easily adapted for cell labeling in cell processing facilities under current Good Manufacturing Practices (cGMP). The iron oxide-labeled NPs can be used for cellular monitoring of in vitro cultures and in vivo transplantation.

Keywords: Pluripotent stem cell, Neural progenitor, Iron oxide, Cell tracking

1 Introduction

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have unlimited proliferation ability and the capability to differentiate into all cell types in three-germ layer (1). Due to the ability of indefinite cell proliferation, PSCs are suitable for large scale production of "off-the-shelf" therapeutic products and the patient-specific autologous therapeutics in the case of human iPSCs (2–5). Phase I clinical trials using human PSC-derived cells produced under current good manufacturing practices (cGMP) have been performed for spinal cord injury and macular degeneration (6–8). These clinical applications require cell imaging tools to monitor transplanted cell survival and distribution.

Magnetic resonance imaging (MRI) can provide high resolution and noninvasive cell tracking for in vivo study (9, 10). For MRI, the cells are usually labeled with superparamagnetic iron

oxide (SPIO, 50–120 nm), ultra-small iron oxide (USPIO, 10–50 nm), or micron-sized particles of iron oxide (MPIO, 0.75 μm and larger), which serve as contrasting agents (11–13). The advantage of MPIO is that the particles contain high level of iron which allows the detection of a single particle (14). While PSCs can serve as the starting cells to obtain the differentiated cell types, such as neural progenitors (NPs), for the applications in drug screening, disease modeling, and regenerative medicine (15), one challenge of PSC-based cell therapy is cell tracking in the patient to monitor the transplanted cell fate including survival, proliferation, migration, and distribution.

Here, PSC-derived NPs are used as an example for labeling the PSC derivatives with MPIO toward cell tracking by MRI (16). The whole procedure includes thawing cells from cell banks, expansion, differentiation, and cell labeling with iron oxide before MRI analysis (16, 17). NP differentiation is performed through the formation of embryoid body (EB) in low attachment surface and the treatment with retinoic acid (18). After 8 days of differentiation for mouse ESCs, the NP spheres are either replated on Geltrex-coated surface for 24 h before MPIO labeling or directly labeled in suspension. After 12 h of incubation, the NP cultures are extensively washed to remove the unlabeled particles. Labeling efficiency and particle localization can be examined by fluorescent imaging and the detectability can be performed using in vitro MRI analysis. For human iPSCs, the differentiation is induced with retinoic acid and fibroblast growth factor-2 for 18–20 days before MPIO labeling. This protocol provides the basic steps for intracellular labeling of PSC-derived neural cells toward cellular monitoring of in vitro cultures and in vivo transplantation, and can be easily adapted in cGMP cell processing facilities.

2 Materials

2.1 Materials for PSC Expansion

1. *One vial of frozen undifferentiated PSCs*: The example of PSC line used here is mouse ES-D3 line (American Type Culture Collection, Manassas, VA), which is stored in liquid nitrogen tank. For transplantation study, a well-characterized cell bank needs to be established first.
2. *0.1 % gelatin-coated surface*: EmbryoMax® ES Cell Qualified 0.1 % gelatin solution is obtained from Millipore (ES-006-B) and can be stored at room temperature. The culture surface is coated with 0.1 % gelatin at 0.1 mL/cm^2.
3. *Preparation of undifferentiated PSC culture medium*: The culture medium for ES-D3 cells is composed of Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10 % ESC-screened fetal

bovine serum (FBS, Hyclone, Logan, UT), 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) (all from Life Technologies), and 1,000 U/mL leukemia inhibitory factor (Millipore, ESG1106). This medium is referred as Culture Medium.

2.2 Materials for PSC Differentiation into NPs

1. *Preparation of NP differentiation medium*: The differentiation medium is composed of DMEM-F12 plus 2 % B27 serum-free supplement (Life Technologies, #17504-044). This medium is referred as Differentiation Medium.
2. *Preparation of Geltrex-coated vessels*: The major component in Geltrex (Life Technologies, #A1413202) is laminin, which can promote neural differentiation (**Note 1**). The frozen lactose dehydrogenase elevating virus (LDEV)–free growth factor reduced Geltrex is thawed at 2–8 °C overnight. A 1:2 dilution by cold DMEM can be made and stored at −20 °C. Additional 1:50 dilution is then made with cold DMEM with complete mixing before coating. The vessels are coated at 0.1 mL/cm^2.
3. *Preparation of growth factors for NP differentiation*. The differentiation is induced using *all trans*-retinoic acid (RA) (Sigma, #R-2625). RA stock solution is prepared at 20 mM. The working concentration is 1 μM.

2.3 Materials for NP Labeling

1. *Micron-sized particles of iron oxide (MPIO)*: Fluorescent (flash red, 660/690 nm) MPIOs (Bangs Laboratories, Part number ME03F/9772) are encapsulated with carboxyl modified polystyrene (P(S/V-COOH)) and have an average diameter of 0.86 μm. The stock solution contains 18.2×10^{-2} g (Fe)/mL particles and can be diluted with Differentiation Medium into various concentrations (2.5–10 μg Fe/mL for replated NPs, 5–50 μg Fe/mL for NP aggregate in suspension).
2. *Agarose gel for* in vitro *MRI analysis*: 2 % (w/w) low-temperature agarose gel (VWR, Suwannee, GA) is prepared to create a tissue mimicking phantom.

3 Methods

3.1 Expansion of Undifferentiated PSCs

3.1.1 Thaw the Cells from the Bank

1. Remove one cryovial from a bank in liquid nitrogen storage tank and place the vial on dry ice.
2. Quickly thaw the vial in a 37 °C water bath. When all ice crystals melt, sterilize the outside of the vial with 70 % ethanol.
3. Collect the cell suspension in a sterile 50 mL centrifuge tube and carefully add 10–15 mL of thawing medium drop-wise in the tube. Centrifuge cells at 200 g for 5 min.

4. The cell pellet is resuspended with appropriate volume of Culture Medium and the cells are plated onto gelatin-coated vessels (for mouse ESCs) at 0.5–1 × 10^5 cells/cm^2. Gently shake the vessel back and forth to evenly distribute the cells in a 37 °C, 5 % CO_2 incubator.
5. Culture Medium is changed daily until the cells reach 70 % confluence (about 3–5 days).

3.1.2 Perform Cell Count to Determine the Cell Number

1. Aspirate the spent medium from the culture vessels and wash once with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS).
2. Add 0.05 % Trypsin-EDTA (0.1 mL/cm^2) and incubate for 3–5 min. Collect the detached cells and dilute the suspension in culture medium (5–10 mL).
3. Centrifuge at 200 ×*g* for 5 min and resuspend the pellet in fresh medium
4. Take a sample and count the cells using a hemocytometer. Based on cell count, determine the number of vessels to be harvested and the number of vessels to be seeded.

3.1.3 Prepare the New Vessels and Cell Seeding

1. Prepare gelatin-coated vessels at room temperature and remove the free gelatin solution after coating (30–60 min).
2. Pipette the calculated volume of Culture Medium into the culture vessels. For example, add 2 mL of fresh medium per well of 6-well plate for seeding 0.5 × 10^6 cells.
3. Mix the cell suspension up and down 3–5 times. Based on the calculated volume, seed each culture vessel with a certain amount of cell suspension. The seeding density is around 0.5 × 10^5 cells/cm^2 for this cell line (the seeding density could vary for different cell lines). For example, if the resuspended cell suspension is at 0.5 × 10^6 cells/mL, then seed 1 mL of cell suspension into each well of 6-well plate. Repeat the cell passage before NP differentiation can be initiated.

3.2 Generation of the Differentiated NPs

The cells expanded in Section 3.1 are used as the starting cells for NP differentiation. NPs are differentiated from ES-D3 cells as previously reported (18).

3.2.1 Prepare EBs on Day 0

1. *Perform a cell count to determine the number of vessels to be harvested*. Cell count is performed as described in Section 3.1. Based on the cell count, determine the number of the harvested vessels to seed cells in low attachment 6-well plate (Corning Incorporated) at the density around 0.5–1 × 10^5 cells/cm^2.
2. *Harvest the cells and seed the cells to low attachment vessels*. Incubate the culture with 0.05 % Trypsin-EDTA (0.1 mL/cm^2) for 3–5 min. Collect the harvested cells into a sterile centrifuge tube containing the Differentiation Medium. Centrifuge and

resuspend the cells at a fixed concentration. Seed the cells into low attachment 6-well plate at 1×10^6 cells/well. Incubate the vessels in a 37 °C, 5 % CO_2 incubator.

3.2.2 Maintaining EBs During Day 1–8

1. *Medium change at day 2.* Perform a complete medium exchange with Differentiation Medium. Collect the EBs into sterile 15 or 50 mL centrifuge tubes. Centrifuge EBs at $150 \times g$ for 5 min (or by gravity settling). Carefully aspirate the supernatant and resuspend the pellets in the fresh Differentiation Medium (**Note 2**). Distribute the EBs back into culture vessels.
2. *Medium change at day 4 and 6.* As described for Day 2, perform a complete medium exchange with Differentiation Medium plus 1 μM RA.

3.3 Labeling Replated NP Aggregates

1. *NP plating.* At day 8, collect NP spheres from the low attachment culture vessels and pool them into a sterile centrifuge tube. Centrifuge the spheres at $200 \times g$ for 5 min. During spinning, aspirate Geltrex solution from the prepared Geltrex-coated culture vessels. After centrifugation, gently resuspend the cell pellet with the Differentiation Medium. Take a small sample of NP spheres, dissociated with 0.05 % Trypsin/EDTA, and count the cells. Based on cell count, seed 5×10^5 cells into each well of Geltrex-coated well plates. Evenly distribute the EBs in culture vessels.
2. *MPIO labeling.* After 24 h, the replated cells are washed with PBS and incubated with 1 mL of fresh Differentiation Medium containing 2.5, 5, or 10×10^7 fluorescent MPIOs/mL, corresponding to concentrations at 2.5, 5, and 10 μg Fe/mL (**Note 3**).
3. *Wash away free particles.* The cells are incubated with MPIO for 12 h and then are extensively washed with PBS (10 times) to remove the free particles (**Note 4**). After that, the labeled cells can be characterized for various assays. Example of MPIO-labeled NP aggregates can be seen in Fig. 1a–c.

3.4 Labeling NP Aggregates in Suspension

1. *Collecting NP spheres.* The NP spheres are collected in 15 or 50 mL centrifuge tubes. By centrifuge or gravity settling to remove the spent medium, the cells are washed with PBS.
2. *MPIO labeling.* NP spheres are incubated with 1 mL of fresh Differentiation Medium containing 5, 25, or 50×10^7 fluorescent MPIOs/mL, corresponding to concentrations at 5, 25, and 50 μg Fe/mL (**Note 5**). The cells are incubated with MPIO for 12 h and then are extensively washed with PBS (10 times). The labeled cells can be characterized for various assays (**Note 6**).

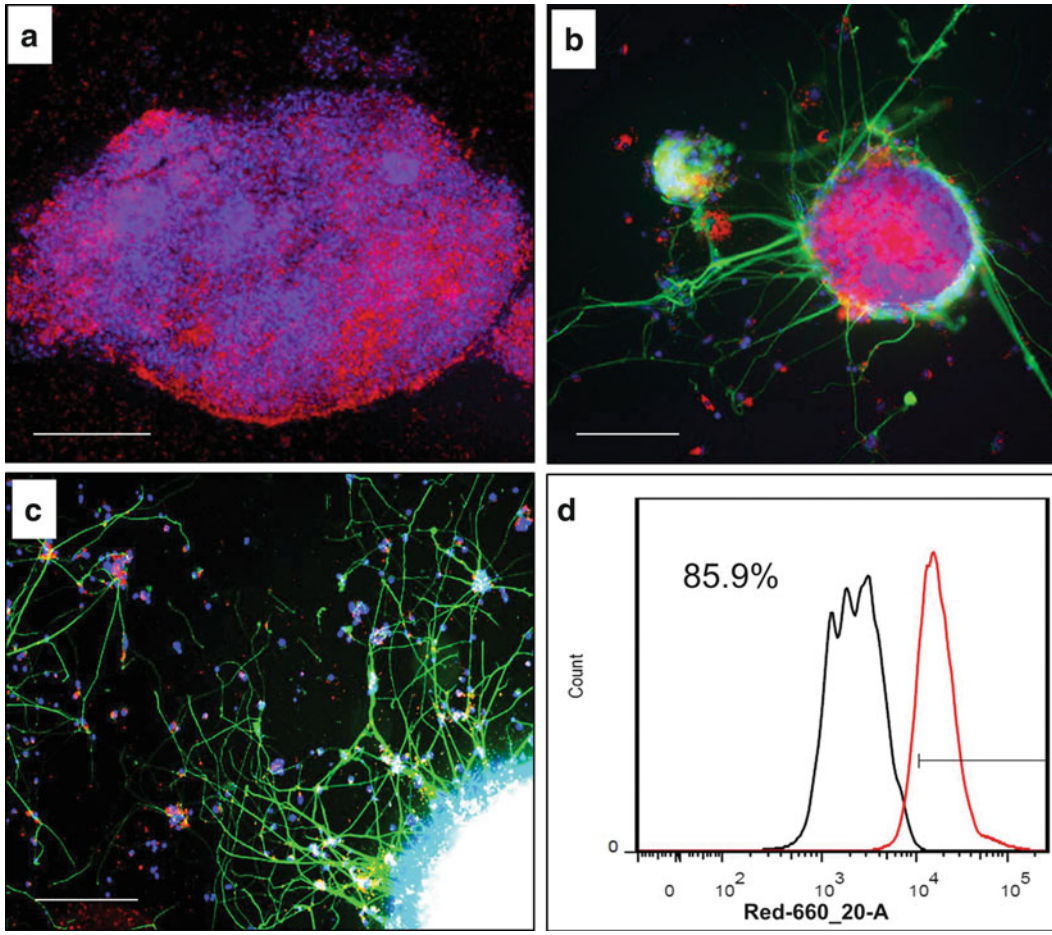

Fig. 1 Illustration of labeled NPs. The replated NP spheres are labeled with MPIO and visualized by confocal microscopy. (**a**) Confocal images of NP spheres labeled with MPIO. *Blue* = cell nuclei; *Red* = MPIO. Scale bar: 100 μm. The replated NP spheres are also differentiated on Geltrex-coated surface for 3 days. Extensive neuron outgrowth is observed. (**b**) Fluorescent image of NP spheres with neuronal outgrowth; (**c**) Neuronal outgrowth from the NP sphere. In (**b**) and (**c**), *Blue* = cell nuclei; *Red* = MPIO. *Green* = β-tubulin III (neurons). Scale bar: 100 μm. (**d**) Flow cytometry histogram of labeling efficiency for NPs derived from human iPSK3 cells

3.5 In Vitro MRI Analysis

1. *Harvest and dissociate the labeled cells.* MPIO-labeled cells are harvested with trypsin and then resuspended at 2×10^5 cells per layer in a tissue mimicking phantom made with agarose gel (**Note 7**).
2. *Prepare samples for* in vitro *MRI analysis.* Agarose-cell layers are formed by mixing an equal volume of cell suspension with a 2 % low-temperature agarose to form a 1 % agarose solution containing cells with final concentration. The suspended cells are layered in a 10-mm NMR tube (Wilmad Glass, Buena, NJ) with a 1 % agarose layer separating the cell-containing layers.

3. In vitro *MRI analysis*. An 11.75-T (500 MHz) vertical magnet (Magnex Scientific, Oxford, UK) with an 89-mm widebore equipped with a Bruker Avance console and Micro2.5 gradients (Bruker Corp, Billerica, MA, USA) is utilized to acquire images from all samples. A 3D gradient recalled echo (GRE) is acquired with 50-μm isotropic resolution using TE (echo time)/TR (repetition time) = 5.3/70 ms. Data are analyzed by means of regions of interest drawn over the magnitude images for every cell layer. Signal intensity for each sample's layer is averaged and T_2 relaxation is obtained using Levenberg–Marquardt algorithm (Sigma Plot 7.101, SPSS Inc, Chicago, IL, USA) (**Note 8**).

3.6 Protocol Adaptation to Human Induced Pluripotent Stem Cells (hiPSCs)

The above procedure is readily adapted to the NPs derived from hiPSCs.

1. *Undifferentiated hiPSC cultures*. The example cell line is human iPSK3 cells (kindly provided by Dr. Stephen Duncan, Medical College of Wisconsin) (19). The cells are maintained on Geltrex-coated tissue culture 6-well plate in mTeSR™ 1 medium (Stem Cell Technologies Inc., #0580). Culture media are replaced every day and the cells are subcultured every 4–6 days. For passaging, the cells are incubated with Accutase (Life Technologies, #A1110501) for 5 min and cell clumps are dissociated by gentle pipetting. The cell suspension is spun down at 300 × *g* for 5 min and after removing the supernatant, the cell pellet is resuspended in fresh medium and plated on Geltrex-coated 6-well plate in the presence of Y27632 (10 μM) (Sigma, #Y0503) for the first 24 h. The seeding density is in the range of 1–1.5 × 10^5 cells/cm^2.
2. *NP differentiation*. Accutase-dissociated undifferentiated iPSK3 cells are seeded into low attachment 6-well plate at 1 × 10^6 cells/well in differentiation medium containing Y27632 (10 μM) for the first 24 h. The differentiation medium is composed of DMEM-F12 plus 2 % B27 supplemented with RA (10 μM) and fibroblast growth factor (FGF)-2 (10 ng/mL) (Life Technologies, #PHG0264) (20). After 5 days of daily medium change, RA is removed and FGF-2 concentration is reduced to 5 ng/mL. The cultures are fed every other day until day 18–20. The resulting NP spheres are used for MPIO labeling (**Note 9**).
3. *MPIO labeling of NP aggregates in suspension*. NP spheres are incubated with 1 mL of fresh Differentiation Medium containing 50 × 10^7 fluorescent MPIOs/mL, corresponding to the concentration at 50 μg Fe/mL. The cells are incubated with MPIO for 12 h and then are extensively washed with PBS (10 times). The labeled cells are characterized with about 80 % labeling efficiency by flow cytometry (Fig. 1d).

4 Notes

1. *Uniform coating with Geltrex*: The quality of Geltrex coating would affect the cell expansion performance and the EB replating efficiency. The diluted Geltrex should be well-mixed before coating.
2. *EB aggregation*: During the initial 4 days of EB formation, the EBs may have high tendency to merge with each other to form large EBs, known as aggregation. EB aggregation should be minimized by evenly distributing the EBs in the vessels (i.e., shaking the vessels back and forth) and gentle pipetting during feeding.
3. *Optimization of MPIO exposure*: MPIO concentration affects the labeling efficiency and cell viability. The current labeling efficiency is about 70–80 % in this procedure. Cell viability is about 80–90 %. The MPIO exposure needs to balance the detectability and cell viability/biological functions. The cell uptake of MPIO may vary for different cell type; thus the concentration needs to be optimized for each cell type.
4. *Washing the MPIO labeled cells*: Excessive free particles usually exist in the culture and extensive wash (more than 10 times) needs to be performed to remove the free particles. Some free particles may still exist in the culture after washing and will be gradually washed away during the feeding.
5. *Labeling the replated NP spheres or labeling NP spheres in suspension*: Labeling the replated cells appears to be more efficient than labeling cells in suspension. To achieve the same labeling efficiency, higher MPIO concentration is required for labeling cells in suspension. The simple incubation method of MPIO labeling is based on the cell endocytosis. Labeling NP spheres in suspension can be used for studies that require cell aggregates.
6. *NP characterization after labeling*: More characterizations can be performed in addition to the labeling efficiency and cell viability, such as cell proliferation, cytoskeleton distribution, oxidative stress, cell phenotype, and differentiation into mature neural cells (16).
7. *Artifacts for* in vitro *MRI analysis*: The complete cell dissociation provides better signals for in vitro MRI analysis compared to the small cell clusters (Fig. 2). Bubbles also need to be avoided in the cell layer solution. The good images should have homogeneous signal distribution in the layer (see Fig. 2a).
8. *Dilution effect of MPIO labeling due to cell division*: With each cell division, the MPIOs in each cell are divided into half which

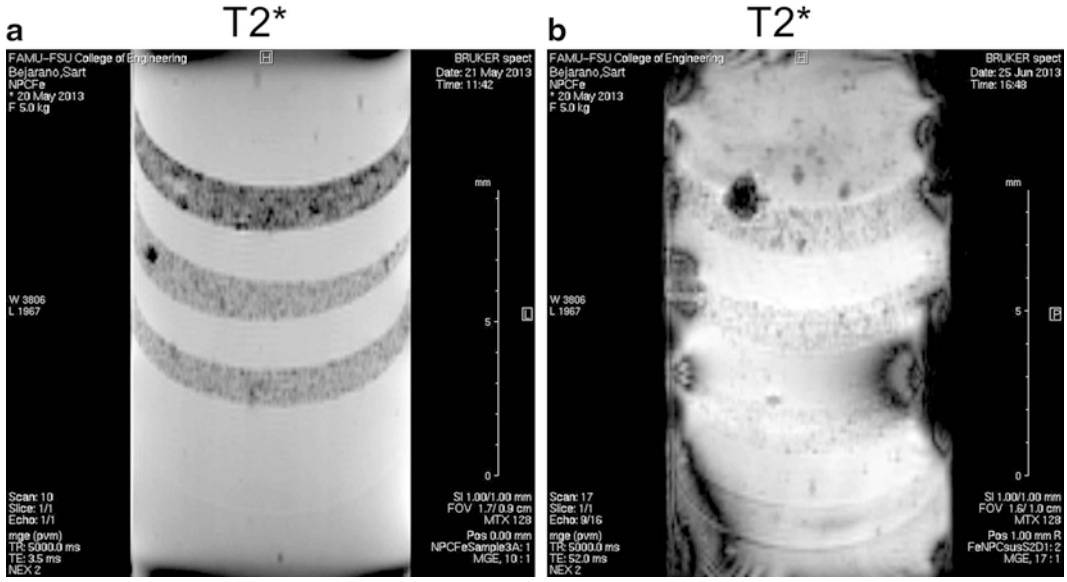

Fig. 2 Example of images from in vitro MRI analysis. In vitro MRI analysis is performed on the cell-agarose layers in an NMR tube, which provides a tissue mimicking phantom. The cell layer is in the sequence from the high MPIO exposure on top to the low MPIO exposure at bottom. (**a**) Representative image showing homogenous cell layer. (**b**) Representative image showing cell layer with the artifacts

causes the decrease in detectability of iron oxides. This phenomenon is called dilution effect and would limit the time window for cell tracking. For high proliferating cells, high detectability of single particle (such as MPIO) is important. This dilution effect could correlate well with cell doubling time, providing an inverse measure of cell growth in vitro and a cautionary note for in vivo interpretation.

9. *Improvement for the differentiation*: Improvements for in-process monitoring (e.g., analysis of secreted proteins or metabolites) may be required in order to predict the differentiation outcome especially for lengthy protocol when human PSCs are used. Although it is challenging, such in-process monitoring will significantly reduce the failure frequency and increase the successful rate of the differentiation for cell tracking.

Acknowledgments

This work was supported by FSU start-up fund and FSU GAP award. Partial supports from National Science Foundation (No.1342192 and DMR-0084173) and National High Magnetic Field Laboratory User Collaboration Grant Program were also acknowledged.

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Methods in Molecular Biology (2015) 1283: 53–63
DOI 10.1007/7651_2014_164

Published online: 20 December 2014

Using the Quantum Cell Expansion System for the Automated Expansion of Clinical-Grade Bone Marrow-Derived Human Mesenchymal Stromal Cells

Gema Martin-Manso and Patrick J. Hanley

Abstract

Bone marrow-derived human mesenchymal stromal cells (hMSCs) constitute a promising therapeutic approach. However, the extremely low frequency of hMSCs in bone marrow makes the translation of these regulatory cells to clinical therapies difficult for large patient populations. Here, we describe a good manufacturing practices-compliant procedure for the expansion of hMSCs using the Quantum Cell Expansion System. This closed and automated system allows the large-scale expansion of hMSCs while maintaining their multipotency, immunophenotype, morphology, and karyotype.

Keywords: Quantum, Flask-based culture, Human mesenchymal stromal cells, Current good manufacturing practices (cGMP)

1 Introduction

Human mesenchymal stromal cells (hMSCs) represent a promising therapeutic resource for cell-based therapies ranging from regenerative medicine and tissue engineering to immunomodulation. hMSCs are known to be present in vascularized tissue in adults, bone marrow, adipose tissue, menstrual blood stem cells, umbilical cord blood, placental, and amniotic fluid (1). Interestingly, hMSCs from different sources have unique characteristics (2). Despite the heterogeneity, the International Society of Cellular Therapy (ISCT) has established the standard characteristics for hMSCs: immunophenotype (>90 % $CD105^+$, $CD90^+$, and $CD73^+$; <2 % $CD45^+$, $CD34^+$, $CD14^+$, and $CD19^+$; and <5 % $HLA\text{-}DR^+$), fibroblast-like morphology, and tri-lineage differentiation capacity (i.e., osteoblasts, chondroblasts, and adipocytes) (3).

The estimated frequency of hMSCs in bone marrow is about 0.001–0.01 % of total nucleated cells. Thus, the translation of hMSCs to clinical therapies for large patient populations presents numerous challenges, including the need for large-scale cell expansion and a controlled environment for propagation (4). The use of bioreactors enables the expansion of human stromal cells while

Table 1
A comparison of automated and manual cell culture processes for the expansion of clinical-grade bone marrow-derived hMSCs

Quantum	Flask-based culture
Automated, customizable process (70 % reduction in labor)	Manual process
Reproducible process, scalable	High degree of variability (manual techniques differ between operators, temperature fluctuations)
Functionally closed system from cell loading through harvest	Numerous open steps throughout the process (risk of contamination)
Cells harvested in ~30 min	Cells harvested in hours
2.1 m^2 (6.9 ft^2) of bioreactor surface area	Area equivalent to 120 T175 flasks
0.3 m^2 (1 ft^2) footprint in class 100,000 clean room	Expensive GMP class 10,000 facility recommended, biosafety cabinet, incubator, and storage for flasks

keeping their multipotency and karyotype (5, 6). Currently, many bioreactors such as the Quantum® Cell Expansion System (Terumo BCT), the WAVE bioreactor (GE Healthcare Life Sciences), and the Xpansion™ Multiplate bioreactor (Pall Life Sciences) are available and provide large surface areas over a small footprint (7). Here, we will focus on the expansion of hMSCs using the Quantum Cell Expansion System (Terumo BCT) (8–10). The Quantum bioreactor contains 11,500 hollow fibers that provide a high cell culture surface area-to-volume ratio and allow the expansion of 500–1,000 million hMSCs per run with a starting population of 10–20 million hMSCs. Thus, this system reliably produces a cellular therapeutic dose in a functionally closed disposable bioreactor with automated temperature control and rate of medium perfusion (Table 1). Bone marrow-derived hMSCs expanded in the Quantum show no malignant neoplastic formation in athymic mice 60 days posttransplant and demonstrate comparable quality to hMSCs cultured on flasks in terms of genetic stability, immunophenotype, morphology, and tri-lineage differentiation capacity (11, 12).

2 Materials

It is essential to adhere to proper procedures to prevent misidentification or contamination of patient samples (*see* **Note 1**).

2.1 D-5 Medium Components

Human platelet lysate (5 %) prepared as described (4, 13) from outdated apheresis platelets pooled from eligible donors (Children's National Blood Bank, Washington, DC, USA) (*see* **Note 2**), Dulbecco's Modified Eagle's Medium with high glucose and without L-glutamine, heparin (2.1 U/mL) (APP Pharmaceutical Schaumburg), GlutaMAX (2 mmol/L), *N*-acetylcysteine (10 mmol/L), VacuCap filters (0.2 μm), and filter storage receiver bottle.

2.2 Other Reagents and Buffers

Fibronectin (5 mg) (BD Biosciences), sterile deionized water, phosphate-buffered saline (PBS) without calcium and magnesium, human bone marrow (LONZA), trypan blue, and TrypLE select.

2.3 Expansion Components

Luer-Lok syringes, needles (16 g, 21 g, spinal 19 g), and caps, sterile conical centrifuge tubes and culture flasks, Quantum Cell Expansion System, cell expansion set, medium bags, cell inlet bags, in-line filter, waste bags, TSCD II Sterile Tubing Welder (Terumo BCT), peristaltic pump (Masterflex), handheld RF sealer (SEBRA), gas tank (90 % N_2, 5 % CO_2, 5 % O_2), and plasma transfer set.

2.4 Lactate and Glucose Measurement

Lactate Plus (Nova Biomedical) and Ascensia Contour BGM (Bayer).

2.5 Release Testing

BacT/Alert anaerobic culture bottle, BacT/Alert aerobic culture bottle, and isolator tube (fungal culture) for sterility tests; MycoAlert (LONZA) and microplate reader (BioTek) for *Mycoplasma* detection; Endosafe-PTS for endotoxin testing (Charles River); trypan blue or 7AAD for viability tests; Human MSC Phenotyping Kit with CD73, CD90, CD105, CD45, CD34, CD14, CD19, and HLA-DR; and MACSQuant Analyzer (Miltenyi Biotec).

2.6 Cryopreservation Medium Components

DMSO at 2× (20 %), Cryovials, Plasma-Lyte A (75 %), and human serum albumin (5 %) (Baxter). American Fluoroseal Corporation (AFC) cryopreservation and cell culture bags.

3 Methods

Perform all steps in a certified biological safety cabinet (BSC), using aseptic technique and following universal precautions.

3.1 Prepare Medium Bags

1. Wipe peristaltic pump with ethanol and place it in the BSC.
2. Open the medium bag set in the BSC and place the hard tubing tip inside the bottle of PBS or medium.
3. Turn on the pump and set the speed to low. Hold the 0.2 μm filter from the medium bag set upright until it is filled with fluid. Then increase the flow rate to medium and pour medium

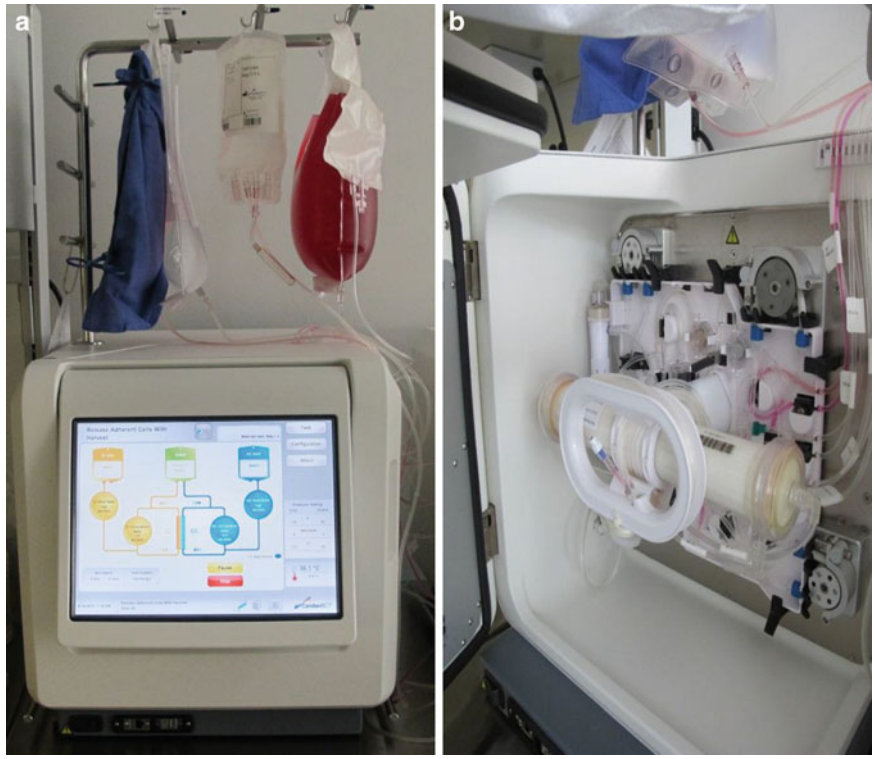

Fig. 1 The Quantum Cell Expansion System. (**a**) Illustrates the workstation with the instrument touch screen and medium bag, cell inlet bag, and waste bag on the bag pole. (**b**) Shows the hollow fiber bioreactor, the peristaltic pumps, and valves

or PBS into the bottle with the hard tubing tip until the medium bag has approximately 4 L.

4. Seal the tubing between the medium bag and the 0.2 μm filter. Disconnect the 0.2 μm filter from the bag.

3.2 Load the Cell Expansion Set

1. On the Quantum screen (Fig. 1a), touch "Task," "Set Management," "Load Cell Expansion Set," and "Start." This step will open all valves (*see* **Note 3**).
2. Open the incubator door. Unlock the rotor latches and open the rotor covers on the pumps. Open the five external mounting clips. Confirm that the internal mounting clips are in the correct horizontal position. If needed, grasp the top internal mounting clip, bend it to the side, and rotate it a quarter turn clockwise to open the clip. Repeat this step with the bottom internal mounting clip.
3. Remove the tray cover from the cell expansion set. Place the tubing organizer on the mounting plate. Rest the bioreactor with the attached rocker assembly on the spill tray of the Quantum. Press on the sides of the tubing organizer to snap

it into place. Ensure that all five mounting clips are locked over the tubing organizer. Grasp the top internal mounting clip and bend it up and rotate the base of the clip a quarter turn counterclockwise. Repeat this step with the bottom mounting clip. Ensure that the rotor latches are unblocked and are open on all the pumps.

4. Grasp the blue collar at the base of the tubing that is not attached to the tubing organizer and pull the tubing over the center of the pump rotor, through the prong, and past the notch in the edge of the tubing organizer. Ensure that the tubing is centered on the top of the pump rotor.

   Close the rotor cover and lock the rotor latch. Repeat this for all four pumps.

5. Load the extracapillary (EC) inlet line located on the right side of the EC inlet pump into the EC fluid detector. Align the notch of the rocker arm to the groove on the rocker assembly. Push the rocker assembly into the rocker arm until it is completely inserted. The bioreactor will be in the home position with the EC inlet and EC outlet ports facing upward and the sample port facing downward (Fig. 1b).
6. Verify that the lines are inserted into the valves correctly. Check for kinked lines.
7. Hang the waste bag and the harvest bag on the bag pole.
8. Insert the tubing line guide onto the pegs located at the top right side of the incubator door frame.
9. Remove the two blue end caps and the one red end cap from the cell expansion set. Connect the gas inlet line (*see* **Note 4**). Ensure all lines are clear of the incubator door and close the incubator door. Touch "Finish" to complete the task and then "Yes" to confirm.

3.3 Prime the Cell Expansion Set

1. Turn on the external gas supply at a pressure of 40–60 psi.
2. Attach the medium bag filled with PBS to the cell inlet line using the sterile tubing welder (*see* **Note 5**) and hang the bag on the pole.
3. Touch "Task," "Set Management," "Prime Cell Expansion Set," and "Start."
4. Upon completion of the prime cell expansion set task, remove a small sample (2–3 mL) from the sampling port using a syringe.
5. Touch "Finish" and then "Yes."
6. Separate the tubing lines by sealing the midpoint of the connected lines. Separate the lines using sterile scissors at the sealed point.

7. Transfer the PBS bag from the cell inlet line to the wash line using the sterile tubing welder and sealer. Ensure that all welds are open.
8. Check the intracapillary (IC) and (EC) header for air bubbles. If air bubbles are notably present, use the "Remove IC/EC Air Task" to remove them.

3.4 Coat the Bioreactor

1. Equilibrate fibronectin at room temperature for at least 30 min.
2. Reconstitute fibronectin with 5 mL of deionized water. Allow it to dissolve for 1 h at room temperature. Then, add 5 mL of PBS using a syringe and transfer the 10 mL to a 50 mL centrifuge tube. Add another 10 mL of PBS to the vial to wash out any residual fibronectin. Remove the 10 mL from the vial and transfer to the same tube. Bring the volume to 40 mL with PBS.
3. Open a cell inlet bag in the BSC and connect a syringe with the plunger removed. Add the 40 mL in the centrifuge tube to the syringe and allow it to flow into the bag. Add an additional 60 mL of PBS to the syringe connected to the cell inlet bag. Push plunger through syringe, adding at least 40 mL of air to the cell inlet bag. Seal the cell inlet bag and remove the syringe.
4. Connect the cell inlet bag containing fibronectin to the reagent line using the sterile tubing welder and sealer. Ensure that all welds are open.
5. Touch "Task," then "System Management," "Coat Bioreactor," and then "Start." Run the task for 4–48 h (*see* **Note 6**). When the task is completed and a message box appears, touch "Finish" and then "Yes."

3.5 Attach Cells

1. Connect the medium bag to the IC or EC medium line and perform an IC EC Washout to eliminate the PBS from the system (*see* **Note 7**). Touch "Task," "Washout," "IC EC Washout," and "Start." Check the status line to confirm that the task has started or has been completed (i.e., Idle).
2. Perform the "Condition Medium" task to homogenize the gas in the medium.
3. Transfer 25 mL of bone marrow into the cell inlet bag (*see* **Note 8**). Bring the total volume of bone marrow up to 100 mL with D-5 medium. Inject a minimum of approximately 40 mL of air into the cell inlet bag. Connect the in-line filter to the cell inlet line and the cell inlet bag containing the bone marrow using the sterile tubing welder and sealer.
4. Run the "Load Cells without Circulation" task. Leave the system at this stage for 48–96 h to allow the hMSCs to adhere to the hollow fibers in the bioreactor.
5. Perform a "High Density Washout" to remove the red blood cells in the bioreactor. Add bolus (200 mL of D-5 medium).

3.6 Feed Cells

1. Begin the "Feed Cells" task by touching "Task," "Feed and Add Cells," and "Feed Cells." Initially, the feed rate is set at 0.1 mL/min (from the IC inlet). This can be adjusted to 0.2 mL/min if necessary, according to lactate or glucose measurements.
2. Check lactate and glucose levels at least once every 2 days. These readings will determine the feed rate for the system. In order to take a sample, use a syringe to remove 2–3 mL of medium from the sampling port and discard. Using a new syringe, remove 1 mL of medium to be tested. If lactate levels reach 4 mmol/L, increase the IC inlet rate to 0.2 mL/min. For the first expansion, the cells are ready to be harvested 24 h after lactate levels reach 4 mmol/L with an IC inlet rate of 0.4 mL/min (i.e., around 11 days) (*see* **Notes 9–11**).

3.7 Release and Harvest Adherent Cells

1. To harvest the cells, fill the cell inlet bag with 180–200 mL of TrypLE select using a 60 mL syringe. After adding the dissociation reagent, add at least 40 mL of air to the cell inlet bag. Connect the cell inlet bag to the reagent line using the sterile tubing welder and sealer.
2. Make sure that there is at least 1.5 L of PBS connected to the wash line and at least 500 mL of medium connected to either the IC or EC inlet line. Run a "Rapid IC Washout" task for cells in the first passage.
3. To begin the "Release Cells with Harvest" task, touch "Task," "Release and Harvest," and "Release Cells with Harvest." Change the time of harvest from 4 min (default) to 15 min (*see* **Note 12**).
4. Upon completion of the task, seal and remove the harvest bag. Turn off the external gas supply.

3.8 Unload the Cell Expansion Set

1. To unload the cell expansion set, seal and disconnect all lines and bags. Then touch "Task," "Set Management," and "Unload Cell Expansion Set." Remove all tubing from pumps, open rotor covers, disconnect the gas inlet line, remove the bioreactor from the rocker arm, turn the internal mounting clips to their original position, unlock the external mounting clips, and remove the expansion set. When finished, touch "Finish."

3.9 Cryopreservation (If Creating a Cell Bank or a Backup Freeze)

1. Mix the cell harvest bag. Insert the plasma transfer set into the spike port of the cell harvest bag and begin draining the content of the bag into conical centrifuge tubes.
2. Centrifuge the cells at 500 × *g* for 15 min at room temperature. The supernatant should be saved for sterility, *Mycoplasma*, and endotoxin testing.

3. Resuspend the cell pellet in a wash medium containing Plasma-Lyte A (95 %) and human serum albumin (5 %). Take samples for phenotyping and cell counting.
4. Resuspend the pellet in wash medium at "half" the freezing concentration (e.g., if freezing at 1×10^7/mL in 1 mL, add 0.5 mL of wash medium per 1×10^7 cells). Place the cells on ice for 10 min. Add freeze medium (2×) and aliquot the cells into Cryovials or AFC cryopreservation bags at a concentration of $1–2.5 \times 10^7$ cells/mL. If a subsequent expansion of preselected hMSCs is needed, remove $15–30 \times 10^6$ cells (ideally 25×10^6 cells) and add these cells to an appropriate AFC cell culture bag. Bring the total volume to 50 mL using D-5 medium.
5. Freeze the MSCs using a controlled-rate freezer with a program intended for cryopreservation of bone marrow products. Then, transfer the frozen product to liquid nitrogen storage.

3.10 Preselected MSC Expansion

1. Place the AFC cell culture bag on a qualified rocking platform, rocking at room temperature (moderate rocking motion), while a new cell expansion set is loaded, primed, and coated with fibronectin.
2. Follow the protocol described above for bone marrow hMSCs expansion with slight modifications that are listed in Table 2.

Table 2
The expansion protocol for bone marrow and preselected hMSCs using the Quantum system

Step #	Bone marrow MSC expansion	Preselected MSC expansion
1	Load Cell Expansion Set	Load Cell Expansion Set
2	Prime Cell Expansion Set	Prime Cell Expansion Set
3	Coat Bioreactor (~18 h)	Coat Bioreactor (~12 h)
4	IC EC Washout	IC EC Washout
5	Condition Medium	Condition Medium
6	Load Cells without Circulation	Load Cells with Uniform Suspension
7	Inlet Line Washout	
8	Attach Cells (48 h)	Attach Cells (24 h)
9	High Density Washout	Rapid IC Washout
10	Add Bolus	
11	Feed Cells (~11 days)	Feed Cells (~4 days)
12	Rapid IC Washout	
13	Release Adherent Cells	Release Adherent Cells
14	Harvest Cells	Harvest Cells

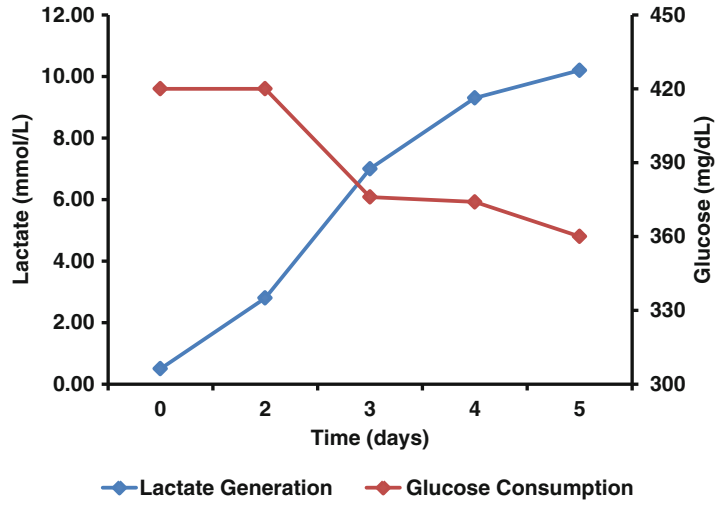

Fig. 2 Lactate and glucose measurements. Proliferating cells rely on aerobic glycolysis to sustain growth, which is characterized by glucose consumption and lactate generation. A representative expansion of preselected MSCs is shown and illustrates the increase in lactate generation (mmol/L) and decrease in glucose concentration (mg/dL) over a period of 5 days

3. Lactate levels should be above 8 mmol/L (for passages >1) for optimal harvest when the flow rate is 1.6 mL/min (i.e., around 4 days) (Fig. 2). Perform an "IC EC Washout" task if lactate levels are above 10 mmol/L.

4 Notes

1. All culture vessels and centrifuge tubes must be labeled with cell line information, component number, lot number, date, and passage number. Unlabeled material should be discarded. Never work with more than one cell line at one time. In addition, all supplies and reagents should be sterile and GMP-grade, qualified, and released prior to use.
2. Alternatively, GMP-grade platelet lysate prepared as described (14) can be purchased from Mill Creek Life Science, Minnesota.
3. For more detailed steps, parameters, and questions, refer to the Quantum user's manual.
4. Ensure that the external gas supply is sufficient and is properly connected to the Quantum. However, do not turn it on until you are ready to prime the cell expansion set. Otherwise the waste bag will be filled with air. Users can determine what gas mixture to use; we recommend 5 % CO_2, 5 % O_2, and 90 % N_2.
5. The sterile tubing welder will be used to sterile-connect all bags and inlet lines. Be sure that the weld is complete and that it has

been opened prior to starting the system. If the weld is not opened, fluid cannot travel through the system.

6. If the adherent cells do not attach to the hollow fibers in the bioreactor, incubate the fibronectin solution longer (i.e., overnight) to promote its adequate deposition on the hollow fiber walls.
7. IC EC Washouts can be performed at any time during the cell expansion if needed to flush old medium out of the system.
8. Reserve 5 mL of bone marrow for archiving, phenotyping, cell counts, and sterility testing.
9. Medium and waste bags should be changed as needed by pausing the system and sterile docking a new bag onto the appropriate line.
10. The feed rate can be increased more aggressively to ensure that fresh medium is available. This will support cell growth and lower the lactate levels.
11. The Quantum can be configured to automatically switch to a second medium bag connected to the EC medium line (but through the IC inlet) if running out of medium in the IC medium bag is a concern. Additionally, automated feed increments can be set up so that GMP Technologists do not need to come in during weekends or holidays.
12. If the adherent cells have not been exposed adequately to the cell dissociation reagent, the cells will not detach completely from the hollow fibers in the bioreactor during harvest. An additional "harvest cells" task can be performed increasing the concentration of the release agent and/or the incubation time.

Acknowledgments

This work was supported by a Board of Visitors grant from Children's National awarded to Catherine M. Bollard, M.D., David Jacobsohn, M.D., and Patrick J. Hanley, Ph.D. The authors would like to thank our collaborators at Baylor College of Medicine, Dr. Adrian Gee, Dr. Zhuyong Mei, and Dr. Helen Heslop. We would also like to thank the Program for Cell Enhancement and Technologies for Immunotherapy (CETI) director, Dr. Catherine Bollard, for her support and guidance.

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Methods in Molecular Biology (2015) 1283: 65–71
DOI 10.1007/7651_2014_116

Published online: 24 August 2014

Isolation and Expansion of Mesenchymal Stromal/Stem Cells from Umbilical Cord Under Chemically Defined Conditions

Heba Badraiq, Liani Devito, and Dusko Ilic

Abstract

From a perspective of manufacturer, procurement of bone marrow aspirates for isolation of mesenchymal stromal/stem cells (MSC) is challenging. The MSC isolated from adult donors have lower proliferation capacity than the cells isolated form young donors of pediatric age. To obtain more MSC from young healthy donors for allogeneic therapy on multiple patients, umbilical cord (UC) seems to be the best alternative. Here, we describe an easy, cost-effective and reproducible protocol of isolation of the MSC from Wharton's Jelly (WJ) in UC.

Keywords: Umbilical cord, Mesenchymal stem cells, Explant culture

1 Introduction

The use of animal derived supplements, such as fetal bovine serum (FBS), should be eliminated for clinical grade cells to avoid the risks in transmitting animal pathogens and to avoid immunogenic reactions after clinical transplantations. The optimization of a xeno-free (XF) culture represents a positive step for the future production of these cells in a clinical grade scale. In addition, content and concentration of cytokines, growth, and other soluble factors greatly varies from batch to batch of FBS and even for purpose of basic research, the trend is to culture cells in serum-free medium. Different research groups have established a range of protocols for isolation and characterization of stromal cells from WJ. However, defined XF culture systems allow for a better multipotent differentiation and expansion rates of adipose tissue- and BM-MSCs, serving as a preferred alternative to FBS containing medium for the production of large scale, functionally competent, clinical grade MSCs (1–3).

Isolation of MSCs from WJ has been poorly investigated and the techniques vary depending on the laboratories. The enzymatic treatment with a combination of various collagenases is widely used; however this treatment fluctuates in the literature. Trypsin

and other proteases such as hyaluronidase are frequently added in different concentrations, and the incubation time also changes from 4 to 24 h at room temperature or at 37 °C (4–8). The yield of MSC often varies and enzymes containing no animal product are difficult to obtain and they are expensive.

The method of WJ MSCs isolation from UC explants possesses advantages over the enzymatic method: (a) cell damage by enzymes is avoided, (b) WJ contains growth factors that support the proliferation of MSC, and (c) it is a low cost protocol. The primary cultures obtained by UC explants appear to be heterogeneous (9, 10), with a population of fibroblast-like cells with a different cell shape and size. In our experimental setting, MSCs outgrowing from explants in chemically defined, animal product-free medium were also relatively heterogeneous initially. However, heterogeneity was substantially decreased after the first passage and it was gone after several population doublings (PD), yielding uniform population of the MSC.

2 Materials

2.1 UC

The UC is collected only from patients who gave their informed consent (*see* **Note 1**). Only one UC should be collected at a time to avoid and minimize risk of contamination (*see* **Note 2**). After delivery and inspection of placenta and UC by a nurse, the UC is cut and placed immediately inside a sterile 500-ml bottle containing PBS supplemented with Gentamicin + Amphotericin B (Gibco). The bottle is placed inside the tissue carrier bag and transported to the laboratory (*see* **Note 3**).

2.2 CELLstart-Coated Dishes

Survival of anchorage-dependent cells is determined by signals from their environment. These signals are primarily derived from soluble factors present in serum and from cell interactions with extracellular matrix (ECM). When serum factors are withdrawn, cells can still survive on certain matrices, whereas they undergo apoptosis on others. Fibronectin is the most effective survival-promoting ECM ligand for both primary fibroblasts and endothelial cells after serum withdrawal (11, 12). CELLstart, xeno-free coating reagent, is mostly fibronectin. Cell culture dishes should be coated the day of use following the manufacturer's recommendation (*see* **Note 4**).

2.3 Equipment

1. 100-μl pipetman.
2. 1,000-μl pipetman.
3. Autoclaved surgical tweezers.
4. Biosafety cabinet.

5. Cryobox/"Mr Frosty"/(Nalgene; Cat. No. 5100-001) (*see* **Note 5**).
6. Disposable scalpels (Swann-Morton, Cat. No. 0503).
7. Hemocytometer (Hausser).
8. Phase contrast microscope.
9. Pipette gun.
10. Tissue culture incubator.
11. Tube rack.

2.4 Plasticware and Other Disposables (See Note 6)

1. 100-mm Cell culture dish (Corning, Cat. No. 430293).
2. 100-µl Filter tips (SLS, Cat No. 171403).
3. 1,000-µl Filter tips (SLS, Cat No. 171703).
4. 500-ml Storage bottle (Corning, Cat. No. CLS430282).
5. 75-cm^2 Flask (Corning, Cat No.430641).
6. γ-irradiated individually wrapped polystyrene 5-ml pipettes (Falcon; Cat. No. 357543).
7. γ-irradiated individually wrapped polystyrene 10-ml pipettes (Falcon; Cat. No. 357551).
8. γ-irradiated individually wrapped polystyrene 25-ml pipettes (Falcon; Cat. No. 357525).
9. γ-irradiated 15-ml conical tube (BD Falcon; Cat no. 352096).
10. γ-irradiated 50-ml conical tube (BD Falcon; Cat no. 352070).
11. Cryotube (Thermo Scientific; Cat No. 377224).
12. Isopropyl alcohol (IPA) wipes.
13. Waste container.

2.5 Reagents and Medium

1. CELLstart (Life Technologies, Cat. No. 07930).
2. Cryostor CS10 (STEMCELL Technologies, Cat. No. A10142-01).
3. DMEM (Life Technologies, Cat No. 41965-039).
4. DPBS with Ca^{2+}/Mg^{2+} (Lonza, Cat No. BE17 513F).
5. Gentamicin/Amphotericin B 500X (Gibco/Life Technologies, Cat. No. R-015-10).
6. Isopropanol (Shield Medicare; Cat. No. 3035300).
7. PBS, Ca^{2+}/Mg^{2+}-free (Life Technologies Cat No. 10010-023).
8. Stemgro (Corning, Cat. No. 40-410-KIT).
9. Tryple (Life Technologies, Cat No. 12604-021).

3 Methods

3.1 Receiving the UC

1. Assure that the UC is accompanied with signed donor consent and a proof that the donor has no blood-borne diseases.
2. The UC is received inside a storage bottle containing sterile PBS supplemented with Gentamicin/Amphotericin B (*see* **Note** 7). The bottle should be wiped with IPA cleaning wipes and placed inside the biosafety cabinet.

3.2 Plating UC Explants

1. The cords should be then transferred into a non-coated 100-mm dish.
2. The cord can be cut into 3–5 cm pieces and thoroughly washed with PBS to eliminate any remains of blood (a new storage bottle can be used to wash the cord pieces).
3. Place one piece in a new non-coated 100-mm dish. While holding the UC piece with surgical tweezers, cut it longitudinally with a sterile scalpel to expose the Wharton's jelly (WJ) and then slice it into 0.5–1 cm^2 pieces.
4. Ten to fifteen pieces (explants) can be transferred per dish into a pre-labeled 100-mm dish coated with CELLstart.
5. Plate the umbilical cord explants on the bottom of the plate and wait 2–5 min before covering slowly with 10 ml Stemgro medium supplemented with Gentamicin/Amphotericin B.
6. Set as many explants as possible. Unused UC discard as medical waste.
7. Place dishes into tissue culture incubators at 37 °C.

3.3 MSC Derivation

1. The medium (Stemgro with Gentamicin/Amphotericin B) in explant culture needs to be exchanged every 2–3 days.
2. The cells are ready for subculture about 10–14 days after initial outgrowths were observed.
3. Remove remaining explants and discard them into medical waste.
4. Wash outgrowths in each 10-mm dish with 5 ml PBS, Ca^{2+}/Mg^{2+}-free
5. Add 5 ml TripLE in each 10-mm dish. Incubate 3–5 min at 37 °C. Detach the cells with vigorous pipetting or, if needed, scrape the cells off with cell scraper. Add equal amount of DMEM and transfer into 15- or 50-ml conical tube. Pool cell suspension from up to four plates in one 50-ml tube.
6. Spin down for 5 min at 700 × *g*. Pool pellets from all tubes and resuspend in 10-ml Stemgro with Gentamicin/Amphotericin B.

7. Count cells using a hemocytometer. This number is population doubling (PD) = 0.
8. Plate the cells in T75 coated with CELLstart in 10 ml Stemgro with Gentamicin/Amphotericin B. Approximate density for optimal growth should be ≈7,000 cells/cm^2 (≈525,000 cells/T75). Lesser density may slow cell proliferation (*see* **Note 8**).
9. Change medium every 2–3 days. Use Stemgrow w/o Gentamicin/Amphotericin B (*see* **Note 9**).
10. Once when the cells reach ≥80 % density, repeat steps 4–8 if needed. Target is to have ≈2 × 10^7 cells in total.
11. When the cell number reaches near ≈2 × 10^7, proceed with cryopreservation.

3.4 MSC Cryopreservation

1. For cryopreservation, cell monolayer is washed with PBS and incubated in TrypLE for 3–5 min at 37 °C. TrypLE is then diluted with an equal amount of pre-warmed DMEM. Separate 20 μl for cell counting before centrifuging the cells at 700 × *g* for 5 min.
2. The cell pellet is then resuspended at a concentration of ≈1 × 10^6 cells/ml with Cryostor CS10. Add 0.5 ml in each cryovial (≈500,000 cells in 0.5 ml/vial). Cryovials should be placed inside precooled Mr Frosty (*see* **Note 10**)
3. Place Mr Frosty at −20 °C for 2 h and then in −80 °C for another 2 h. After that, transfer the Cryovials from the freezer into liquid nitrogen dewar.

4 Notes

1. Assure that you have the following information:
   (a) Copy of signed consent.
   (b) Time of delivery.
   (c) Hospital ID number.
   (d) Results of tests for: HIV1/HIV2, HepA, HepB, HepC, HTLV1/2, and Syphilis tests. The mandatory tests are different from country to country; assure that you follow regulations set for your country.
   (e) Ask if there is clean medical history. If not, write down the conditions.
2. UC can be collected from healthy donors either after vaginal delivery or from Caesarean section deliveries. UC obtained from Caesarian sections are sterile and risk of contamination in the culture is low in comparison with UC collected from natural vaginal deliveries.

3. The tissue carrier bag should not be carried in public transport. Private car, hired car, or specialized courier services should be used to transport the tissue carrier bag containing the UC between the collection site and the laboratory.
4. Avoid drying of coated surface.
5. Each isopropanol freezing cryobox can hold 18 cryovials. Check level of isopropanol in each cryobox before usage. If not sufficient, fill it up to the fill line
6. Isopropanol, disposable culture, and plasticware of the equivalent quality can be purchased from different manufacturers without altering the outcome of the procedure.
7. If you are not sure that PBS is supplemented with antibiotic/antimycotic mix, wash the cord 3× in 150 ml of PBS supplemented with antibiotic/antimycotic mix, gently stirring for 5 min each time.
8. Use smaller vessels if needed (i.e., T25).
9. Since all cells were mixed at step 6 (Section 3.3), the medium sample can be taken from any of culture flasks and it will represent the whole population. The following two tests are recommended:
   (a) To assess sterility of the culture, spread 1 ml of spent medium w/o Gentamicin/Amphotericin B in which cells were at least 48 h and spread over agar plate and send to microbiology laboratory for analysis.
   (b) For testing mycoplasma, plate ≈10,000 cells in Maktek dish and stain with Hoechst 24 h later.
10. Do not leave cells in cryopreservant for extended period at room temperature. That will affect their viability upon thawing.

Acknowledgments

The study was supported by the studentship to H.B. from Saudi Arabia government.

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Methods in Molecular Biology (2015) 1283: 73–85
DOI 10.1007/7651_2014_125

Published online: 20 September 2014

Production of Good Manufacturing Practice-Grade Human Umbilical Cord Blood-Derived Mesenchymal Stem Cells for Therapeutic Use

Phuc Van Pham and Ngoc Kim Phan

Abstract

Human umbilical cord blood-derived mesenchymal stem cells (UCB-MSCs) are multipotent stem cells that can be differentiated into several specific cell types such as adipocytes, osteoblasts, and chondroblasts. They also were demonstrated to trans-differentiate into other cell lineages such as muscle cells and neurons. Thus, they are considered a promising stem cell source for therapeutic use. Here, we describe a method for production of good manufacturing practice-grade human UCB-MSCs for therapeutic use. The obtained UCB-MSCs are free of allogenous or xenogenous proteins. In addition, these MSCs could maintain the MSC phenotype in long-term culture.

Keywords: Good manufacturing practice, Mesenchymal stem cells, Therapeutic use, UCB-MSCs, Umbilical cord blood

1 Introduction

Mesenchymal stem cells (MSCs) were first described by Friedenstein et al. as a cell population similar to fibroblasts (1, 2). To date, MSCs have been successfully isolated from various sources such as bone marrow (3, 4), adipose tissue (5, 6), menstrual blood (7), dental pulp, and umbilical cord blood (UCB) (8–10). In comparison to other sources, UCB-MSCs exhibited some advantages such as noninvasive recovery, the abundance of MSCs, and well-known characteristics.

Defined as MSCs, UCB-MSCs can also differentiate into other cell types such as hepatocytes (11, 12), neurons (13–15), and cardiocytes (16–18). UCB-MSCs have been found to exhibit immunomodulatory effects on immune cells (19, 20). They can modulate the activities of T cells, B cells, natural killer cells, and dendritic cells. Thus, UCB-MSCs are not only used in regenerative medicine for chronic diseases but also in immune diseases such as autoimmune diseases (21), inflammation (22), and graft-versus-host disease (23).

Clinically, UCB-MSCs have been used in the treatment of diseases such as autism (24), hereditary spinocerebellar ataxia (25), foot disease in patients with type 2 diabetes mellitus (26), basilar artery dissection (27), and peripheral arterial occlusive disease (28). There have also been some clinical trials performed around the world, including MSC transplantation for engraftment of unrelated hematopoietic stem cell transplantation (NCT00823316), treatment of steroid-refractory acute or graft-versus-host disease (NCT01549665), articular cartilage defect treatment (NCT01733186), and hematologic malignancy treatment (NCT01854567) (retrieved from clinicaltrial.gov).

Although UCB-MSCs have some advantages, they still have not been applied in the treatments of many diseases. The main concern is due to the procedure required to culture and expand the cells, which has some limitations. In almost all cases, cells for clinical application should be produced using a good medical practices (GMP)-compliant procedure. This means that the procedure can be controlled and is free from any viral transmission, especially in the culture medium. Moreover, by using a GMP-compliant procedure, UCB-MSCs are easily expanded and maintain their phenotype and karyotype. In previously published studies, UCB-MSCs were cultured in a fetal bovine serum (FBS)-based medium (9, 8). FBS-based medium presents some risks, as prion and viral transmission or adverse immunological reactions against xenogenic components can occur. In some improved media, FBS is replaced by human serum, especially platelet-rich plasma (PRP).

PRP has been successfully prepared from peripheral blood (29–33) as well as UCB (34–37). By replacing FBS with PRP, MSCs derived from bone marrow (30, 35), adipose tissue (29, 38), and UCB (34, 37) have been successfully cultured. In the medium with PRP, MSCs from these sources exhibited a normal phenotype and maintained their stemness (34–37). Use of allogenic PRP in UCB-MSC isolation and culture allows avoidance of xenogenic immunological reactions and prion and viral transmission. However, UCB-MSCs can still be contaminated through human viral transmission and immunological reactions induced by allogeneic components. Therefore, establishment of a procedure for UCB-MSC culture using autologous PRP could resolve these limitations.

PRP is a pool of at least seven different growth factors, including epidermal growth factor (EGF), platelet-derived growth factor, transforming growth factor beta, vascular endothelial growth factor, fibroblast growth factor (FGF), insulin-like growth factor, and keratinocyte growth factor. The therapeutic influence of PRP is due to the high concentration of these growth factors compared with normal plasma. They play pivotal roles in the stimulation of MSCs in in vitro culture. Moreover, PRP also contains some different

proteins that help MSCs adhere to the flask surface without a protein coating.

This chapter will describe the step-by-step production of GMP-grade human UCB-MSCs for therapeutic use.

2 Materials

2.1 Collection and Processing the Blood

1. Blood collection bags with citrate phosphate dextrose adenine (CPDA) with 20-G syringe.
2. Ficoll-paque PREMIUM (GE Heathcare—Life Science, Piscataway, NJ).
3. Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} (cat. no. A12856-01; Life Technologies, Carlsbad, CA) manufactured in state-of-the-art cGMP, ISO certified facilities.
4. Trypan blue solution 0.4 % (cat. no. T8154-100ML, Sigma-Aldrich, St. Louis, MO).

2.2 Stem Cell Enumeration

1. BD Stem Cell Enumeration Kit (CD45 FITC/CD34 PE) (cat. no. 344563, BD Bioscience, Franklin Lakes, NJ). Kit includes BD Stem Cell reagent (CD 45 FITC/CD34 PE), 7-aminoactinomycin-D (7-AAD) reagent, 10× ammonium chloride lysing solution, 50 BD Trucount tubes.
2. BD Calibrite 3 three-color kit beads (cat. no. 340486, BD Bioscience, Franklin Lakes, NJ) for use on a BD FACSCalibur flow cytometer.
3. Reagent-grade (deionized) water.
4. 12 × 75-mm BD Falcon™.
5. 1× PBS (Dulbecco's modified, pH 7.2 ± 0.2) with 0.5 % bovine serum albumin (BSA), if sample dilution is necessary.
6. BD Stem Cell Control kit (cat. no. 340991, BD Bioscience, Franklin Lakes, NJ).

2.3 Cell Culture Medium

1. 20 % Calcium chloride (meet USP, Ph.Eur, cat. no. 12022, Sigma-Aldrich, St. Louis, MO) prepared in water (injection grade).
2. Iscove modified Dulbecco medium (IMDM) (cat. no. 31980-030, Life Technologies, Carlsbad, CA).
3. Antibiotic Antimycotic Solution (100×) (cat. no. A5955, Sigma-Aldrich, St. Louis, MO).
4. Epidermal growth factor (EGF) (cat. no. PHG6045, Life Technologies, Carlsbad, CA).

5. Basic fibroblast growth factor (bFGF) (cat. no. CTP0261, Life Technologies, Carlsbad, CA).
6. TrypLe (cat. no. A12859-01, Life Technologies, Carlsbad, CA).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Collection of UCB

1. UCB must be anticoagulated and used fresh.
2. UCB samples should be collected with the informed consent of the donors.
3. Carry out infectious diseases screening of the mother for syphilis, hepatitis B & C, and human immunodeficiency virus I & II (*see* **Note 1**).
4. Collect the UCB according to the institutional guidelines (*see* **Note 2**).
5. Collect the UCB in clean aseptic conditions using standard blood bags with anticoagulant CPDA. The suitable ratio of CPDA and UCB is 25 mL:100 mL (*see* **Note 3**).
6. During the UCB collection, the collection bag should be gently shaken to disperse the CPDA into the blood.
7. Transport the blood sample immediately from the hospital to the laboratory. The sample should be kept at 4 °C during transportation (*see* **Note 4**).

3.2 Sample Selection of UCB for Isolation

This step aims to eliminate the samples with low stem cell numbers in order to increase the efficiency of this procedure. We recognized that there is a similar amount of hematopoietic stem cells (HSCs) and MSCs in the UCB. Thus, we can count the number of HSCs in order to determine the number of MSCs.

1. Preparation of 1× lysing solution: dilute the 10× concentrate 1:10 with distilled water. The pH of the 1× solution should fall within the range of pH 7.1–7.4 (*see* **Note 5**).
2. Pipette 20 μL of the BD Stem Cell reagent (cocktail of CD45-FITC/CD34-PE) into the bottom of the tube.
3. Pipette 20 μL of 7-AAD reagent into the tube (*see* **Note 6**).
4. Pipette 100 μL of the UCB onto the side of the tube just above the retainer.
5. Cap each tube and vortex gently to mix.
6. Incubate for 20 min in the dark at room temperature (20–25 °C).

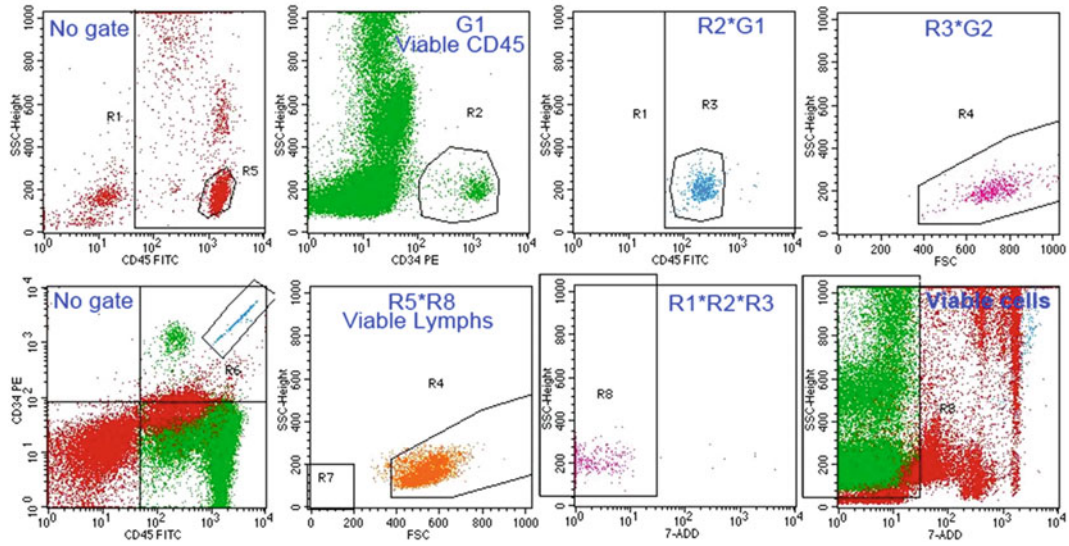

Fig. 1 Absolute count of HSCs in the UCB samples. HSCs were counted using the TruCount Kit on the FACSCalibur™ system

7. Add 2 mL of 1× ammonium chloride lysing solution to each tube to lyse the red blood cells.
8. Cap each tube and vortex gently to mix.
9. Incubate for 10 min in the dark at room temperature (20–25 °C) (*see* **Note 7**).
10. Immediately place tubes on wet ice in the dark until ready to acquire samples.
11. The HSC count is determined based on the number of $CD34^+CD45^+$ cells and the number of beads in the TruCount tube. It is easy to get the template for analysis from BD Bioscience. The template is also presented in Fig. 1. The $CD34^+CD45^+7\text{-}AAD^-$ cell population gated in R3 is defined as the number of viable HSCs in the UCB sample. R5 is defined as the number of beads that already exist in the Trucount Tube. Based on the relative ratio of counted beads to the total count number (added into the Trucount tube) and the HSC count, the absolute number of HSCs can be calculated as: (Viable $CD34^+CD45^+$ × Trucount × Dilution factor)/(Beads × Sample Volume) = HSCs/μL (*see* **Note 8**). UCB samples with $\geq 1 \times 10^3$ HSCs/μL or $\geq 1 \times 10^6$ HSCs/mL were used in the experiments.

3.3 Separation of Blood Cells and Plasma

In this step, blood cells are separated to isolate mononuclear cells (MNCs), whereas plasma is used to produce activated PRP.

1. Pipette 50 mL of an anticoagulated blood sample into a 50-mL centrifuge tube, then centrifuge at 300 × *g* for 15 min in a

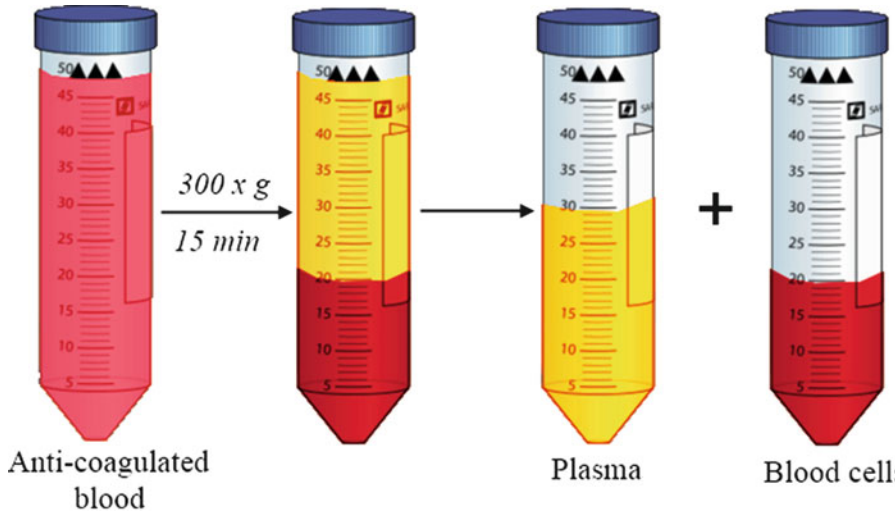

Fig. 2 Separation of blood cells and plasma from the same UCB sample

swinging bucket rotor centrifugation at room temperature (*see* **Note 9**).

2. After centrifugation, collect the plasma in the upper layer (yellow to pink in color) in a new 50-mL centrifuge tube to produce activated platelet-rich plasma (aPRP). Keep the blood cell pellet to isolate the MNCs in the next step (Fig. 2).

3.4 Production of Activated PRP

1. Centrifuge the obtained plasma at 400 × *g* for 15 min in a swinging bucket rotor at room temperature.
2. Discard the upper two thirds (2/3) of the supernatant in the centrifuge tube and keep the bottom one third of the plasma containing the platelet pellet (*see* **Note 10**).
3. Resuspend the pellet in the remaining plasma by pipetting with an air-pipette or vortex. This suspension is called the PRP.
4. Add 100 μL of sterile $CaCl_2$ and 20 % per 1 mL of PRP to form the gel fibril and activate growth factor release. Incubate the samples at 37 °C for 30 min or until the occurrence of clotting (Fig. 3) (*see* **Note 11**).

3.5 Isolation of MNCs from Blood Cells

MNCs can be separated from UCB by density-gradient centrifugation with Ficoll (Fig. 4).

1. Dilute the obtained blood cells with phosphate-buffered saline (PBS) (without Mg^{2+} and Ca^{2+}) at a 1:1 blood:PBS ratio. For example: 20 mL of blood with 20 mL of PBS.
2. Add 10 mL of Ficoll to a 50-mL centrifuge tube, and put this tube in the centrifuge tube holder.

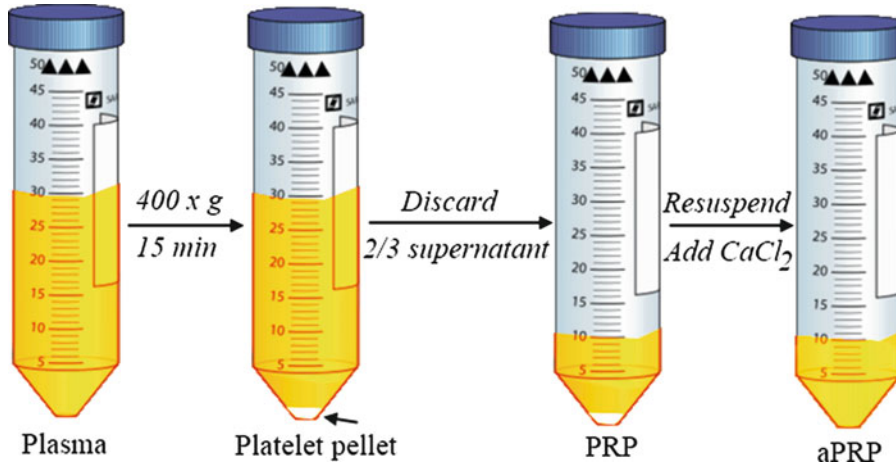

Fig. 3 Preparation of PRPs from UCB-derived plasma

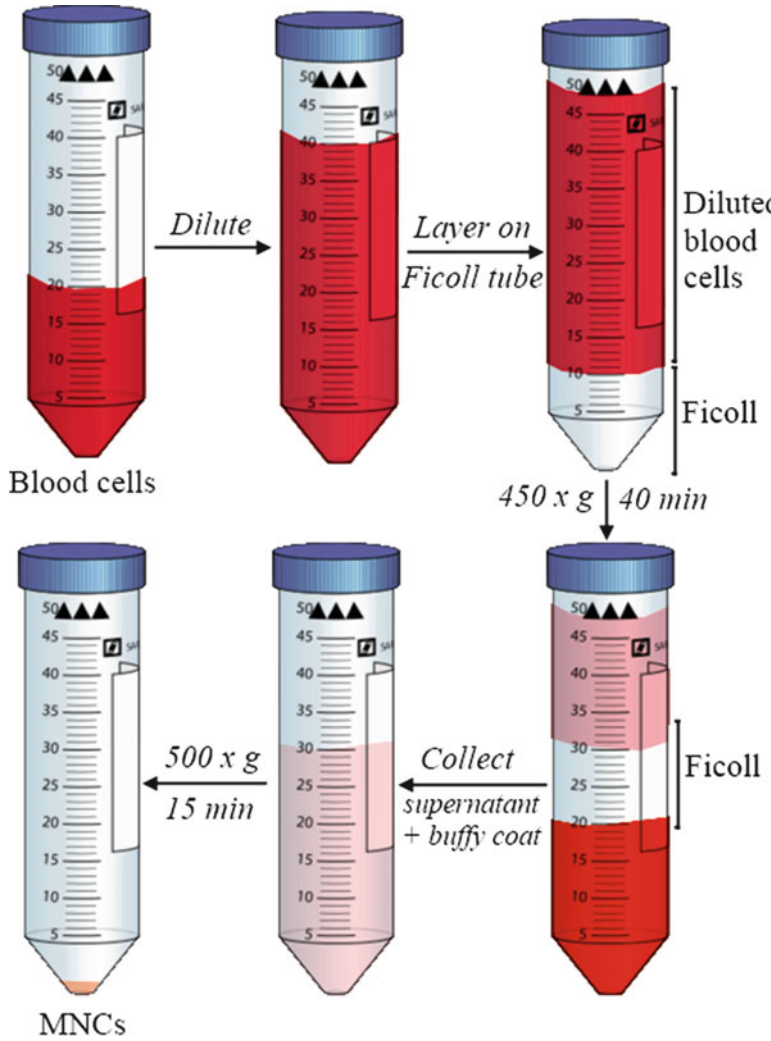

Fig. 4 Isolation of MNCs from blood cells diluted with PBS

3. Slowly layer 10–30 mL of diluted blood on top; be careful to not disturb and mix the blood and Ficoll.
4. Centrifuge these tubes by swinging bucket rotor centrifugation at room temperature at 450 × *g* for 40 min.
5. Carefully extract the supernatant including the buffy coat layer (the cloudy interface layer) with a serological pipette and place into a new 50-mL centrifugation tube (*see* **Note 12**).
6. Centrifuge the tube at 500 × *g* for 15 min to obtain the MNC pellet.
7. Wash the MNCs with 20 mL PBS without Mg^{2+} and Ca^{2+} twice. After each wash, re-pellet the MNCs by centrifugation at 500 × *g* for 15 min.
8. Finally, resuspend the cell pellet in primary culture medium.

3.6 Selective Primary Culture of MSCs from MNCs

1. Preparation of primary culture medium: Iscove modified Dulbecco medium (IMDM) supplemented with 1 % antibiotic-mycotic, 10 ng/mL EGF, 10 ng/mL basic fibroblast growth factor (bFGF), and 10 % activated PRP (aPRP) (*see* **Note 13**).
2. Dilute the cell suspension with primary culture medium to a concentration of 10^6 MNCs/mL.
3. Pipette 7.5 mL of the diluted cell suspension into a 75-cm^2 T-flask. Gently shake the flask so that the medium flows over the surface of the flask.
4. Incubate the flask at 37 °C with 5 % CO_2 and saturated humidity.
5. After 3 days of incubation, add 6 mL of fresh media to each flask (*see* **Note 14**).
6. After 7 days, replace the media with fresh media.
7. Replace the media every 4 days until the cells reach 70–80 % confluence (Fig. 5).

Fig. 5 UCB-MSC shape. UCB-MSCs adhered to the surface of T75 flasks after 3 days (**a**), 10 days (**b**), and 15 days of incubation. (Magnification ×100)

3.7 Expansion of MSCs

1. Preparation of primary culture medium: IMDM supplemented with 1 % antibiotic-mycotic, 10 ng/mL EGF, 10 ng/mL bFGF, and 5 % aPRP (*see* **Note 15**).
2. After the primary cells reach 70–80 % confluence, subculture by using TrypLe™ (*see* **Note 16**).
3. Completely remove the medium in the flask using a serological pipette or aspirating pipette.
4. Wash the cells with 7.5 mL PBS without Mg^{2+} and Ca^{2+}.
5. Pipette 7.5 mL TrypLe™ into the T-75 flask.
6. Keep the flask in the biosafety cabinet and incubate for 5 min.
7. Observe and monitor the detached cells under an inverted microscope. The detached cells will be round in shape and float in the solution.
8. Pipette using a 5-mL serological pipette to disassociate the cell clumps into single cells until about 70–80 mL of cells are detached.
9. Pipette the cell suspension into 15-mL centrifugation tubes, and centrifuge these tubes at 500 × *g* for 10 min at room temperature.
10. Discard the supernatant and keep the cell pellets.
11. Resuspend the cell pellet with 22.5 mL of the fresh expanding culture medium. Divide the cell suspension into 3 new T-75 flasks with 7.5 mL in each T-75 flask.
12. Incubate these flasks at 37 °C with 5 % CO_2 and saturated humidity. Replace the media every 4 days until the cells reach 70–80 % confluency. These cells are considered the first passage of MSCs.
13. Repeat steps 3 through 12 to expand the MSCs to the desired passage (*see* **Note 17**).

3.8 Characterization of MSC Phenotypes

UCB-MSCs should be characterized according to the minimal standards of MSCs that Dominici et al. (2006) suggested: (1) exhibit a fibroblast-like shape during plastic adherence; (2) express CD73, CD90, and CD105; (3) be negative for CD14, CD34, CD45, CD19, and HLA-DR; (4) be capable of successful in vitro differentiation into osteoblasts, adipocytes, and chondroblasts (39).

4 Notes

1. Donors should be checked for syphilis, hepatitis B & C, human immunodeficiency virus I & II before birth. Testing must be performed at a hospital or medical center with confirmation tests.

2. UCB should be collected and processed according to the local ethical guidelines and approved by the ethical review committee.
3. CPDA is a suitable anticoagulant for UCB collection. However, heparin can also be used. Heparin can cause cells to clump so that the separation efficiency is lower. In this case, cells should be washed twice with PBS without Mg^{2+} and Ca^{2+}.
4. Although UCB-derived stem cells can survive at 4 °C for 48–72 h, it is better if the UCB is fresh. If UCB is transferred over a long distance, a temperature recorder should be used to monitor the temperature of the box during the transportation. Do not freeze the UCB.
5. A 1× lysing solution should be warmed to room temperature prior to use.
6. Pipette tips must be changed at each step to avoid cross-contamination between antibodies and other chemicals.
7. At the end of this step, almost all red blood cells are lysed and the color of the solution becomes clear with a slight pink tint. If the solution is still turbid, the tube needs to be incubated for 5–10 min more to completely lyse the red blood cells.
8. For accuracy in determining the absolute counts, it is critical to use good pipetting technique when adding the UCB to each BD Trucount tube. Trucount represents the number of beads of the Trucount tube that can read in the pouch of the Trucount tube. The sample volume in this case is 100 μL.
9. A lower rate of centrifugation can be applied for a longer time; however, this is not useful. In fact, more platelets will break and release growth factors during high-speed centrifugation.
10. Because two thirds of the plasma supernatant (platelet-poor plasma) is discarded, the plasma is enriched in platelets by three times.
11. After PRP forms a clot, the clot will adhere to the tube wall. To facilitate clot shrinkage, the clot should be separated from the tube wall.
12. In this step, the upper supernatant with PBS can be discarded first. The buffy coat (MNCs) can then be collected into the new tube.
13. If using IMDM without L-glutamine, it should be supplemented with L-glutamine at a 2 nM working concentration.
14. In the medium with aPRP, there are fewer adherent proteins than in the medium with FBS. Three days is the required time to permit the stem cells to adhere to the surface of the flasks.
15. Expanding culture medium contains a half of aPRP compared to the primary culture medium. In fact, the expanding culture

medium can be similar to the primary culture medium. However, using 50 % aPRP is economic and sufficient for cell expansion.

16. TrypLe™ is used to replace trypsin/EDTA. Almost all trypsin used in cell culture originates from the porcine or bovine pancreas. Using trypsin in this procedure can cause contamination of exogenous proteins in the cultured cells.
17. The number of subcultures will affect the quality of the MSCs. Some previous studies showed that when MSCs are expanded in vitro for a long time they can become mutated, especially in karyotype. Therefore, MSC expansion should be restricted to 3–7 passages.

Acknowledgment

This study was supported by the grant number DTDL.2012-G/23, Ministry of Science and Technology, Vietnam.

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Methods in Molecular Biology (2015) 1283: 87–92
DOI 10.1007/7651_2014_122

Published online: 19 October 2014

Xeno-Free Culture of Human Periodontal Ligament Stem Cells

Oriana Trubiani and Francesca Diomede

Abstract

The possibility of transplanting adult stem cells into damaged organs has opened a new prospective for the treatment of several human pathologies. Currently, in vitro expansion and culture of mesenchymal stem cells is founded on supplementing cell culture and differentiation medium with fetal calf serum (FCS) or fetal bovine serum (FBS) that contain numerous growth factors inducing cell attachment to plastic surfaces, proliferation, and differentiation. Mesenchymal stem cells (MSCs) cultured with medium containing FCS or FBS are unusable in the cell therapy; in fact the central issues regarding limitations in using animal sera for cell therapy is that its components are highly variable and often unknown and may trigger a xenogenic immune response, immunological reactions, and the potential transmission of prion diseases and zoonoses.

Here we describe the culture system protocols for the expansion and production of human Periodontal Ligament Stem Cells (hPDLSCs) using a new xeno-free medium formulation ensuring the maintenance of the stem cells features comprising the multiple passage expansion, mesengenic lineage differentiation, cellular phenotype, and genomic stability, essential elements for conforming to translation to cell therapy.

Keywords: Adult stem cells, Periodontal ligament, Xeno-free cell culture

1 Introduction

The current literature has described six different human dental stem cells derived from the oral tissues: dental pulp stem cells (DPSCs) (1, 2), exfoliated deciduous teeth stem cells (SHED) (3), periodontal ligament stem cells (PDLSCs) (4–6), apical papilla stem cells (SCAP) (7), dental follicle stem cells (DFSCs) (8), and gingiva stem cells (GSCs) (9, 10).

Dental stem cell populations present properties similar to bone marrow mesenchymal stem cells (BM-MSCs), such as the ability to self-renew and the potential for multilineage differentiation (2). They have the ability to give rise to odontogenic cells and regenerate oral tissue as dental pulp and periodontal ligament. They have the capacity to differentiate into all three germ line cells, proving that a population of stem cells is present in the dental tissues (11–13). Then, their isolation and characterization is crucial to develop new methods for dental applications and for the treatment of human bone degenerative diseases (9). Currently, in vitro

expansion and culture of mesenchymal stem cells is founded on supplementing cell culture and differentiation medium with fetal calf serum (FCS) that contains numerous growth factors inducing cell attachment to plastic surfaces, cell proliferation, and differentiation (14). MSCs cultured with medium containing FCS or FBS were unsuitable in the cell therapy; in fact the presence of FCS or FBS can trigger an immune response and the potential transmission of prion diseases and zoonoses (15–17). Moreover, one of the central issues regarding limitations in using animal sera for cell therapy is that its components are highly variable and often unknown, Moreover, one of the central issues regarding limitations in using animal sera for cell therapy is that its components are highly variable and often unknown, then the consistency between different lots cannot be assured. (18). A right alternative is represented by human serum, but their limits were the high quantity of autologous serum indispensable to expand MSCs for cell therapy (19). Then, cell therapy application needed a standard protocol to isolate and expand stem cells without FBS or FCS supplemented medium. Here, we describe an efficient xeno-free culture system of hPDLSCs using a commercial medium that assures the maintenance of the stem cells features, including the multiple passage expansion, mesengenic lineage differentiation, cellular phenotype, and genomic stability, which are essential to be utilized in cell therapy.

2 Materials

Prepare all solution under sterile conditions at room temperature (RT).

2.1 Cell Culture Medium

1. Prepare TheraPEAK™ Chemically Defined Mesenchymal Stem Cell Growth Medium (MSCGM-CD™) (LONZA Walkersville Inc., Walkersville, MD, USA). MSCGM-CD™ is a kit that is composed of MSCGM-CD™ basal medium (store at 4 °C) and MSCGM-CD™ SingleQuots™ (store at −20 °C).
2. Add MSCGM-CD™ SingleQuots™ (5 mL) to MSCGM-CD™ basal medium.
3. Store completed medium at 2–8 °C in a dark place.
4. Before every use place the culture medium in 37 °C water bath.

2.2 Solution to Detach Cell Culture

1. TryPLE™ Select Enzyme (Gibco, Life Technologies, Eugene, OR, USA).
2. Store at RT.
3. Is ready to use.

2.3 Solution to Wash Cell Culture

1. Dulbecco's Phosphate Buffered Saline (0.00095 M) (Lonza) without Ca^{++} and Mg^{++}.
2. Store at RT.

3 Methods (*See* Fig. 1)

3.1 Surgical Procedures

1. Patients sign written consent for clinical research and for the processing of personal data before surgical procedure.
2. Prepare patient (*see* **Note 1**), for surgery by pretreatment for 1 week with professional dental hygiene and decontamination of the oral cavity with chlorhexidine 0.2 %.
3. Carry out periodontal ligament biopsies from human teeth (*see* **Note 2**).
4. Collect the periodontal ligament tissue, in particular horizontal fibers, by scraping the coronal part of dental root using Gracey's curette.

3.2 Laboratory Procedures

1. Put the specimens of periodontal tissue in a sterile tube (15 mL) with DPBS.
2. Wash gently the tissue five times in DPBS (10 mL) supplemented with 5 % of Gentamicin (Lonza).
3. Place the tissue in Corning® BioCoat™ dish (Corning Inc., Corning, NY, USA) (diameter 10 mm).
4. Wash gently twice with DPBS supplemented with 5 % of streptomycin (Lonza) (5 mL).
5. Dissociate mechanically the periodontal tissue into small clumps using sterile scalpel.
6. Add appropriate volume (5 mL) of completed medium (MSCGM-CD™) to the dish.
7. Put the dish in the incubator at 37 °C and 5 % CO_2.

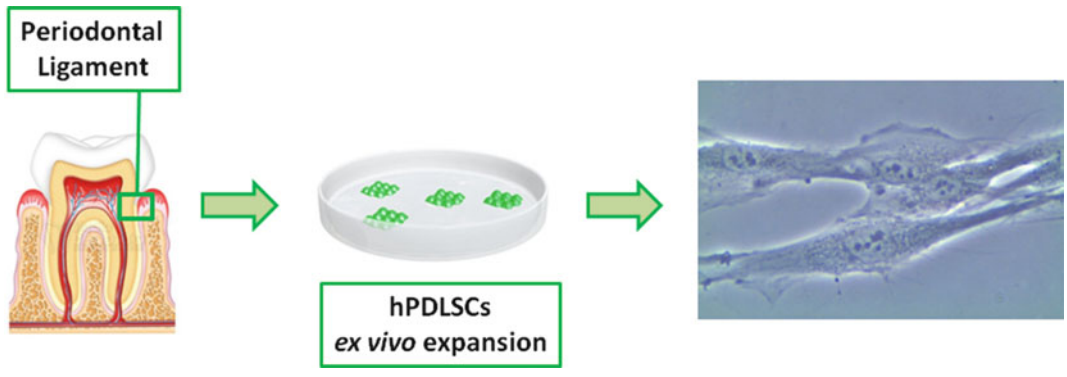

Fig. 1 Flow chart of the protocol of xeno-free culture of human periodontal ligament stem cells. Light microscopy image represents cell culture stained with toluidine blue. hPDLSCs showed a homogeneous fibroblast-like appearance with a stellate shape and long cytoplasmic processes. Nuclei appeared as round or oval, with one or more nucleoli and a signified secretory apparatus (Mag: 40×)

8. Change medium three times a week.
9. Wait 7–20 days of culture the spontaneous cell migration from the explants.
10. Detach the cells at 80 % of confluence.
11. Remove medium.
12. Wash gently twice with DPBS (5 mL).
13. Add TryPLE™ Select to the dish (1 mL).
14. Put dish in the incubator at 37 °C and 5 % CO_2 for 10 min.
15. Control cell morphological changes at light microscopy.
16. Transfer solution from dish to a sterile tube.
17. Centrifuge at 210 × *g* for 8 min at 25 °C.
18. Remove supernatant.
19. Resuspend the cell pellet in a minimal volume of temperature equilibrated MSCGM-CD™.
20. Remove a sample for counting.
21. Count the cells with a hemocytometer or cell counter and calculate the total number of cells.
22. Assess cell viability using Trypan Blue exclusion dye test.
23. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of viable cells} = \frac{(\text{Total cell count} \times \text{percent viability})}{100}.$$

24. Determine the total number of dishes to seed by using the following equation. The number of dishes needed depends upon cell yield and seeding density.

$$\text{Total \# of dishes to inoculate} = \frac{\text{Total \# of viable cells}}{(\text{Growth area} \times \text{Rec. seeding density} : 15\text{–}25 \times 10^3 \text{cells/mL})}.$$

25. Use the following equation to calculate the volume of cell suspension to seed into your dishes. Determine the volume of MSCGM-CD™ to add to each flask so that the final culture volume is 0.2–0.4 mL/cm^2.

$$\text{Seeding volume} = \frac{\text{Total volume of diluted cell suspension}}{\text{\# of dishes as determined in step 2}}.$$

26. Prepare dishes by labeling each dish with the passage number, strain number, cell type, and date.
27. Add the MSCGM-CD™ at RT to the dish (5 mL).

28. Incubate at 37 °C and 5 % CO_2.
29. After 3 days remove completely the medium.
30. Replace with an equal volume of MSCGM-CD™.
31. Cultures will be near confluence by day 5 or 6 and ready to subculture.

4 Notes

1. Patient inclusion criteria:
   - Age range: 18–35 years,
   - nonsmoking,
   - oral and general healthy conditions.
2. Teeth must be noncarious and inflammation-free.

Acknowledgements

This work has been supported from PRIN 20102ZLNJ5 "Stem cells and 3D scaffolds: a novel construct in bone regeneration" financed by the Ministry of Education, University and Research (M.I.U.R.), Rome, Italy.

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Methods in Molecular Biology (2015) 1283: 93–107
DOI 10.1007/7651_2014_112

Published online: 20 August 2014

GMP-Compliant Human Adipose Tissue-Derived Mesenchymal Stem Cells for Cellular Therapy

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Abstract

Stem cells, which can be derived from different sources, demonstrate promising therapeutic evidences for cellular therapies. Among various types of stem cell, mesenchymal stem cells are one of the most common stem cells that are used in cellular therapy. Human subcutaneous adipose tissue provides an easy accessible source of mesenchymal stem cells with some considerable advantages. Accordingly, various preclinical and clinical investigations have shown enormous potential of adipose-derived stromal cells in regenerative medicine. Consequently, increasing clinical applications of these cells has elucidated the importance of safety concerns regarding clinical transplantation. Therefore, clinical-grade preparation of adipose-derived stromal cells in accordance with current good manufacturing practice guidelines is an essential part of their clinical applications to ensure the safety, quality, characteristics, and identity of cell products. Additionally, GMP-compliant cell manufacturing involves several issues to provide a quality assurance system during translation from the basic stem cell sciences into clinical investigations and applications. On the other hand, advanced cellular therapy requires extensive validation, process control, and documentation. It also evidently elucidates the critical importance of production methods and probable risks. Therefore, implementation of a quality management and assurance system in accordance with GMP guidelines can greatly reduce these risks particularly in the higher-risk category or "more than minimally manipulated" products.

Keywords: Adipose-derived stem cell, Cell therapy, Good manufacturing practice, Quality assurance

1 Introduction

Stem cells, which can be derived from different sources, demonstrate promising therapeutic evidences for cellular therapies (1). On the other hand, cellular therapy as an interesting arena in biologic sciences has grown dramatically (2, 3). Among different types of stem cell, mesenchymal stem cells (MSCs) have increasingly used for cell therapy trials. MSCs are multipotent with the ability to differentiate into mesoderm-derived cells and can be isolated from almost all tissues, for instance bone marrow, umbilical cord tissue and blood, adipose tissue, dental pulp, synovial membrane and fluid, skin, etc. (4, 5). Furthermore, they have some proven immunomodulatory, hematopoiesis, and regeneration capabilities that have led their utilization in clinical cell transplantation trials (1, 6). Although bone marrow is the most common source

for MSCs, the number of bone marrow-derived MSCs and their differentiation potential dramatically decrease with age (only 0.002 % of total stromal cell population). In addition, the isolation of MSCs from bone marrow is an invasive and painful procedure. Therefore, scientists tried to find and develop an alternative source of MSCs. Human subcutaneous adipose tissue provides an easy and repeatable access to adipose tissue while the simple isolation procedures provide a clear advantage (1, 7). Therefore, adipose-derived mesenchymal stem cells (ADSCs) seem as a superior choice for cellular therapy because of several advantages such as the large number of isolated cells using a minimally invasive procedure (8). Furthermore, various basic, experimental, and clinical researches have revealed enormous potential of ADSCs in regenerative medicine (9–16). Significantly, increasing clinical transplantation of ADSCs has shown the importance of several concerns regarding clinical application of these cells and also has been accompanied by the regulations governing cellular therapies (2). Accordingly, it is recommended that the clinical-grade preparation of ADSCs should be performed in accordance with current good manufacturing practice (cGMP) guidelines that are used for facility or manufacture, processing, and packing controls (17, 18) to ensure the safety, quality, characteristics, and identity of cell products (19, 20). Additionally, cell manufacturing in accordance with GMP standards involves several issues similar to drugs manufacturing guidelines (6). Bridging between stem cell basic science and clinical practice is one of the most complex organizational and regulatory areas (21). Consequently, advanced cellular therapy requires extensive validation, process control, and documentation and clearly speculates the critical importance of production methods as well as the potential risks (2). Therefore, implementation of a quality management system in accordance with international standards such as GMP can greatly reduce these risks (22). The Food and Drug Administration (FDA) has defined human cells, tissues, and cellular and tissue-based products (HCT/Ps) in two categories; the lower risk category or "minimally manipulated" products and the higher-risk category or "more than minimally manipulated" products. Accordingly, manufacturing the first ones must be complied with good tissue practice (GTP) regulations as a level of control similar to that already practiced by most clinical laboratories and the second ones must be complied with both GTP and GMP (2, 23). As we described previously we established a clean room facility as the most tangible aspect of cGMP, affiliated to Brain and Spinal Cord Injury Research Center to perform (stem) cell transplantation trials according to cGMP standards (22, 24). Now, our purpose is to demonstrate GMP-complaint and clinical-grade ADSCs manufacturing for clinical cell therapy trials.

2 Materials

2.1 Adipose Tissue Trimming and Digestion, Cell Isolation, Culture, Subculture, and Cryopreservation

1. Clean room (GMP) facility (Fig. 1).
2. Laminar air flow cabinet (biological safety cabinet).
3. Shaking water bath (Memmert, Germany).
4. Weighing balance (Sartorius, Germany).
5. Centrifuge (Swing-out with buckets for 50 and 15 ml tubes).
6. CO_2 Incubator (set at 5 % CO_2, 37 °C, and 100 % humidity).
7. Optical inverted microscope.
8. NucleoCounter® NC-100™ (Chemometec, Denmark).
9. 100 mm Petri dish (TPP, Switzerland).
10. Sterile scissors and tweezers.
11. Falcon tubes 50 ml (TPP, Switzerland).
12. Pipette (TPP, Switzerland).
13. Sterile clean room garments (hood, face masks, coveralls, powder-free gloves, and boots) (Fig. 2).
14. 70 % ethanol (Merck, Germany).

Fig. 1 Clean room facility

Fig. 2 Clean room garments

15. Phosphate buffer saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
16. Collagenase NB6 (Serva Electrophoresis GmbH, Germany, Cat. No. 17458) (*see* **Note 8**).
17. TrypLE Select (recombinant trypsin-like substitute) (Invitrogen, USA).
18. Dulbecco's Modified Eagle Medium–low glucose 1 g/100 ml (DMEM-LG) (PAA, Austria).
19. Fetal bovine serum (FBS) (Pharma grade, Australian origin and gamma irradiated, PAA, Austria, Cat. No. A15-512, Lot. No. A51210-2738) (*see* **Note 13**).
20. Trypan blue (Invitrogen, USA).
21. 0.22 μm syringe filter (Orange Scientific, Belgium).
22. 70 μm cell strainer (Becton Dickinson, USA).
23. Motorized pipette (Orange Scientific, Belgium).
24. Filter cap cell culture flasks (175 and 25 cm^2) (TPP, Switzerland).
25. CryoSure-Dimethyl-sulfoxide (DMSO) USP Grade (Wak Chemie, Germany).
26. Cryo vial (Thermo Scientific, USA).

27. Nalgene™ cryo freezing container (Cat. No. 5100-0001, Thermo Scientific, USA).
28. Ultralow temperature freezer.
29. Liquid nitrogen Tank (Statebourne Cryogenics, UK).

3 Methods

All procedures should be accomplished in a GMP facility (clean room) using laminar air flow cabinet and in accordance with cGMP guidelines.

3.1 Controls Prior to Adipose Tissue Processing

1. Check obtained informed consent and tissue container at arrival time for labeling, probably leakage, transfer medium, amount of adipose tissue and write all data according to documentation SOPs (*see* **Notes 1** and **2**).
2. Put the tissue container in the refrigerator (+4 °C) till processing time (*see* **Note 3**).
3. Provide all documents that are required for tissue trimming and cell isolation.
4. Check instruments' maintenance records and documentation. All equipment servicing and maintenance programs should be done perfectly (*see* **Note 1**).
5. Check facility environmental monitoring documents. Be sure that environmental parameters are within their defined ranges (*see* **Note 4**).
6. Facility and all instruments (e.g., laminar air flow cabinet, CO_2 incubator) and working area should be cleaned and/or sterilized according to the organization-specific SOPs (*see* **Notes 1** and **2**).
7. Prepare all labeling and coding requirements before starting the processing procedures according to SOPs.
8. Prepare all medium, enzymes, and supplements prior to processing.

3.2 Tissue Digestion and Cell Isolation

1. Spray 70 % ethanol on the outer side of the tissue container and place it into laminar air flow cabinet.
2. Send a sample of the tissue or transfer medium for microbiological testing.
3. Put adipose tissue in 100 mm Petri dish.
4. Wash the adipose tissue prior to processing by PBS to remove excess blood from the tissue.
5. Repeat step 2 twice (*see* **Note 5**).

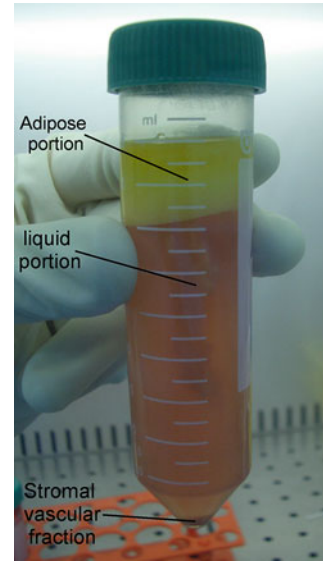

Fig. 3 Collagenase digestion of adipose tissue. Three different portions can be seen: adipose portion, liquid portion, and Stromal Vascular Fraction (SVF)

6. Trim the adipose tissue using tweezers and scissors, to remove small vessels and also connective tissues and then cut it in very small pieces and put into two or three 50 ml Falcon tubes (*see* **Note 5**).
7. Add each falcon tube 3 mg/ml collagenase-NB6 followed by incubation at 37 °C for 30 min (*see* **Notes 6–10**).
8. Stop digestion by adding PBS-EDTA (in equal volume).
9. Centrifuge the suspension (500 × *g* for 10 min at room temperature) (*see* **Note 8**).
10. Discard adipose and liquid portion and save the stromal vascular fraction (SVF) (Fig. 3) (*see* **Note 11**).
11. Resuspend SVF in PBS-EDTA (1 mM of Ethylene Diamine Tetra Acetic Acid, per 1 ml PBS).
12. Place 70 μm cell strainers on top of 50 ml falcon tubes considering a small air gap between the bottom of the cell strainer and the top of tube.
13. Filter the suspension through the cell strainer.
14. Spin filtered suspension (350 × *g* for 5 min at 20 °C).
15. Count cells by NucleoCounter.

3.3 ADSCs Culture and Subcultures

1. Place 175 cm^2 filter cap flasks into laminar air flow cabinet.
2. Insert a label on each culture flask (*see* **Note 12**).
3. Transfer around 80 × 10^3 mixed SVF cells per cm^2 into each culture flask (14 × 10^6 in a 175 cm^2 filter cap flask).

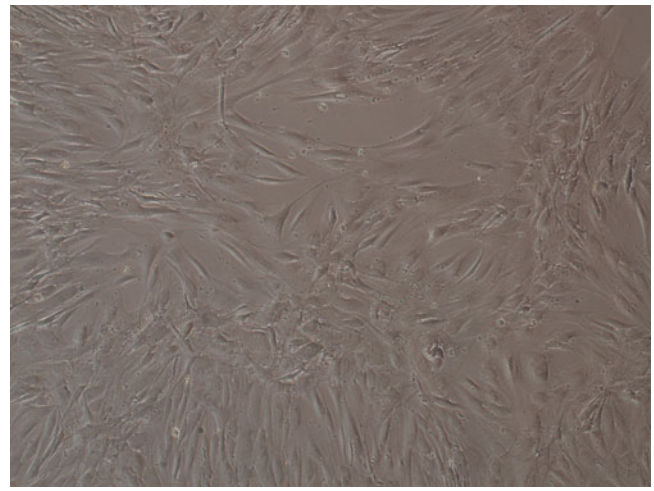

Fig. 4 ADSCs morphology. Light inverted microscopy photographs of ADSCs in culture ×20

4. Add complete culture medium (DMEM supplemented with 10 % FBS Pharma Grade) into each 175 cm^2 flask up to 22 ml (*see* **Note 13**).
5. Transfer flasks into the incubator (37 °C, 5 % CO_2, humidified) (*see* **Note 14**).
6. After 48 h, wash the culture medium and discard non-adherent cells and then renew complete culture medium.
7. Replace culture medium every 72 h.
8. During cell culture period, you should check the morphology of cells (Fig. 4) and their culture medium for every probable undesired event (medium color changes, bacterial contamination, morphology changes, cellular death, etc.). In addition, microbiological evaluation is a fundamental procedure before cell isolation, during, and also after cell culture for safety assurance (*see* **Note 15**).
9. At approximately 85–90 % confluence, transfer culture flasks into laminar air flow cabinet.
10. Collect and discard all culture medium using motorized pipette.
11. Wash cells gently with PBS-EDTA (1 mM of EDTA, per 1 ml PBS).
12. Remove and discard PBS-EDTA solution.
13. Add 5 ml TrypLE Select to each 175 cm^2 flask (*see* **Note 16**).
14. Transfer flasks into incubator (37 °C and 5 % CO_2) and leave them horizontally for around 10–12 min.
15. Then check flasks for cell detachment and agitate them gently.

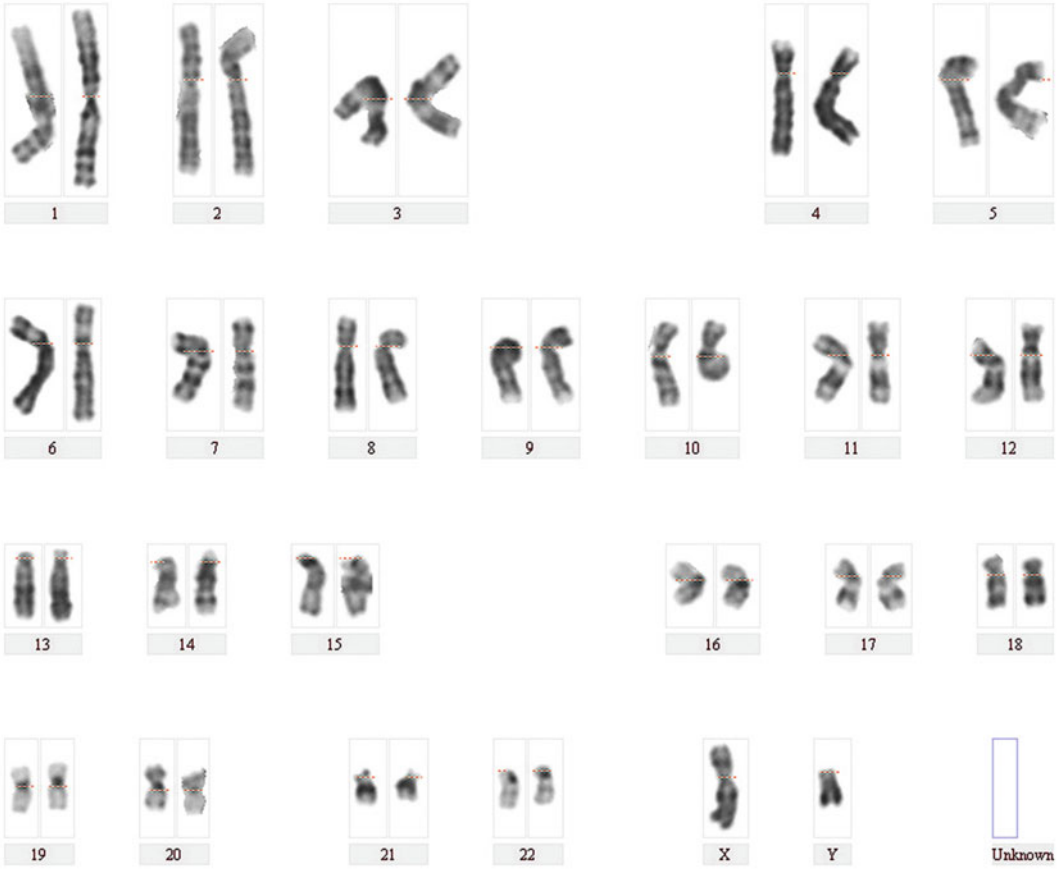

Fig. 5 Karyotyping analysis on the adipose tissue-derived stromal cells demonstrated that the cells were grossly normal at third subculture

16. Provide a single-cell suspension using pipette flow.
17. Transfer the cell suspension into 50 ml conical tubes.
18. Add PBS to falcon tubes.
19. Spin tubes at 300 × *g* for 5 min at 20 °C.
20. Transfer tubes into flow cabinet and remove and discard supernatant (*see* **Note 15**).
21. Resuspend cell pellets in complete culture medium by gently pipette flow.
22. Count resuspended cells and calculate their viability and purity using trypan blue (1:1dilution) and hemocytometer. Document all parameters according to SOPs (*see* **Notes 1** and **2**).
23. Aliquot cells into the labeled 175 cm^2 flasks (see **Note 17**).
24. Prepare a 25 cm^2 flask for karyotyping (Fig. 5).
25. Prepare cells from second or third subcultures for cryopreservation (*see* **Note 5**).

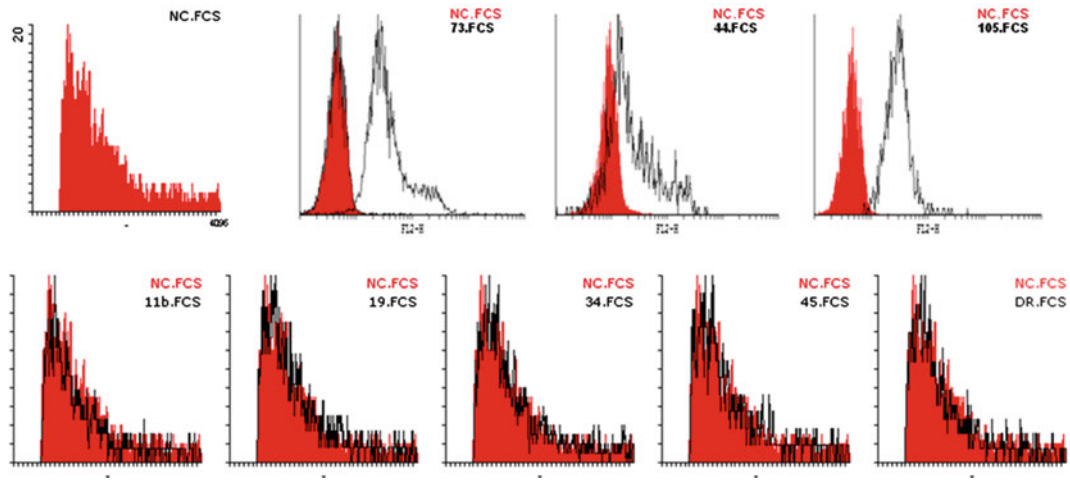

Fig. 6 Cell surface markers of ADSCs. Histogram analysis of cell surface markers. ADSCs are positive for CD73, CD44, and CD105, and negative for CD11b, CD19, CD34, CD45, and HLA-DR

3.4 Characterization of Cultured ADSCs

1. Flow cytometric analysis should be performed to evaluate cell surface markers (CD markers) (Fig. 6) (*see* **Note 18**).
2. Differentiation potential for adipogenic and osteogenic differentiation should be evaluated (Fig. 7) (*see* **Note 18**).

3.5 Cryopreservation

1. Based on obtained ADSCs, estimate amount of freezing medium (40 % DMEM, 50 % FBS, and 10 % DMSO) (*see* **Notes 19** and **20**).
2. Label appropriately cryo vials (*see* **Note 12**).
3. Harvest cultured ADSCs using enzymatic technique (TrypLE select) and the same protocol for passaging.
4. Wash cell pellet with DMEM.
5. Spin cell suspension at 250 × *g* for 5 min at 20 °C.
6. Remove and discard supernatant and save cell pellet.
7. Transfer the labeled cryo freezing container, cryo vials, and DMEM (40 %)-FBS (50 %) mixture into an ice tray and then place them in the cleaned (with 70 % ethanol) laminar air flow cabinet (*see* **Note 21**).
8. Resuspend cell pellet gently using pipette flow in DMEM (40 %) and FBS (50 %).
9. Add DMSO (10 %) into cell suspensions.
10. Fill each cryo vial with 1 ml of cell suspension with freezing Medium (*see* **Note 22**).
11. Transfer cryo vials into the cryo freezing container.
12. Transfer cryo freezing container (containing cryo vials) into the ultralow temperature freezer for 24 h.

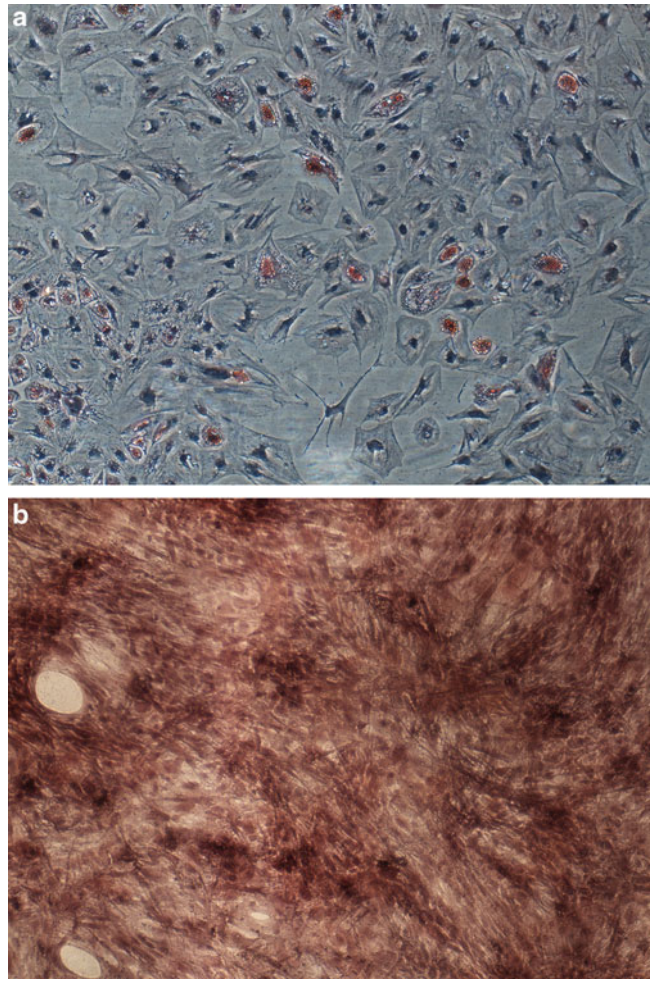

Fig. 7 Light inverted microscopy photographs of ADSCs induced to differentiate into different lineages. (**a**) Adipogenic differentiation is demonstrated by Oil Red O staining. (**b**) Osteogenic differentiation is demonstrated by Alizarin Red staining

3.6 Banking and Releasing for Clinical Transplantation

1. After 24 h, transfer cryo vials into a liquid nitrogen tank for long-term storage (in the vapor phase) (*see* **Note 23**).
2. After final inspection by the director, release ADSCs for transplantation (*see* **Notes 24–26**).

4 Notes

1. To achieve a reasonable safety and to avoid contamination, immune reaction, and undesirable events in clinical applications of ADSCs, all manufacturers should implement a quality management system (QMS) in their organization by adhering to the principles of cGMP guidelines and appropriate quality

assurance programs including organization and personnel, standard operating procedures (SOPs), facilities, environmental control, equipment monitoring, supplies and reagents, process controls and changes, process validation, labeling design and control, storage requirements, records, tracking, nonconformances and complaints management, risk assessment, reporting and reviewing, documentation for all procedures, record keeping, education, and training (25, 26).

2. For all the procedures, standard operating procedures (SOPs) should be written and accessible for relevant staff and personnel to conduct the procedures independently, with minimal oversight (18).
3. Be careful, adipose tissue after retrieval should be processed as soon as possible. Although several methods showed the feasibility of ADSCs isolation after 6, 12, and 24 h or more from tissue retrieval, in clinical grade and GMP scale-up, long ischemic time can affect cell viability, potency, and quality and consequently, patient's outcome.
4. The clean room facility must be monitored for air change, temperature, humidity, and sterility. Sterilization process must be done according to documented SOPs.
5. The manufacturing processes should be kept to a minimum to decrease the risk of manipulation as contamination (27).
6. For GMP-grade preparation of cells, you should avoid animal-origin supplements if it is possible (1), if not, some GMP-grade, clinical-grade, and pharma-grade reagents, enzymes, and serums are available.
7. As terminal sterilization of cells is not possible, the quality of raw materials, medium, instruments, and everything that comes into direct contact with the product should be check more seriously.
8. Collagenase NB6 GMP grade is designed for human tissue digestion and cell isolation in clinical cellular therapy trials. cGMP guidelines can guarantee the stability, reproducibility, traceability of final product batches, and also viral and TSE safety.
9. The body of literature and previous studies have suggested various collagenase concentration and also different methods for centrifugation.
10. To achieve a good result and higher cell quality, during enzymatic digestion shake the tubes in a water bath.
11. After tissue digestion and centrifugation, three different phases can be seen in the tube: adipose portion, liquid portion, and SVF.
12. Labeling must be performed by a trained staff who is responsible for documentation and processing procedures. The label

should include donor's unique code, date of processing and cell isolation, and passage number (25).

13. The European Medicines Agency (EMEA) recommends that "when manufacturers have a choice the use of materials from 'non transmissible spongiform encephalopathy (TSE) relevant animal species' or non-animal origin is preferred" (6). In addition, the Australian Therapeutic Goods Authority (TGA) (equivalent of the FDA in the USA) permits the use of FBS for clinical-grade cell manufacturing as long as its source is from an area free of BSE such as Australia or New Zealand (28). Accordingly, FBS Pharma Grade is produced according to regulations and guidelines that comprise the EMEA guideline 1793/02 of the committee for proprietary medical products (CPMP), EMEA guideline 743/00 of the committee for veterinary medical products (CVMP), the Ph. Eur. (European Pharmacopoeia) current edition monograph of Bovine Serum (2262), and US Code of Federal Regulation (9CFR). Furthermore, an extensive test panel of mycoplasma and ten different viruses, 35 kGy effective gamma irradiation, as well as biochemical and cell culture parameters can minimize the risks.
14. Ensure that the base of all flasks is covered with culture medium. Also in clinical and GMP scale-up, you should have some backup flasks for each case in another incubator with a separate CO_2 supply.
15. Microbiological tests should be performed for aerobic, anaerobic, and fungi prior, during, and after cell processing and culture. Furthermore, prior to clinical application, the director must check the results of Mycoplasma and endotoxin tests.
16. TrypLE™ Select is a serum-free and nonanimal-derived enzyme with similar kinetics and cleavage specificity to trypsin. On the other hand it has significantly better stability than trypsin, gentle on cells, stable at room temperature, and easy to use. TrypLE™ Select is manufactured at a cGMP-compliant facility registered with the US Food and Drug Administration.
17. We usually expand around 8×10^3 cells per cm^2 (1.4×10^6 cells per each 175 cm^2 flask) at cell passages. Accordingly, we obtain around 1×10^6 ADSCs from 1 ml processed pure adipose tissue.
18. A most uniform characterization criteria is needed. The mesenchymal and tissue stem cell committee of the international society for cellular therapy (ISCT) proposes minimal criteria to define human mesenchymal stem cells including ADSCs; (1) these cells must be plastic-adherent, (2) must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules, (3) must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro (29).

19. CryoSure-DMSO USP-Grade is sterile, pyrogen-free, endotoxin-free, and also free of mycoplasma.
20. Although DMSO is relatively nontoxic but the use of frozen-thawed cells for clinical application can cause some adverse effects and toxic reactions such as cytotoxicity and undesired differentiation to cardiac or neuronal-like cells. Accordingly, removal of DMSO prior to clinical transplantation is strictly necessary. However, it is too difficult and time consuming to remove it totally; thus, formulating a cryopreservation protocol using lower concentrations of DMSO is crucially important in a GMP and clinical-grade scale-up. Several investigations have recommended the minimal concentrations of DMSO for cryopreservation (2–10 %) (1).
21. Cryo freezing container should be cleaned and filled with isopropyl alcohol before placing cryo vials into it.
22. Ensure that freezing procedure be carried out quickly.
23. Some samples should be thawed after cryopreservation for stability test and evaluating the potency of frozen cells and also validating the cryopreservation procedures.
24. Each added supplement must be omitted from the final cell product according to a validated method.
25. Final product must be specified for several parameters at least sterility, cell count, purity, viability, transfer medium, stability throughout the isolation, storage, and shipping process (27).
26. The final inspection prior to release for cell transplantation includes checking of sterility (gram stain and 2-week microbiology culture), purity, viability, Karyotype, Mycoplasma, and endotoxin.

Acknowledgement

This work was supported by Brain and spinal Cord Injury Research Center, Tehran University of Medical Sciences (research grant # 85/2160). The authors would like to acknowledge Dr. Abbas Norouzi-Javidan, Dr. Seyed Majid Manavi, Dr. Ramin Heshmat, and Dr. Mohsen Khorshidi for their considerable assistance. We also thank Hanieh Rostam Abadi, Shokouh Salimi, Faezeh Nouraei, and Maryam Kavousi.

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Methods in Molecular Biology (2015) 1283: 109–122
DOI 10.1007/7651_2014_105

Published online: 10 August 2014

cGMP-Compliant Transportation Conditions for a Prompt Therapeutic Use of Marrow Mesenchymal Stromal/Stem Cells

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Abstract

We recently described conditions for safe 18-h manufacturer-to-patient transportation of freshly harvested hBM-MSC expanded under cGMP protocols using human platelet lysate (hPL), that allowed prompt use as an advanced therapeutic medicinal product. Here we outline important considerations when comparing different transportation conditions, highlighting that although cell transportation may involve a reduction in viability, this did not undermine the ultimate bone-forming regenerative potential of the cGMP-hBM-MSC population.

Keywords: MSC, Transportation, Fresh, Platelet lysates, Saline solution, cGMP-compliant, Bone regeneration

1 Introduction

Successful outcomes using multipotent stromal cells derived from the human bone marrow, coined human Mesenchymal Stromal/Stem cells (hBM-MSC), in ex vivo and in vivo preclinical studies have encouraged clinical trials wherein the entire cell manufacturing process, from the tissue isolation, to the amplification phase to the delivery in patients must comply with current good manufacturing practice (cGMP). The discrepancy between the limited hMSC yield obtainable from a bone marrow harvest (typically 2.7 –8 million cells within the first 20 mL of aspirated tissue biopsy) (1) and current estimates of the number required for a clinical dose (typically >100 million or 1–3 million MSC/kg) can be resolved by ex vivo cell expansion under controlled conditions (2). The final cell number harvested for therapeutic purposes can be influenced by both

*Author contributed equally with all other contributors.

isolation procedure (3) and cell culture microenvironment (4) constituting an advanced therapy medical product (ATMP).

In Europe, these procedures are subject to Regulation (EC) No. 1394/2007 on Advanced Therapies with guidelines requiring specialized facilities optimized for maintaining the highest quality environment within which to grow the autologous cells (5). Relatively few Universities and Hospitals are currently equipped with suitable on site local cell-expansion facilities and safe transportation enables multicentric clinical trials, thereby facilitating recruitment of selected populations for longitudinal studies. As pharmaceutical companies introduce centralized large-scale manufacture of autologous cell-derived products (6), guidance with regard to a safe ATMP transportation protocol is likely to become increasingly important when isolated viable stem cells become accepted advanced therapy medical products (7). Autologous therapeutic strategies require transportation of both the initial bone marrow biopsy from which the hBM-MSC are derived and the subsequent final ATMP. Compared to the successful transportation of blood transfusion products, requirements for the safe transportation of ATMP are less well defined. Even so, cell context-dependent differences are apparent, since the majority of fresh blood products and sterile BM samples benefitted from transportation at room temperature (8, 9), whereas the recommendation for isolated peripheral blood progenitor cell products at high cell concentrations ($>200 \times 10^9$/L) was transportation at 4 °C (10). Given that inappropriate cGMP-hBM-MSC transportation risks undermining the whole treatment process, it is important to carefully evaluate the relevant procedures.

Protocols adopted for short-term shipment of viable cells between laboratories can vary (11), nonetheless the main consideration is to preserve cell viability, differentiation, and secretion potential for all processes including isolation from tissues, population expansion, transportation, and application. Although cryopreservation effectively stabilizes cells (12) and there are permissible cryoprotective reagents that avoid adverse host reactions (13), freshly thawed hBM-MSC need acclimatization before full function is restored (14) and this may be problematic for a prompt therapeutic use. In particular, our clinical procedures for osteogenic therapy required prompt adhesion of freshly arrived cGMP-hBM-MSC on osteoconductive biomaterial one hour before co-administration. Thus, beyond cell viability, we needed to explore the influence of transporting nonfrozen ATMP on cell adhesion. Overall, the factors influencing our protocol for transportation of expanded cGMP-hBM-MSC cultures included the cell preservation, need for prompt cell adhesion to a supportive scaffold upon arrival and concern for retaining the function of osteoblastic differentiation and bone formation. The selectable procedures involved included: (1) Harvest method and transfer to the

shipment vessel, (2) Transportation medium, (3) Cell concentration, (4) Holding Temperature, and (5) Duration of Shipment.

Methods for propagation and harvest of cGMP-BM-MSC (2) include use of TrypZean® (Sigam-Aldrich) or TrypLE™ (Life Technologies) as animal origin-free alternatives to traditional bovine or porcine Trypsin for disassociating adhered cells from the culture surface, thus avoiding xenobiotic risk. Trypsin treatment may also be substituted through use of a temperature-responsive poly (*N*-isopropylacrylamide) platform, recently shown to be appropriate for use with mesenchymal stem cells (15). Use of a CE-labeled syringe made of low cell-adhesion gas-impermeable polypropylene conformed to guidelines for a sterile small volume vessel that was readily disposable and of low toxicity (16). Moreover, a syringe allowed prompt cell application upon arrival without need for additional instrumentation and conveniently enabled exclusion of air bubbles from the transport buffer before shipment.

A cell transportation medium incorporating growth factors would require re-definition of the ATMP constituents and introduce a potential clinical risk requiring further assessment. This introduced a compromise between admissible transportation buffer ingredients and inclusion of factors that could help sustain optimal cell performance. Minimizing complexity, we compared transportation buffers of injectable sterile saline solution (0.9 % NaCl v/v) versus saline plus 4 % human serum albumin (HSA) as an admissible reagent that could help improve liquid storage of marrow stromal cells (17). The outcome of comparing a saline only transportation buffer with one containing 4 % HSA was to some extent surprising, since ex vivo HSA introduced a slight impairment of adhesion to the 3D-scaffold, yet better subsequent proliferation upon subsequent culture. Notably however, following osteogenic differentiation of transportation buffer-exposed cells grown on 3D-scaffold, Von Kossa staining to detect mineralization could be more intense in cells exposed to the saline-only transportation buffer. In these conditions, although sample size was relatively small, the use of HSA in the transportation buffer did not provide a significant advantage with regard to the amount of bone formed in experimental xenografts (18). Although a relatively high cell density for cGMP-hBM-MSC transportation could induce cell aggregation, MSC were found in the bone marrow as cell aggregates (19, 20) and ex vivo, cell clustering can promote viability (21). Pilot studies confirmed that a cell density of 20×10^6 cells/mL cGMP-hBM-MSC for 18 h of storage in saline was crucial for maintaining the regulatory requirement of over 70 % viability for clinical use, since a tenfold dilution led to an inadequate viability of only about 50 %. Notably, cell density influenced the flow behavior of a concentrated stromal cell suspension (22) and slow ejection to minimize damage from shear forces was used when preparing a nonaggregated cell suspension for seeding the osteoconductive

scaffold, adopting cell trituration procedures shown not to reduce cell adhesion (23).

Although room temperature is preferred for the transportation of whole blood (24), cultured hBM-MSC stored in suspension for extended periods beyond 1 h become increasingly dependent on a low storage temperature to maintain more than 80 % viability (25–27). Thus complying with cold chain management to maintain a transportation temperature close to 4 °C is an important consideration resembling needs for vaccine shipment (28) with additional concerns regarding vibration during transportation (29). Favorable consequences of hypothermic storage, including a reduced metabolism and lower oxygen demand to increases ischemic tolerance, are compromised by the negative stressful reactions to cold. There is a principal need to maintain a normal isotonic osmotic balance whereby extruded ions counteract the osmotic pressure from intracellular macromolecules and organelles. However, this can be compromised by the hypothermically-induced outer membrane lipid phase transition that inactivates adenosine triphosphate (ATP) driven Na^+/K^+ or Mg^{2+}/Ca^{2+} ionic pumps. Transportation conditions within a sealed syringe can exacerbate cold stress-induced conformational changes that impair citric acid cycle enzymes, since anaerobic respiration produces less ATP. Low-level glycolytic activity and disrupted oxidative phosphorylation make the cells prone to produce lactic acid, reducing the threshold level of glucose that can cause intracellular acidosis and cellular degradation. Cytoskeletal support involves temperature dependent chemical bonds that can be weakened by cold, rendering the cell more vulnerable to mechanical damage. Increased levels of intracellular calcium from ionic leakage can activate calcium dependent proteases and phospholipidases further damaging the cytoskeleton. Hypothermia in the absence of an optimized preservation solution that does not mimic the hyperosmotic situation of low temperature cells leads to an ionic imbalance. Subsequent ionic leakage, compensating for an increased intracellular osmolality can lead to cell swelling and cumulative time-dependent edemic damage that is subsequently made evident by cells dying when they are returned to their normal physiological temperature (30). Given that normal saline, mimics normothermic extracellular fluid that is high in sodium and low in potassium compared to intracellular fluid, it was perhaps surprising that most of our cGMP-hBM-MSC survived the hypothermic transportation conditions. However, survival was also cell concentration dependent, perhaps not only because cell aggregates may more readily achieve a higher extracellular osmolarity, but also because hBM-MSC can spontaneously secrete protective factors such as the extracellular antioxidant superoxide dismutase-3 (SOD3) (31) that may contribute to the improved recovery of cells kept at 4 °C (32).

Clinically safe solutions specifically formulated for hypothermic conditions include HypoThermosol®-FRS, that has intracellular-type

ionic concentrations to more closely balance the altered cellular ion concentrations at hypothermic temperatures, as well as impermeant molecules to help redress osmotic imbalances, low levels of glucose to limit cellular acidosis yet provide some source of energy during/after cold storage and HEPES buffer that remains effective at low temperatures. It has been shown to be very effective at preserving hBM-MSC kept at 4 °C for at least 4 days (33), yet full evaluation of bone-forming potential remains to be tested. Its protective effect can in certain contexts be improved by the addition of the chelating agent EDTA or anti-apoptotic molecules (34). The emerging consensus is that the cell stress response needs to be considered as very context specific, reflecting precise cell type, isolation methods, culture medium storage solution, and duration, with a potential for improvement that stands to benefit from further molecular analysis (35).

The shipment time tested was that deemed reasonable for overnight shipment by courier service as confirmed by experience between European laboratories within the Reborne Framework 7 EU consortium. Within 18 h the loss of cell viability remained well within the 70 % viability limit required by regulations, nonetheless this indicated that the cells were undergoing stress. Although concern focuses on cell preservation, the introduction of stress during transportation before implantation might not be entirely detrimental. It provides a preconditioning period that can trigger survival signaling-pathways in the MSC that may be subsequently beneficial for engraftment in vivo (36, 37). Since hypoxic preconditioning can induce secretion of protective factors (38, 39), it was noteworthy that we implanted the entire contents of the transported syringe and the term ATMP includes the potential involvement of all cellular products. Our observation that normal saline alone may suffice as a cost-effective transportation solution for hypothermically preserved cGMP-hBM-MSC is advantageous. Its simpler formulation lends itself to more robust manufacture than HypoThermosol®-FRS or organ perfusion solutions such as Viaspan® and normal saline for injection has an extensive clinical history. It remains to be formally demonstrated whether hypothermic transportation in normal saline provides an optimal degree of preconditioning stress for a positive contribution to bone formation but given that each additional component to a therapeutic protocol can introduce a risk, it already provides a significantly safe baseline for clinical trials.

2 Materials

All solutions described below were prepared using sterile conditions under a HEPA-filtered vertical laminar-flow cabinet at room temperature. 70 % ethanol was used as disinfectant and gloved hand aseptic manipulation was used throughout the preparation of the

expansion culture medium and the reagents needed for the cell transportation. Cells were cultured in 5 % CO_2 humidified 37 °C incubators.

2.1 cGMP-hBM-MSC Culture Conditions

1. ® CellSTACK® culture chambers manufactured under GMP conditions provided a convenient low-maintenance vessel for monolayer culture, minimizing risk of culture contamination. The two layer chambers had a total surface area of 1,272 cm^2 with cell growth monitored by phase-contrast microscopic observation of the lower culture surface.
2. Culture Medium: The sodium bicarbonate buffered basal medium used for culture of the cGMP-hBM-MSC, MEM-α (Gibco® Life Technologies, Lonza or Macopharma) required a 5–10 % CO2 environment to maintain a physiological pH (*see* **Note 1**).
3. When used, Ciprofloxacin (HIKMA) was stored as aliquots at −20 °C until addition to the culture medium at a final concentration of 0.5 % v/v (*see* **Note 2**).
4. L-Glutamine (Gibco® Life Technologies) was stored as aliquots at −20 °C and added to the medium at a final concentration of 1 % v/v (*see* **Note 3**).
5. Heparin Sodium (B. Braun) 5,000 I.U./mL was dissolved in sterile ddH_2O and added at a final concentration of 0.02 % (*see* **Note 4**).
6. For consistent culture conditions between laboratories and to allow multicentric clinical trials, batches from pooled human Platelet Lysate (hPL) were distributed from a central source prepared from pooled batches of hPL from 50 donors (*see* **Note 5**). The final concentration of hPL in the culture medium was 8 % (*see* **Note 6**).

2.2 Cell Harvest and Transfer to the Transportation Syringe

1. Cells were washed prior to enzymatic dissociation from the CellSTACK® culture surface with 200 mL Phosphate Buffered Saline, (PBS) pH 7.2 (PAA) replacing the vented cap with a solid cap whilst treating the cells with the air-buffered PBS for about 15 s (*see* **Note 7**).
2. After removing the PBS, the cells were detached using TrypZean® (Sigma-Aldrich) (*see* **Note 8**).
3. Sterile 0.9 % Normal Saline (NS) for injection was sourced from USP (Baxter) or from S.A.L.F. Spa; Laboratorio Farmacologico) 308 mOsm/L, pH 7.0.
4. For experiments exploring the outcome of adding serum albumin (HSA) in the transportation medium, HSA was purchased from CSL Behring or Kedrion and diluted to a concentration of 4 % in NS (*see* **Note 9**).

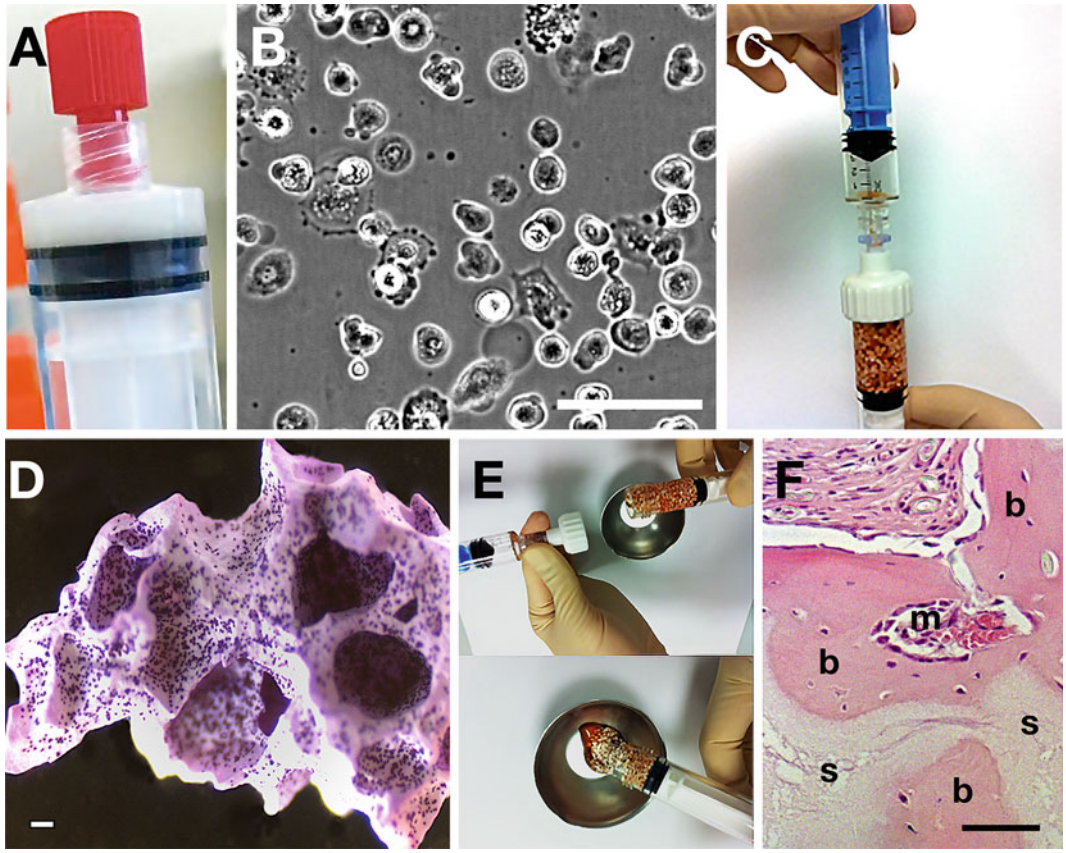

Fig. 1 Transportation conditions of cGMP-hBM-MSC for use in osteogenic therapy. (**a**) A white cell pellet of 200×10^6 cells transported in a minimal volume of 5 mL in a 20 mL syringe sealed with a screw to Luer Lock cap. (**b**) Phase-contrast photomicrograph of cGMP-hBM-MSC placed into culture after 18 h in NS at 4 °C at a concentration of 20×10^6 cells/mL. Within 20 min cells were already observed adhering to the plastic culture surface. (**c**) A female Luer lock adapter was used to transfer cells directly from the upper transportation syringe to the lower syringe used to transport the 3D scaffold MBCP+™ biomaterial. For the purpose of image clarity, the cells were suspended in phenol red-containing medium. (**d**) After the 1 h incubation period with MBCP+™ crystal violet staining revealed cells adhered to the 3D scaffold granule surface. (**e**) Removing the male Luer cap of the syringe facilitated application of the ATMP cell/biomaterial slurry. For the purpose of image clarity, the cells were suspended in phenol red-containing medium. (**f**) A representative histological section of pink stained osteoid bone (b) formed on *gray areas* of demineralized scaffold (s) surrounding marrow-like regions (m) after cGMP-hBM-MSC subjected to NS transportation conditions were tested for bone forming potency in a 6-week immune compromised mouse model. For (**b**), (**d**), (**f**) bar = 100 μm

5. Risk of contamination was kept to a minimum by using CellSTACK® compatible MacoPharma cell culture seeding, medium exchange and passage kits to maintain a cGMP grade closed system within the ISO7 clean room environment before the final packaging of the cells for distribution in a 20 mL syringe (B. Brown) (Fig. 1a).

2.3 Transportation of the MBCP+™ 3D Scaffold Biomaterial

1. MBCP+™ (Biomatlante) is a 100 % synthetic (no human or animal origin) biphasic ceramic composed of 20%HA (Hydroxy apatite) and 80 % β-TCP (Tricalcium Phosphate Beta) with a complete interconnected porosity of 70 %, with 2/3 macropores >10 μm and 1/3 micropores, that has been extensively used in the clinic for its known osteoconductive properties.
2. The e-beam sterilized 14 mL open-ended barrel transportation syringe was fitted with a silicon tip plunger and sealed with a removable male Luer Lock fitted with a female Luer Lock thread cap. A typical clinical dose of 2.5 g of Biomatlante MBCP+™ 1–2 mm Ø granules occupied a total volume of 5 mL within the syringe. The syringe was designed with wing grips and detachable male Luer cap that helped deliver the eventual cell/MBCP + ™ slurry constituting the ATMP.

2.4 Combining Cells with 3D Scaffold Biomaterial

1. Prompt combination of cells with MBCP+™ granules upon arrival in the operating theater was achieved by having the dedicated Biomatlante 14 mL biomaterial-transporting syringe sealed by a female Luer thread cap that could be replaced by a female Luer adaptor allowing direct connection to the cGMP-hBM-MSC-containing 10 or 20 mL transportation syringe (B. Braun), without resorting to needles or lengthy connection tubing (Fig. 1c) (*see* **Note 10**).

3 Methods

The following protocol describes the procedures for growing cGMP-hBM-MSC, their transportation conditions and subsequent combination with an osteoconductive scaffold biomaterial for direct clinical use in bone regeneration.

3.1 Expansion of Bone Marrow-Derived hBM-MSC

1. Bone marrow-derived hBM-MSC from cGMP facilities; Etablissement Français du Sang, Toulouse (France), Institute of Clinical Transfusion Medecine and Immunogenetics, Ulm (Germany) and Cell Factory, Fondazione IRCCS Ca´Granda Ospedale Policlinico, Milan (Italy) were isolated and expanded to single clinical doses of at least 100×10^6 cGMP-hBM-MSC using a two-step protocol.
2. The 5 % CO_2-buffered culture medium was prepared under sterile flow cabinet conditions at room temperature. To alpha-MEM was added Na-Heparin, Glutamine and hPL in that order, ensuring hPL was preceded by heparin to avoid its coagulation.
3. To improve consistency within a multicentric trial, cGMP-hBM-MSCs were cultured in centrally produced hPL pooled from 50 donors.

4. Unprocessed bone marrow cells were seeded with an initial density of 50,000 white blood cells/cm^2 in 300 mL complete medium in 2-level CellStack™ (Corning) tissue culture chambers in medium containing 5 % hPL.
5. After 3 days of allowing the cells to settle and adhere to the culture surface, supernatant with nonadherent was removed and 300 mL of freshly prepared culture medium was added to each 2-level CellSTACK® chamber. Medium was fully replaced twice a week.
6. After 10 days, the cells were rinsed with PBS and harvested using TrypZean®, accordingly named passage 0 and promptly seeded at a density of 4,000 hBM-MSC/cm^2 in 2-level CellStack™ chamber vessels, this time using a slightly higher concentration of hPL (8 %) with a final concentration of 1 i.u. Na-heparin per mL. The culture medium was replaced twice per week.
7. After a 5 or 7-day period the cells were rinsed with PBS and harvested by treating the cells at 37 °C in the 5 % CO_2/95 % humidified air incubator for about 4 min with 50 mL TrypZean® per 2-level CellSTACK® chamber. When microscopic observation confirmed 75 % of the cells were detached, the TrypZean® treatment was continued for a final 2 min incubation before adding an equal volume of kept supernatant cell culture medium.
8. The cellular suspension was collected by gravity into sterile 50 mL tubes that were centrifuged at 400 × *g* at room temperature for 10 min.
9. The supernatant was removed from the cells gently using a sterile pipette leaving about 3 mL of supernatant in each tube with which to resuspend the cells sequentially transferring the pooled cells into one 50 mL tube. An additional 3 mL of culture medium was used to serially rinse the tubes and complete the pooled cell harvest in the final tube.
10. The cells were washed in NS 4 % HSA transportation buffer and counted with a sample removed for cell counting and quality control tests.

3.2 Transport of cGMP-hBM-MSC from Manufacture Site to Clinic

1. Certificates of analysis, conformity, and compliance were obtained from all suppliers for each apparatus and reagent used in cell manufacture. Release criteria included microbial contamination tests include measuring endotoxin levels using Limulus Amebocyte Lysate (LAL) assays and antibody-based analysis of pathogenic viruses. The final product should have endotoxin levels $\leq$0.25 EU/mL and be negative for tests including Hepatatis A virus (HAV), Human Immunodeficiency virus (HIV), Hepatitis B virus (HBsAg), Hepatatis C virus

(HCV), Cytomegalovirus (CMV), Syphilis (*Treponema pallidum* bacterium).

2. Enumeration of cell proliferation plus a test for colony forming unit fibroblast (CFU-F) efficiency was accompanies by a FACS based analysis of surface antigens, CD45, CD105, CD90, CD73, CD34, HLA-DR with release criteria requiring a cell viability greater than 80 %, expression of canonical mesenchymal antigens (CD105, CD90, and CD73) in over 90 % of the cells and less than 5 % positivity for the haematopoietic cell antigen CD45.
3. Each 20 mL transportation syringe contained cGMP-hBM-MSC cell suspension of 200×10^6 cells in 5 mL of transportation buffer (*see* **Note 11**).
4. Transportation syringes were packaged promptly in a sterile steel container and maintained in a horizontal position during +4 °C shipment monitored by a data logger.

3.3 Transport of MBCP+™ Biomaterial from Manufacture Site to Clinic

1. The e-beam sterilized 14 mL open-ended barrel transportation syringe was loaded with 2.5 g gamma sterilized MBCP+™ granules occupying a volume of approximately 5 cm^3 for transportation at ambient temperature.
2. No pre-treatment of the granules before addition of the cell suspension was required.

3.4 Combining cGMP-hBM-MSC with Scaffold Granules Before Surgery

1. This procedure needed to be performed successfully before surgery could commence.
2. An additional female Luer Lock adaptor was used to connect the vessel containing cGMP-hBM-MSC to the 14 mL sterile syringe directly.
3. The connected syringes were placed in a vertical position, the syringe containing the cells uppermost. By gently pulling on the plunger of the syringe containing 3D scaffold biomaterial, the cell suspension was drawn into the syringe with the biomaterial.
4. Keeping the syringes connected, the plunger of the lower syringe was adjusted so that all the biomaterial well mixed with the cell suspension and saturated without void air pockets.
5. The conjoined syringes were left to stand for one hour vertically to allow cell adhesion to the 3D scaffold.

3.5 ATMP Delivery

1. After the 1-h cell adhesion period, the male Luer cap of the syringe containing the ATMP was removed allowing unimpeded free-flow of the cell/biomaterial slurry from the syringe (Fig. 1e) that assisted application to the diphyseal fracture site in the tibia or femur.

4 Notes

1. This modification of Minimum Essential Medium (MEM) was made with Earle's salts and contained amino acids, sodium pyruvate, lipoic acid, Vitamin B12, Biotin, Ascorbic Acid, phenol red, and 1,000 mg/L Glucose.
2. Ciprofloxacin is an infusion grade quinolone antibiotic effective against a wide variety of Gram-negative anaerobic bacteria, some Gram positive organisms and mycoplasma as well as Penicillin-Streptomycin resistant bacteria that may be found in laboratories where this more common antibiotic combination is used to help prevent cell culture contamination. It was used as a precaution in pre-clinical experiments, however to avoid any risk of side effects was omitted from cultures destined for clinical use.
3. L-Glutamine is not as stable as other amino acids in culture and it was beneficial to add it fresh, using aliquots to avoid freeze–thaw cycles of the stock solution.
4. It was important to reconstitute the culture medium by adding heparin before hPL in order to prevent gel formation. It is noteworthy that higher concentrations of heparin can introduce deleterious effects with regard to hMSC proliferation and differentiation.
5. The precise protocol for the closed system procedures to produce hPL from pooled batches of apheresis platelet concentrates deemed not valid for transfusion and analysis of its major growth factor components influencing hBM-MSC growth is described in detail elsewhere (40). For those without centralized sources, yet able to obtain platelet lysate from local hospital transfusion, we have obtained equivalent growth from platelet concentrates ranging from 5.5×10^8 to 1.1×10^9 (median 7.75×10^8, $n = 20$), subsequently used at a final concentration of 5×10^7/mL. It was better to first freeze the platelet concentrate apheresis bag at −80 °C before thawing at 4 °C overnight before use. The freeze–thaw lysed platelet concentrate could then be isolated by filtration. We found that stepwise incremental filtration through 0.8, 0.45, and 0.22 μm filters was preferred to single step filtration, to avoid blockage of the 0.22 μm filter. Also Polyethersulfone (PES) membranes with a highly asymmetrical pore structure offered a high "dirt" loading capacity, increasing the filtration performance to allow higher throughputs and higher flow rates.
6. The two-step procedure whereby the cells are grown in 5 % hPL for the first 10 days and subsequently in 8 % hPL for the final expansion phase of 5 days was arrived at after experiments testing alternative hPL concentrations, including two-step

procedures using a constant 10 % hPL, but using such higher hPL concentrations did not provide a greater cell yield.

7. Pre-washing in Calcium and Magnesium free PBS removed spent medium, cell wastes and lowers salt concentrations that could otherwise impair trypsin activity.
8. TrypZean® is a recombinant bovine trypsin expressed in corn thus without animal contaminants potentially present from animal pancreas sources or expression of recombinant molecules in animal cells. When exploring different transportation conditions in scaled-down experiments, we also found that cell dissociation equivalent to that of Trypsin/EDTA, was obtained by TrypLE™, an animal-free pure reagent that is stable at room temperature and also gentle on cells with the advantage that unlike Trypsin/EDTA it could be used without need for enzyme activity blocking reagents.
9. When exploring different transportation conditions in scaled-down experiments, we compared HSA from Kedrion with HSA from CSL Behring, in each case diluted to a concentration of 4 % in NS. In most cases near-equivalent performance was obtained regardless of manufacturer. Nevertheless, the extent of Von Kossa stained biomineralization obtained from 3D cultured cells treated with osteogenic culture medium, was lower than controls after cells had experienced transport buffer with 4 % HSA from CSL Behring. Moreover, the ultimate outcome of bone formation was not necessarily improved by the use of HSA, thus NS alone may suffice as a usefully simple transportation buffer when cells are at a high concentration and kept cold.
10. When performing scaled-down experiments to test different transportation conditions, the same cell concentration was maintained using one fifth of the total clinical dose in a smaller 5 mL syringe, i.e. 20×10^6 cells in 1 mL.
11. For ease of handling with a reduced risk of contamination it was pragmatically advantageous to use a generously sized 20 mL syringe despite the small volume to be transported. It was essential to make sure all void air was expunged from the syringe before transportation.

Acknowledgments

We are very grateful to the members of the European consortium REBORNE in providing the cGMP grade cells used to set up the protocol, in particular to Rosaria Giordano, Philippe Burin, and Hubert Schrezenmeier. We thank Biomatlante that provided the MBCP+™ scaffolds. This work was supported by a grant

(HEALTH-2009-1.4.2-241879) from the 7th Framework Programme of the European Commission REBORNE (REgenerating BOne defects using New biomedical Engineering approaches).

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Methods in Molecular Biology (2015) 1283: 123–136
DOI 10.1007/7651_2014_101

Published online: 05 August 2014

GMP-Grade Human Fetal Liver-Derived Mesenchymal Stem Cells for Clinical Transplantation

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Abstract

Stem cell therapy seems a promising avenue in regenerative medicine. Within various stem cells, mesenchymal stem cells have progressively used for cellular therapy. Because of the age-related decreasing in the frequency and differentiating capacity of adult MSCs, fetal tissues such as fetal liver, lung, pancreas, spleen, etc. have been introduced as an alternative source of MSCs for cellular therapy. On the other hand, using stem cells as advanced therapy medicinal products, must be performed in compliance with cGMP as a quality assurance system to ensure the safety, quality, and identity of cell products during translation from the basic stem cell sciences into clinical cell transplantation. In this chapter the authors have demonstrated the manufacturing of GMP-grade human fetal liver-derived mesenchymal stem cells.

Keywords: Fetal stem cells, Good manufacturing practice, Karyotyping, Mesenchymal stem cells, Therapeutic abortion

1 Introduction

The essential purpose of regenerative medicine is to restore the normal structure or function of damaged cell, tissue, or organ (1). Stem cell research is one of the novel technologies in regenerative medicine that promises many benefits for humanity (2, 3). Accordingly, mesenchymal stem cells (MSCs) have increasingly introduced for cellular therapy in recent years. Almost every tissue can be used as the source of MSCs from adults and fetuses (4, 5). Even though bone marrow is the most common used tissue, the number of bone marrow derived MSCs and also their differentiation capacities are limited. Therefore, alternative sources have been nominated by investigators. On the other hand, the age-related decreasing in the frequency and differentiating capacity of adult MSCs, has proposed several fetal tissues including liver, bone marrow, umbilical cord tissue and blood, lung, spleen, pancreas, etc. from legally aborted fetuses (6–9). Moreover, fetal stem cells have some advantages over their adult counterparts as greater differentiation, homing, and engraftment potency while lower immunogenicity (10, 11).

According to the mentioned properties resulted from several basic and preclinical investigations, human fetal MSCs, are ideal candidates for clinical stem cell transplantation trials. Throughout the translation from the basic or preclinical researches to the clinical-grade stem cell manufacturing and advanced cellular therapy, accurate definition and implementation of all procedures and resources in a quality management and assurance system including extensive validation, process control and documentation is compulsory (12–14). Accordingly, MSCs that are considered as advanced therapy medicinal products (ATMPs) by the European Medicines Agency (EMEA) must be manufactured in compliance with current Good Manufacturing Practices (cGMP) as a quality assurance system to ensure the sterility, safety, potency, quality, and all of preset specification (12, 15). On the other hand, the Food and Drug Administration (FDA) uses a risk-based approach to regulate cell-based products in two classes: minimally manipulated (cryopreservation, thawing, density gradient isolation, washing, and dilution) and effectively or more than minimally manipulated (ex vivo expansion and genetic modification). Minimally manipulated production should follow the current Good Tissue Practice (cGTP) guidelines, while effectively or more than minimally manipulated cell manufacturing should follow the cGMP guidelines, in addition to cGTP (16, 17). The purpose of this chapter is to demonstrate GMP-grade human fetal liver-derived mesenchymal stem cells (hFL-MSCs) manufacturing for cellular therapy.

2 Materials

2.1 Fetal Liver Harvesting, Trimming, Cell Isolation, Culture, Subculture, Cryopreservation, and Banking

1. Clean room (GMP) facility (*see* **Notes 1–3**).
2. Sterile clean room garments (hood, face masks, coveralls, powder-free gloves, and boots).
3. Laminar vertical air flow cabinet (biological safety cabinet).
4. Weighing balance (Sartorius, Germany).
5. Sterile hand homogenizer (Sartorius, Germany).
6. Centrifuge (Swing-out rotor with buckets for 50 and 15 mL tubes).
7. Optical inverted microscope equipped with phase-contrast option.
8. CO2 Incubator (set at 5 % CO_2, 37 °C, and humidified).
9. Ultralow temperature freezer.
10. Liquid nitrogen tank (Statebourne Cryogenics, UK).
11. Nalgene™ cryo freezing container (Cat. No. 5100–0001, Thermo Scientific, USA).
12. NucleoCounter® NC-100TM (Chemometec, Denmark).

13. Sterile stainless steel surgical tray.
14. 100 mm petri dish (TPP, Switzerland).
15. Sterile scissors and tweezers.
16. Motorized pipette (Orange Scientific, Belgium).
17. Pipette (TPP, Switzerland).
18. 0.2 μm sterile and endotoxin-free syringe filter (Orange Scientific, Belgium).
19. 70 μm cell strainer (Becton Dickinson, USA).
20. Falcon tubes 15, 50 mL (TPP, Switzerland).
21. Filter cap cell culture flasks (175, 75, and 25 cm^2) (TPP, Switzerland).
22. 70 % ethanol (Merck, Germany).
23. Povidone–iodine.
24. Phosphate buffer saline (PBS) (CliniMACS®, Miltenyi Biotec, Germany).
25. Ficoll-Paque™ PREMIUM (GE Healthcare Life Sciences, USA).
26. Dulbecco's Modified Eagle Medium—low glucose 1 g/100 mL (DMEM-LG) (PAA, Austria).
27. Fetal bovine serum (FBS) (Cat. No. A15-512, Lot. No. A51210-2738, Pharma grade, Australian origin and gamma irradiated, PAA, Austria).
28. TrypLE Select (recombinant trypsin-like substitute) (Invitrogen, USA).
29. Trypan blue solution 0.4 % (Invitrogen, USA).
30. CryoSure-Dimethyl Sulfoxide (DMSO) USP Grade (WAK-Chemie, Germany).
31. Cryo vial (Greiner Bio-One, Germany).

3 Methods

The entire processing procedures including fetal liver harvesting, tissue trimming, tissue disruption, cell isolation, and the rest of processes should be performed in a laminar vertical air flow cabinet which is located in a GMP (clean room) facility and compatible with the relevant quality assurance guidelines (*see* **Notes 1–3**).

3.1 Controls Prior to Adipose Tissue Processing

1. All fetuses must be procured from therapeutic and legally abortions at the gestational age of 6–12 weeks after obtaining informed consent, in accordance with ethical committee approval and therapeutic abortion act, local and national legislation, regulations, and guidelines (*see* **Notes 4** and **5**).

2. Donor eligibility (mother) should be appraised using medical history and complete physical examination.
3. Check and record abortion time.
4. Put the donated fetus in a sterile container.
5. Obtain a blood sample from the mother at the abortion time for serological tests (HBV antibody, HCV antibody, HIV-1, 2, HTLV-1-2, and RPR for syphilis). Additionally, nucleic acid tests should be done for HIV and HCV.
6. After procurement, transfer the fetus to the cGMP facility (*see* **Note 6**).
7. Check all accompanying documents (e.g., written informed consent, past medical history, physical examination, permission for therapeutic abortion) (*see* **Note 7**).
8. Check the container at arrival time for appropriate labeling, probably leakage, volume of transfer medium, maternal blood sample, and write all data according to documentation SOPs (*see* **Notes 7** and **8**).
9. Put the container in a home refrigerator till processing time (*see* **Note 6**).
10. Provide all documents, labeling and coding requirements prior to processing in accordance with organization specific SOPs (*see* **Notes 7** and **8**).
11. Ensure that environmental parameters are within their preset ranges and have been documented according to SOPs (*see* **Notes 1** and **2**).
12. Ensure that all equipment servicing and maintenance programs have been performed and documented perfectly (*see* **Note 7**).
13. Ensure that the facility, equipment (e.g., laminar vertical air flow cabinet, CO_2 incubator), and working area have been cleaned and/or sterilized in accordance with the organization specific SOPs (*see* **Note 7**).
14. Turn on the laminar air flow cabinet 15 min before starting medium preparation and processing.
15. Prepare all enzymes, culture mediums, and reagents prior to processing.

3.2 Fetal Liver Preparation

1. Record starting time of processing according to documentation SOPs (*see* **Note 7**).
2. Spray 70 % ethanol on the outer side of the fetus container and open the container in the laminar air flow cabinet and transfer the fetus onto a sterile surgical tray.
3. Take a sample of transfer medium for microbiological testing.
4. Weight the fetus and record it.

5. Rinse it with 5 % povidone–iodine solution once and follow washing with PBS two times.
6. Put it onto another sterile surgical tray.
7. Harvest the fetal liver by a midline laparotomy following subcostal extensions (*see* **Note 9**).
8. Place the liver in a 100 mm petri dish and remove the adjacent tissues carefully.
9. Cut the liver into small pieces using tweezers and scissors.

3.3 Liver Tissue Disruption and Mononuclear Cells (MNCs) Isolation

1. Transfer the liver small pieces into a hand homogenizer.
2. Disrupt the liver pieces by homogenizer gently.
3. Wet cell strainer (70 μm) with PBS before use.
4. Pass the disrupted tissue through 70 μm cell strainer to remove connective tissues adding PBS–EDTA (Ethylenediaminetetraacetic acid, 1 millimolar per 1 mL PBS (CliniMACS®)) (*see* **Note 10**).
5. Dilute filtered suspension with the PBS–EDTA.
6. Carefully layer 7 mL of diluted cell suspension over 3 mL of Ficoll-Paque PREMIUM in 15 mL Falcon tubes (*see* **Note 11**).
7. Spin at 500 × *g* for 30 min at 22 °C in a swing-out rotor without brake.
8. Aspirate the upper layer leaving the mononuclear cells (MNCs) layer intact (Fig. 1).
9. Carefully collect the MNCs layer using 10 mL pipette.
10. Transfer the MNCs at the interphase to a 50 mL Falcon tubes.
11. Wash the cells by adding up to 45 mL of PBS.

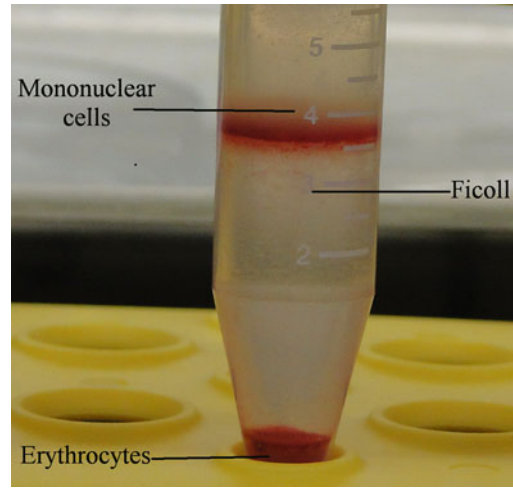

Fig. 1 Isolation of mononuclear cells from human fetal liver by density gradient. Mononuclear cells phase, ficoll, and erythrocytes phase can be seen

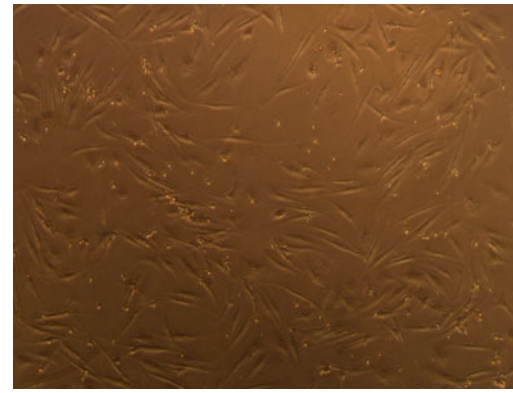

Fig. 2 Morphology of hFL-MSCs. Light inverted microscopy photographs of cells in culture medium. Magnification ×200

12. Centrifuge at 300 × *g* for 5 min at 22 °C.
13. Remove supernatant gently.
14. Count the cells by NucleoCounter.

3.4 Fetal Liver-Derived MSCs Culture and Subcultures

1. Place 75 cm^2 filter cap flasks into laminar vertical air flow cabinet.
2. Insert an appropriate label on each flask (*see* **Note 8**).
3. Transfer around 1×10^4 cells per cm^2 into each flask (75×10^4 in a 75 cm^2 filter cap flask).
4. Add complete culture medium (DMEM supplemented with 15 % FBS Pharma Grade) into each 75 cm^2 flask up to 12 mL (*see* **Note 12**).
5. Put the flasks in the incubator (37 °C, 5 % CO_2, humidified) (*see* **Note 13**).
6. 48 h later, wash and discard the culture medium containing non-adherent cells and then renew complete culture medium.
7. Renew culture medium every 72 h regularly.
8. Throughout the culture period, check the morphology of cells (Fig. 2) and also culture medium for medium color changes, bacterial contamination, unsought morphology changes, and cellular death.
9. Perform microbiological evaluation before cell isolation, during and also after cell expansion.
10. At approximately 80 % confluence, transfer the flasks into laminar vertical air flow cabinet.
11. Collect and discard all culture medium.
12. Carefully wash adherent cells with PBS–EDTA.
13. Collect and discard PBS–EDTA solution.

14. Add 3 mL of TrypLE Select to each 75 cm^2 flask (*see* **Note 14**).
15. Transfer flasks into CO_2 incubator (set at 37 °C) and leave them horizontally for around 10–12 min.
16. Check flasks for cell detachment during incubation period.
17. Transfer the flasks into laminar air flow cabinet following cell detachment.
18. Provide a single cell suspension using pipette flow.
19. Transfer cell suspension into 50 mL conical tubes.
20. Add PBS to cell suspension up to 45 mL.
21. Spin at 300 × *g* for 5 min at 22 °C.
22. Transfer tubes into safety cabinet and remove supernatant gently.
23. Resuspend cell pellet in culture medium by pipette flow softly.
24. Count resuspended cells and estimate cell viability and purity using trypan blue 0.4 % solution (1:1 dilution) and hemocytometer.
25. Confirm the mentioned parameters by using NucleoCounter.
26. Record all parameters according to documentation SOPs (*see* **Note 7**).
27. Aliquot cells into the labeled 75 cm^2 flasks (see **Note 8**).
28. Provide 25 cm^2 flasks for karyotyping and characterization (Fig. 3) (*see* **Note 15**).
29. At 2^{nd} or 3^{rd} subcultures prepare cultures cells for cryopreservation.

3.5 Characterization of Cultured hFL-MSCs

1. Immunophenotypic characterization of hFL-MSCs should be performed to evaluate cell surface markers (CD markers) by flow cytometry (*see* **Note 16**).
2. The differentiation potential of hFL-MSCs can be provided by using differentiation specific mediums to induce osteogenic and adipogenic differentiation (Fig. 4) (*see* **Note 16**).
3. Perform Reverse transcriptase polymerase chain reaction (RT-PCR) to appraise pluripotency gene expression (e.g., Oct4, Nanog, Sox2, and Rex1) (Fig. 5).

3.6 Cryopreservation

1. Prepare freezing medium (40 % DMEM, 50 % FBS, and 10 % DMSO) (*see* **Notes 17** and **18**).
2. Insert appropriate labels on cryo vials (*see* **Notes 8** and **19**).
3. Harvest cultured hFL-MSCs at second or third subculture using the same protocol for passaging.
4. Wash cell pellet adding DMEM (low glucose) and using pipette flow gently.

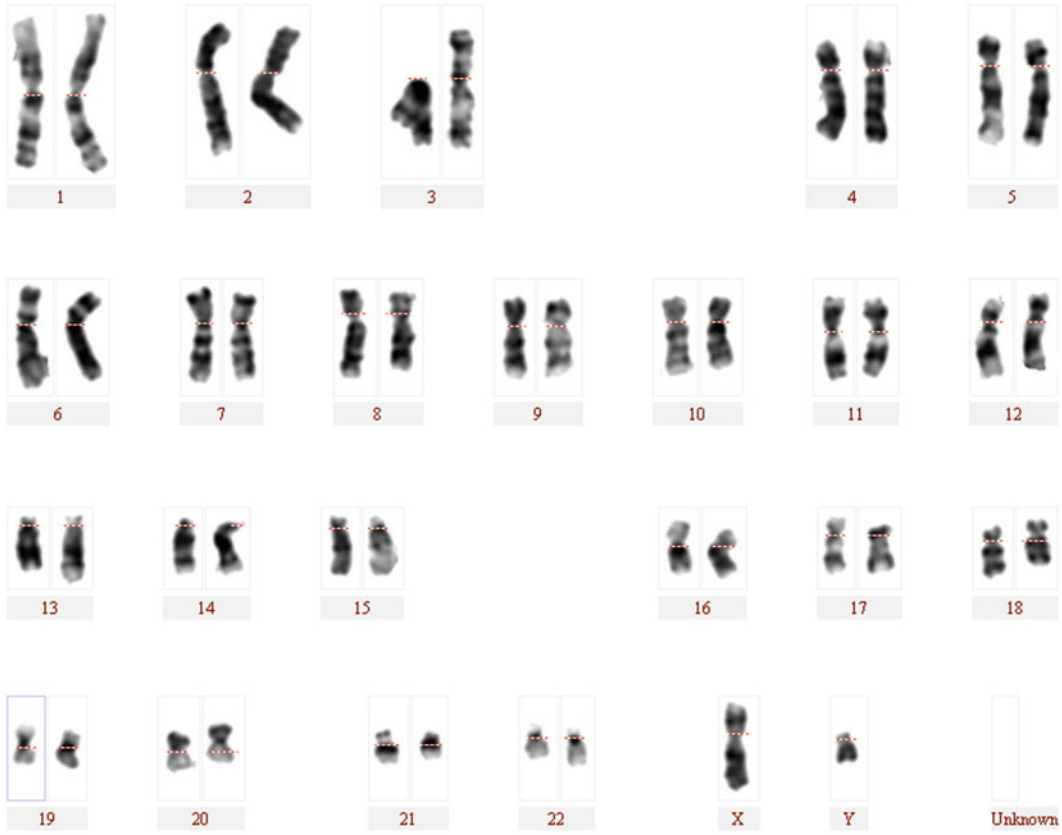

Fig. 3 Karyotyping analysis on the hFL-MSCs demonstrated that the cells were grossly normal at 10th subculture

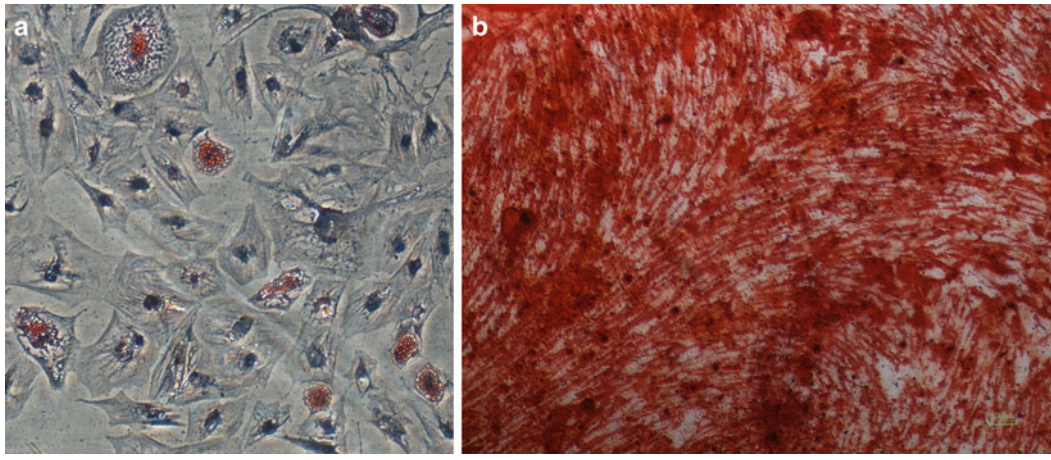

Fig. 4 Light inverted microscopy of hFL-MSCs induced to differentiate into different lineages. (**a**) adipogenic differentiation is demonstrated by Oil Red O staining. (**b**) osteogenic differentiation is demonstrated by Alizarin Red staining

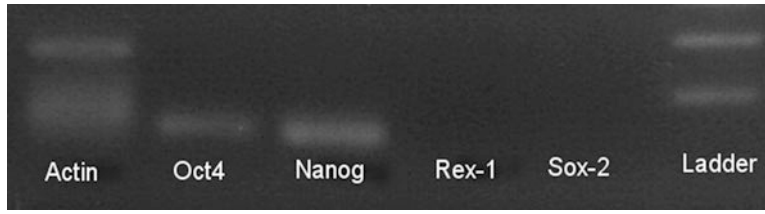

Fig. 5 Expression of Oct-4 and Nanog and lack of expression of Rex1 and Sox2 as pluripotency markers has been demonstrated by reverse transcription-polymerase chain reaction

5. Spin cell suspension at 300 × *g* for 5 min at 22 °C.
6. Collect and discard supernatant and save cell pellet.
7. Transfer the labeled cryo freezing container, cryo vials, and DMEM (40 %)–FBS (50 %) mixture into an ice tray and then, place them in the cleaned laminar air flow cabinet (*see* **Note 20**).
8. Resuspend cell pellet in DMEM (40 %)–FBS (50 %) mixture using pipette flow softly.
9. Count resuspended cells using trypan blue solution and hemocytometer.
10. Add DMSO (10 %) into cell suspensions very slowly (*see* **Note 18**).
11. Estimate cell count per 1 mL of freezing medium.
12. Fill each cryo vial with 1 mL of suspended cells in freezing medium.
13. Record number of cryo vials and cell count in accordance with documentation SOPs (*see* **Note 7**).
14. Put cryo vials in the cryo freezing container (it must be cleaned and filled with isopropyl alcohol before transferring cryo vials into it).
15. Place cryo freezing container (containing cryo vials) into the ultralow temperature freezer and leave them for 24 h.

3.7 Banking and Releasing for Clinical Transplantation

1. 24 h later, transfer cryo vials into the vapor phase of liquid nitrogen tank, for long-term storage.
2. Add the specification of preserved vials precisely to the list of stocked cell products according to documentation SOPs (*see* **Note 7**).
3. Before releasing preserved hFL-MSCs for clinical applications, the director must check relevant documentations as final inspection (*see* **Note 21**).
4. Ensure that stem cell product is washed carefully to remove all reagents prior to transplantation (*see* **Note 22**).

Fig. 6 Automated clean room environmental controls for air pressure, humidity, and temperature

4 Notes

1. Clean room facility is the most tangible aspect of cGMP with controlled temperature, humidity, easily cleanable surfaces, and a positive pressured air supply filtered through high efficiency particulate air (HEPA) filters (18, 19).
2. The clean room facility must be monitored for air pressure, temperature, humidity (Fig. 6), and sterility. Particle counting must be done regularly according to the clean room facility maintenance, monitoring, and documentation SOPs.
3. An air shower isolates processing area from other unclassified spaces (support room, gowning and scrubbing area) (Fig. 7).
4. In Iran, induced abortion is permitted only when mother's life is threatened (therapeutic abortion) or the fetus is diagnosed with a disease or defect (14, 20). The therapeutic Abortion Act was signed by the Iranian parliament on June 21, 2005. This Act allows therapeutic abortion after a distinct diagnosis by three experts and confirmation by the legal medicine organization. It also covers cases of fetal diseases and anomalies leading to afflictions for the mother. Additionally, these circumstances must be carried out prior to ensoulment (fourth month of gestational age), with the mother's consent (21).
5. The gestational age should be estimated from the first day of the mother's last menstrual period (LMP) and also ultrasonography reports.
6. After fetus procurement, start the liver processing and cell isolation as soon as possible. Ischemic time (from the tissue retrieval to processing and cell isolation) up to 12 h to achieve a suitable viability, potency, and rest of preset specification.

Fig. 7 Air shower; isolates classified processing area from other unclassified spaces (e.g., support room, gowning, and scrubbing area)

7. Translation from the basic stem cell sciences to the clinical cellular therapies needs a higher level of safety in a quality management and assurance framework such as cGMP. cGMP covers all of stem cell manufacturing processes including organization, facilities, machines and instruments, man power and staff, standard operating procedures (SOPs), environmental control and monitoring, equipment maintenance, supplements and reagents, process controls, validation, labeling and tracking design and control, storage requirements, documentation and records, non-conformances and complaints management, risk management, reporting and reviewing, audit plan, education and training (19, 22–24).
8. A labeling, coding, and traceability system must be implemented in the cGMP facility (22).
9. You can harvest other organs (e.g., lung, pancreas) if you need (Fig. 8).
10. For GMP-grade manufacturing of hFL-MSCs, you need to use xeno-free supplements if it is possible (25), if not some GMP grade, clinical grade, and pharma grade reagents are commercially

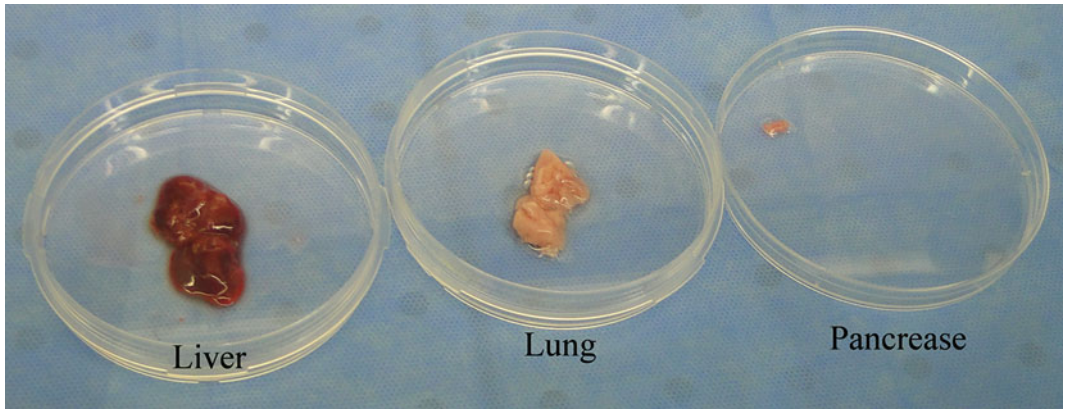

Fig. 8 Human fetal liver, lung, and pancreas have been harvested through laparotomy

available. When the clinical grade reagents and supplements are not available, the manufacturer should provide sufficient documentation about the supplement production to minimize probable risks.

11. Ficoll-Paque^{TM} PREMIUM is a sterile and non-cytotoxic density gradient medium which is suitable for clinical-grade isolation of MNCs and mesenchymal stem cell expansion (26). It is manufactured in accordance with GMP and ISO 13485 standards and the recommendations of the US Pharmacopeia with low levels of endotoxin (<0.12 EU/mL).
12. According to the EMEA recommendation, "non-transmissible spongiform encephalopathy (TSE) relevant animal species" or xeno-free supplement is preferred' (27). Accordingly, FBS Pharma Grade is manufactured in accordance with the EMEA guideline 1793/02 of the committee for proprietary medical products (CPMP), EMEA guideline 743/00 of the committee for veterinary medical products (CVMP), the Ph. Eur. (European Pharmacopoeia) current edition monograph of Bovine Serum (2262), and US Code of Federal Regulation (9CFR). In addition, an extensive test panel of ten different viruses and mycoplasma follows by 35 kGy effective gamma irradiation can minimize the probable risks.
13. Provide some backup flasks for each case in another incubator with a separate CO_2 supply.
14. TrypLE™ Select is a serum-free and also xeno-free counterpart of trypsin which is manufactured at a cGMP compliant facility registered with the US Food and Drug Administration.
15. Karyotyping should be performed for each batch of stem cell at the same passage number. Ensure that the karyotype is normal prior to release for clinical transplantation.

16. The mesenchymal and tissue stem cell committee of the international society for cellular therapy (ISCT) has suggested a minimal criteria to define human MSCs including fetal MSCs as: (a) these cells must be plastic-adherent, (b) must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules, (c) must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro (28).
17. CryoSure-DMSO USP Grade is sterile, pyrogen-free, endotoxin-free, and also free of mycoplasma.
18. Remove DMSO prior to clinical transplantation. Because, the use of frozen-thawed cells for can cause cytotoxic effects and also unsought differentiation to cardiac or neuronal like cells. In addition, a cryopreservation protocol using lower concentrations of DMSO is increasingly recommended (25).
19. Ensure that all cryo vials as final product container are sterile and their components are non-pyrogenic, non-cytotoxic, DNA-free, DNase and RNase free.
20. Stem cell freezing procedure must be carried out quickly.
21. The medical director must execute final inspection prior to releasing stem cell product for clinical application (Checking of sterility, cell count, purity, viability, karyotype, mycoplasma; and endotoxin tests) (29).
22. Remove all added supplement from the final product according to a validated method.

Acknowledgement

This work was supported by Chronic Diseases Research Center, Endocrinology and metabolism research institute, Tehran University of Medical Sciences. The authors would like to thank Dr. Ramin Heshmat, Dr. Seyed Majid Manavi, Dr. Mohsen khorshidi, Dr. Mohamad Reza Amini, and Shokouh Salimi for their considerable assistance.

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Methods in Molecular Biology (2015) 1283: 137–145
DOI 10.1007/7651_2014_115

Published online: 24 December 2014

Purification of Human Induced Pluripotent Stem Cell-Derived Neural Precursors Using Magnetic Activated Cell Sorting

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Abstract

Neural precursor (NP) cells derived from human induced pluripotent stem cells (hiPSCs), and their neuronal progeny, will play an important role in disease modeling, drug screening tests, central nervous system development studies, and may even become valuable for regenerative medicine treatments. Nonetheless, it is challenging to obtain homogeneous and synchronously differentiated NP populations from hiPSCs, and after neural commitment many pluripotent stem cells remain in the differentiated cultures. Here, we describe an efficient and simple protocol to differentiate hiPSC-derived NPs in 12 days, and we include a final purification stage where Tra-1-60$^+$ pluripotent stem cells (PSCs) are removed using magnetic activated cell sorting (MACS), leaving the NP population nearly free of PSCs.

Keywords: Human induced pluripotent stem cells, Neural commitment, Magnetic activated cell sorting, Neural precursors, Cell purification

1 Introduction

Human induced pluripotent stem cells (hiPSCs) have been recognized as a very powerful tool and numerous applications have been suggested for hiPSC derivatives in the fields of disease modeling (1), drug screening (2), and autologous cell therapy (3). The ability to restore pluripotency to somatic cells, through ectopic co-expression of reprogramming factors (4), permitted the indefinitely expansion and differentiation of these cells into any specialized cell of the human body, without raising ethical issues associated to the manipulation and destruction of human embryos. However, differentiation protocols designed to derive the preferred specialized cells are often deficient and heterogeneous cell populations result from these attempts. Furthermore, in many cases, a significant amount of hiPSCs remains in those differentiated cell populations, which is perturbing because these cells are potentially tumorigenic after transplantation (5). In addition to the risk of teratoma generation, in advanced phases of the differentiation

protocols, hiPSCs can alter the results of in vitro experiments: their presence might influence culture development by compromising differentiation processes or might alter the response of the targeted cells to certain drugs, making it more difficult to interpret the results. Thus, further utilization of hiPSC derivatives would greatly benefit if non-differentiating hiPSCs were removed as early in the differentiation process as possible. This preparative hiPSC depletion would avoid the burden of maintaining and expanding undesired hiPSCs, would expedite scientific discovery, by diminishing heterogeneity and variations between experiments, and would potentially allow a safer application of hiPSC derivatives in cell therapy settings.

Here, we describe an efficient protocol for the generation of hiPSC-derived neural precursors (NPs) that includes a final purification stage, where Tra-1-60$^+$ pluripotent cells are removed using magnetic activated cell sorting (MACS), and resulted in a purer and viable NP cell population with interest for a number of biomedical and regenerative medicine applications. The neural commitment culture system to generate Pax6$^+$ and Nestin$^+$ NPs from hiPSCs has been extensively validated under adherent culture conditions (6–8) and depends on the synergistic action of two SMAD signaling inhibitors—SB431542 and LDN193189—which promote neural conversion of >80 % hiPSCs in culture after 12 days. Finally, since MACS suits for positive and/or negative selection, has a high-throughput, requires no expensive equipments, and is easy to operate (9), this cell separation method can be used to deplete hiPSC-derived populations of Tra-1-60$^+$ PSC after neural commitment.

Knowing that many central nervous system disorders in humans are currently lacking effective treatments, the application of this protocol should facilitate the use of hiPSC-derived NPs in disease modeling, drug screening and, eventually, in functional in vivo integration.

2 Materials

2.1 Reagents

1. 70 % Ethanol (Sigma).
2. Phosphate buffered saline (PBS) solution: dissolve (1×) PBS powder (Life Technologies) in 1 L of distilled water and filter the solution using a 0.22 μm filter and store at room temperature.
3. Accutase solution (Sigma).
4. Trypan blue stain 0.4 % (Gibco).
5. MACS buffer: dilute 0.5 % bovine serum albumin (Invitrogen) and 2 mM EDTA (Gibco) in PBS.

6. Anti-Tra-1-60 MicroBead kit (Miltenyi Biotec): anti-Tra-1-60-PE antibody and anti-PE MicroBeads.
7. Monoclonal mouse IgM-PE antibody (Miltenyi Biotec).
8. Poly-L-ornithine (Sigma).
9. FACSFlow sheath fluid (BD Biosciences).
10. Knockout serum replacement (KO-SR, Invitrogen).
11. Dimethyl sulfoxide (DMSO, Gibco).

2.2 Laboratory Supplies and Required Instruments

1. Cryogenic vials (BD Biosciences).
2. 6-well cell culture plates (BD Biosciences).
3. Polypropylene 15-mL conical tubes (BD Biosciences).
4. FACS tubes (BD Biosciences).
5. KOVA glasstic slide 10 with grids hemocytometers (Hycor).
6. 0.22 μm filters (VWR).
7. LS MACS columns (Miltenyi Biotec).
8. 30 μm nylon mesh filters (Miltenyi Biotec).
9. MidiMACS separator magnet (Miltenyi Biotec).
10. MACS multistand (Miltenyi Biotec).
11. Inverted phase contrast microscope (10×, 20×, and 40× objectives, Olympus CKX31).
12. Micropipette (1–10, 10–100, 100–1,000 μL).
13. Electronic pipettor (Kartell).
14. Laminar flow hood (Bioair Instruments, MAC 2000).
15. Freezing container (Nalgene).
16. Liquid nitrogen tank (StateBourne, Biorack 3000).
17. Cell culture incubator with temperature and CO_2 control (Memmert).
18. Temperature adjustable water bath (Memmert).
19. Cell culture centrifuge (Humle 2400K).
20. Flow cytometer Becton Dickinson Biosciences FACSCalibur.
21. CellQuest software (Becton Dickinson).

2.3 Solutions and Media

1. Matrigel (BD Biosciences).
2. Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F12, Gibco).
3. Y-27632 ROCK inhibitor (StemGent).
4. mTeSR1 hiPSC maintenance medium (Stem Cell Technologies).
5. KO-DMEM/SR medium: 80 % knockout-DMEM (Gibco), 20 % KO-SR (Invitrogen), 1 % MEM-nonessential amino acids

(Gibco), 1 mM L-glutamine (Gibco), 0.1 mM ß-mercaptoethanol (Sigma), and 1 % penicillin/streptomycin (Gibco).

6. DMEM/F12/N2 medium: 97 % DMEM/F12 (Gibco), 1 % N2 supplement (Gibco), 1.6 g/L glucose (Sigma), 1 % penicillin/streptomycin (Gibco), and 20 μg/mL insulin (Sigma).
7. LDN193189 (StemGent).
8. SB431542 (Sigma).
9. Mouse laminin (StemGent).
10. Recombinant human fibroblast growth factor-basic (bFGF, Peprotech).

2.4 hiPSC Line

The hiPSC-line (iLB-C1-30m-r12) used to develop this protocol (6) was derived from foreskin fibroblasts of healthy donors, at Prof. Oliver Brüstle's laboratory (Institute of Reconstructive Neurobiology, University of Bonn, Germany).

3 Methods

3.1 hiPSC Thawing and Expansion Culture Conditions

1. One day before thawing cells, thaw one matrigel aliquot of 200 μL on ice at 4 °C.
2. Dilute Matrigel (1:30) in DMEM-F12 and incubate 1 mL/well of a 6-well plate for at least 2 h at 37 °C.
3. Retrieve a cryogenic vial of hiPSCs from the liquid nitrogen tank and quickly thaw it in a 37 °C water bath (*see* **Note 1**).
4. Use 5 mL of pre-warmed KO-DMEM/SR medium to dilute the content of the cryogenic vial and resuspend cells in a 15-mL conical tube.
5. Centrifuge at 1,000 rpm for 5 min, discard the supernatant, and resuspend the pellet in 1.5 mL of mTeSR1 culture medium supplemented with 10 μM of Y-27632 ROCK inhibitor.
6. Plate thawed cells in one matrigel-coated well (9.6 cm^2) from a 6-well plate.
7. Incubate cells at 37 °C and 5 % CO_2 in a humidified atmosphere.
8. Replace 1.5 mL/well of pre-warmed mTeSR1 culture medium every day without ROCK inhibitor.
9. During expansion conduct single-cell passages (*see* Section 3.2) when cells are 80 % confluent.

3.2 hiPSC Single-Cell Passaging on Matrigel

1. Incubate cells at 37 °C for 1 h with 1 mL/well KO-DMEM/SR medium containing 10 μM Y-27632 ROCK inhibitor.

2. Discard the medium and incubate cells at 37 °C for 5–7 min with 1 mL/well accutase (*see* **Note 2**).
3. Tap cell culture plate gently and add 2 mL/well KO-DMEM/SR medium containing 10 μM Y-27632 ROCK inhibitor.
4. Flush cells from the bottom of the wells and collect them into a 15-mL conical tube.
5. Repeat the washing step once more and centrifuge cells at 1,000 rpm for 5 min.
6. After removing the supernatant resuspend cells in KO-DMEM/SR medium containing 10 μM Y-27632 ROCK inhibitor and count cells using the trypan blue dye exclusion method.
7. Discard the supernatant and resuspend cells in the appropriate amount of pre-warmed mTeSR1 culture medium supplemented with 10 μM Y-27632 ROCK inhibitor and plate 20,000 cells/cm^2 in 1.5 mL/well of medium onto matrigel-coated wells.
8. Incubate cells at 37 °C and 5 % CO_2 in a humidified atmosphere.
9. Replace 1.5 mL/well of pre-warmed mTeSR1 culture medium every day without ROCK inhibitor.

3.3 Neural Commitment of hiPSC

1. Start by performing an hiPSC single-cell passaging and replace mTeSR1 medium every day until cells become approximately 80 % confluent (*see* **Note 3**).
2. When hiPSCs reach the appropriate confluence replace mTeSR1 by 2 mL/well of pre-warmed KO/DMEM-SR medium supplemented with 10 μM SB431542 and 100 ηM LDN193189 every day for 4 days.
3. From the 5th day until the 11th day change medium every 2 days and supplement it with 100 ηM LDN193189. Gradually add DMEM/F12/N2 medium to the final medium formulation until day 11 following this rule: 75 % + 25 % on day 5, 50 % + 50 % on day 7, 25 % + 75 % on day 9, and 0 % + 100 % on day 11, of KO-DMEM/SR and DMEM/F12/N2 media, respectively.

3.4 Depletion of hiPSC Using Magnetic Activated Cell Sorting (MACS)

1. After neural commitment of hiPSC wash cells once with PBS by replacing medium with 2 mL/well of PBS.
2. Aspirate PBS gently, add 1 mL/well accutase, and incubate cells at 37 °C for 7 min.
3. Tap cell culture plate gently, add 2 mL/well KO-DMEM/SR medium, and smoothly flush cells.

4. Collect cells into a 15-mL conical tube and repeat the previous step until all cells are suspended.
5. Dissociate to single-cell suspension by pipetting up and down using a 1,000 μL pipette.
6. Centrifuge at 1,000 rpm for 4 min, discard the supernatant, resuspend up to 10^6 cells in 100 μL of MACS buffer, and add 10 μL of anti-Tra-1-60 antibody.
7. Incubate the cells for 10 min in the dark at 4 °C (*see* **Note 4**).
8. Add 2 mL of MACS buffer, pipette up and down using an electronic pipettor, and centrifuge at 1,000 rpm for 4 min.
9. Discard the supernatant, resuspend cells in 80 μL of MACS buffer, and add 20 μL of anti-PE MicroBeads.
10. Incubate for 15 min in the dark at 4 °C.
11. Add 2 mL of MACS buffer, pipette up and down using an electronic pipettor, and centrifuge at 1,000 rpm for 4 min.
12. Prepare the MACS setup for cell separation while cells are centrifuging by placing an LS MACS column in the MidiMACS separator magnet, add the 30 μm nylon mesh filter to the top of the column and rinse with 5 mL of DMEM/F12/N2 medium (*see* **Note 5**).
13. Discard the supernatant, vigorously resuspend cells in 500 μL of MACS buffer with a 1,000 μL pipette, and apply cell suspension into the filter mesh on top of the column, having the column reservoir empty and a 15-mL conical tube below the column to collect cells.
14. Wash the column 3 times with 3 mL of DMEM/F12/N2 medium by adding medium when the reservoir gets empty (*see* **Note 6**).
15. Gently retrieve the column from the magnet, place it onto other 15-mL conical tube, add 5 mL of DMEM/F12/N2 medium, remove filter, and quickly flush out the magnetically labeled cells by firmly pushing the LS MACS column plunger into the reservoir.

3.5 Replating of the MACS-Purified Neural Precursors (NPs)

1. Prepare the appropriate number of cell culture plate wells in the previous day by incubating with 2 mL/well of 15 μg/mL poly-L-ornithine at 37 °C overnight (*see* **Note 7**).
2. Wash well plates once with 2 mL/well PSB and incubate with 20 μg/mL mouse laminin diluted in PBS (1 mL/well) for 3–4 h at 37 °C.
3. Centrifuge the 15-mL conical tube with the LS MACS column 9 mL washed fraction at 1,000 rpm for 5 min, discard the supernatant, resuspend cells in pre-warmed DMEM/F12/N2

medium supplemented with 100 ηM LDN193189, and count cells using the trypan blue dye exclusion method.

4. Remove laminin from wells.
5. Discard the supernatant and resuspend cells in the appropriate amount of pre-warmed DMEM/F12/N2 medium supplemented with 100 ηM LDN193189 and plate 300,000 cells/cm^2 onto laminin-coated wells in 2 mL/well of medium.
6. Incubate cells at 37 °C and 5 % CO_2 in a humidified atmosphere.
7. After 24 h aspirate conditioned medium and dead cells, withdraw LDN193189, and add 2 mL/well of pre-warmed DMEM/F12/N2 medium supplemented with 20 ηg/mL bFGF.
8. Replace bFGF DMEM/F12/N2-supplemented medium every 2 days.
9. At this stage cells can be cryopreserved, used for phenotype characterization assays or further differentiated using other neuronal differentiation protocols (10–12).

3.6 Flow Cytometry Analysis of MACS-Purified Fractions

1. Take a sample from cell suspension obtained after step 5 in Section 3.4.
2. Prepare this sample as the negative control for flow cytometry in parallel with samples to be purified by MACS (steps 6–11 of Section 3.4) but replace anti-Tra-1-60-PE antibody by 10 μL monoclonal mouse IgM-PE antibody in step 6.
3. Take a sample with at least 400,000 cells from the MACS buffer washed fraction during step 11 of Section 3.4 in order to quantify the percentage of Tra-1-60^+ cells after the neural commitment protocol.
4. After MACS purification, take a sample with at least 400,000 cells from the 15-mL conical tube with the LS MACS column 9 mL washed fraction.
5. Take a sample with at least 400,000 cells from the 15-mL conical tube with the LS MACS column 5 mL eluted fraction.
6. Centrifuge IgM-PE control and the three samples collected at 1,000 rpm for 5 min.
7. Remove supernatants, resuspend cells in 400 μL of MACS buffer, and transfer to four FACS tubes.
8. Analyze cells by flow cytometry to quantitatively determine the percentage of cells expressing the Tra-1-60 cell surface marker in the initial, MACS-washed and MACS-eluted fractions, and evaluate MACS-based purification performance. Collect a minimum of 10,000 events for each sample and use CellQuest software for data acquisition and analysis.

3.7 Cryopreservation of hiPSC-Derived and Purified NPs

1. Incubate cells with 1 mL/well of accutase for 7 min at 37 °C.
2. Tap cell culture plate gently and add 2 mL/well of DMEM/F12/N2 medium.
3. Flush cells from the bottom of the wells and collect them into a 15-mL conical tube.
4. Repeat the washing step once more and centrifuge cells at 1,000 rpm for 5 min.
5. After removing the supernatant resuspend cells in the appropriate volume of 90 % KO-SR and 10 % DMSO to obtain a 10^7 cells/mL concentration.
6. Distribute 500 μL of cell suspension per cryogenic vial.
7. Place cryogenic vials in the freezing container and transfer them to a –80 °C freezer.
8. For long-term preservation cells should be transferred to a liquid nitrogen tank.

4 Notes

1. The cryogenic vial should be thawed in the 37 °C water bath until the content is almost thawed, but not completely. Depending on the cell suspension volume cryopreserved, it should take 30–60 s.
2. Gently tap the cell culture plate and inspect cell colonies under the microscope. Proceed to culture medium addition when cell colony contours become loose.
3. If the neural commitment protocol begins when cells are nearly confluent, primarily central nervous system (CNS) cells, that are $Pax6^+$, are formed. If it begins with lower densities, both CNS and neural crest fates are observed (7). When plated at a seeding density of 20,000 cells/cm^2, hiPSCs should take 3–5 days to reach the ideal 80–90 % confluence.
4. It is very important to dissociate cells effectively without being too harsh in order to reduce cell loss and avoid deficient antibody labeling. MACS buffer and anti-Tra-1-60 MicroBeads kit should be kept at 4 °C during the protocol and cells should be incubated with the antibodies at 4 °C to reduce unspecific labeling. It is important to work fast to maintain cell viability.
5. It is important to maintain a sterile environment in the laminar flow hood and to be especially careful with the MACS column sterility.
6. It is important to wash the column with culture medium to maintain cell viability.
7. For each well plate differentiated prepare approximately 3 wells for replating. However, depending on the cell loss during MACS purification, less wells may be needed to plate 300,000 cells/cm^2.

Acknowledgements

This work was funded by Fundação para a Ciência e a Tecnologia, Portugal (Project PTDC/EBB-BIO/122504/2010). Gonçalo M. C. Rodrigues, Tiago G. Fernandes, and Carlos A.V. Rodrigues were supported by fellowships from Fundação para a Ciência e a Tecnologia, Portugal (SFRH/BD/89374/2012, SFRH/BPD/86316/2012, and SFRH/BPD/82056/2011, respectively). We acknowledge Professor Oliver Brustle for providing the hiPSC cell line.

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Methods in Molecular Biology (2015) 1283: 147–159
DOI 10.1007/7651_2014_100

Published online: 26 July 2014

Scalable Ex Vivo Expansion of Human Mesenchymal Stem/Stromal Cells in Microcarrier-Based Stirred Culture Systems

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Abstract

The clinical demand for human mesenchymal stem/stromal cells (MSC) drives the need for reproducible, cost-effective, and good manufacturing practices (GMP)-compliant ex vivo expansion protocols. Bioprocess engineering strategies, namely controlled stirred bioreactor systems combined with the use of xenogeneic(xeno)-free materials, provide proper tools to develop and optimize cell manufacturing for the rapid expansion of human MSC for cellular therapies. Herein we describe a microcarrier-based stirred culture system operating under xeno-free conditions using a controlled stirred-tank bioreactor for an efficient and controlled ex vivo expansion of human MSC. This culture platform can be applied to MSC from different human sources, as well as different microcarriers and xeno-free medium formulations.

Keywords: Mesenchymal stem/stromal cells, Ex vivo expansion, Stirred bioreactor, Microcarriers, Xenogeneic-free, Scale-up

1 Introduction

The major advances in stem cell research have driven the progress in cell-based therapies made over the past decade targeting the treatment of degenerative, genetic, and immunological diseases. In order to fully realize the therapeutic potential of stem cells, robust, reproducible, and scalable bioprocesses need to be developed to enable the production of cells with well-defined characteristics and in quantities that meets clinical demands (1–3).

Mesenchymal stem/stromal cells (MSC), in particular, are promising candidates for cell-based therapies, which can be readily isolated from a variety of adult and neonatal tissues, including bone marrow (BM), adipose tissue (AT), umbilical cord matrix (UCM), among other sources (4) In addition, MSC have the capacity to differentiate into a variety of cell types of mesodermal origin (cartilage, bone, fat, among others) and display a high in vitro expansion potential (5). Importantly, these cells are also able to migrate to sites

of injury, inflammation, and tumors and can secrete a wide variety of cytokines and other factors presenting immunomodulatory and regenerative capacities (5–7). Based on these unique properties, systemic or local administration of both autologous or allogeneic MSC are currently under investigation in clinical trials for the treatment of different conditions including acute graft-versus-host disease, acute myocardial infarction, autoimmune disorders like Crohn's disease, type I diabetes mellitus, among others (8). Of notice, the possibility of using allogeneic MSC poses an important advantage for cell-based therapy settings as culture-expanded cells are expected to offering the unique features of a safe, GMP-compliant, and "off-the-shelf" product.

Although MSC have been extensively used in clinical studies given their therapeutic potential, minimal and maximal doses required have not been fully defined; nevertheless, over one million MSC per kg of patient body weight have been infused in clinical trials (8).

Since the frequency of MSC in vivo is very low (for instances, in the BM, the most extensively studied MSC source, MSC represent 0.01 % of the mononuclear cells in a newborn, declining with age to 0.001–0.0005 %), an efficient ex-vivo expansion process is required to achieve clinically relevant MSC numbers. In addition, the anticipated success of allogeneic MSC-based cell therapies will demand cell manufacturing at a large scale to generate multiple cell doses, while maintaining the safety and potency of the cellular product.

To date, clinical-scale expansion of MSC has involved cumbersome multilayer planar culture systems, which have serious limitations regarding cell productivity and scalability, as well as culture monitoring. Moreover, it requires extensive handling and long cultivation times, making it susceptible to contamination and genetic instability (9). The large-scale MSC manufacturing process needs to be compliant with GMP guidelines (10), meeting rigorous quality and regulatory standards, able to robustly generate clinical-grade cells in a high-quality and cost-effective manner. These processes must offer optimized growth conditions for MSC, full monitoring and control of culture conditions, while featuring easy scalability.

Stirred and perfused bioreactors offer significant advantages over static cultivation since these produce a more homogeneous culture environment and allow monitoring and control of key culture parameters (e.g. temperature, pH, dissolved oxygen). Therefore, there has been significant advances on the development of microcarrier-based MSC cultures in scalable stirred bioreactors (11–15) alongside with the development and evaluation of well-defined serum- and xeno-free medium formulations (12, 16–19), which represent important milestones towards the clinical-scale production of MSC. In fact, we have previously demonstrated that human adipose tissue-derived stem/stromal cells (ASC) and

BM MSC can be efficiently expanded in a plastic microcarrier-based culture system under xeno-free conditions, using spinner flasks (12) and controlled stirred-tank bioreactors (20).

Herein, we describe a scalable protocol to expand human MSC in a microcarrier-based stirred culture system under xeno-free conditions using a controlled stirred-tank bioreactor. This protocol has the advantage of being potentially adapted to MSC derived from different human sources and different types of microcarriers, including plastic beads (either with or without precoating) and xeno-free medium formulations.

2 Materials

2.1 MSC Thawing and Expansion Under Static Conditions

1. Thawing medium: Iscove's Modified Dulbecco's Medium (IMDM, Life Technologies, Carlsbad, CA) supplemented with 20 % Fetal Bovine Serum (FBS, Life Technologies) (*see* **Note 1**), 1 % of Penicillin-Streptomycin (10,000U/mL Penicillin + 10,000 μg/mL Streptomycin, Life Technologies) and 0.1 % of Fungizone (250 μg/mL (1,000×), Life Technologies). Store at 4 °C.
2. Expansion medium: StemPro® MSC SFM XenoFree complete medium (Life Technologies) with 1 % of GlutaMAX™-I CTS™ supplement (Life Technologies), 1 % of Penicillin-Streptomycin (10,000 U/mL Penicillin + 10,000 μg/mL Streptomycin, Life Technologies) and 0.1 % of Fungizone (250 μg/mL (1,000×), Life Technologies). Store at 4 °C.
3. Phosphate buffered saline (PBS) solution. Prepare solution (1×) by dissolving PBS powder (Life Technologies) in 1 L of distilled water. Filter the solution using a 0.22 μm filter and store at room temperature.
4. Coating substrate: CELLstart™ CTS™ (Life Technologies). Store at 4 °C and prepare working solution diluting the necessary volume (1:100) in PBS.
5. TrypLE™ Select CTS™ (1×) cell dissociating reagent (Life Technologies). Store at room temperature.
6. Trypan blue stain 0.4 % (Life Technologies). Store at room temperature.
7. Laminar flow hood.
8. Temperature adjustable water bath set to 37 °C.
9. Polypropylene conical tubes (15/50 mL, BD Biosciences, San Jose, CA).
10. Cell culture centrifuge.
11. Cell culture incubator with CO_2, temperature, and humidity control.

12. Tissue culture treated plastic ware (T-75/T-175, BD Biosciences).
13. Hemocytometer.
14. Inverted microscope equipped with ultraviolet (UV) light.

2.2 Microcarrier Preparation

1. Nonporous plastic microcarriers (SoloHill Engineering Inc., Ann Arbor, MI). Store at room temperature.
2. Polypropylene conical tube (50 mL, BD Biosciences)
3. Coating substrate.
4. PBS solution.
5. Thermomixer comfort (Eppendorf, Hamburg, Germany).
6. Expansion medium.

2.3 MSC Expansion Under Stirred Conditions

1. Expansion medium.
2. Bellco® spinner flask (Bellco Glass, Inc., Vineland, NJ) with 100 mL working volume, equipped with 90° normal paddles and a magnetic stir bar (*see* **Note 2**).
3. Sigmacote® (Sigma-Aldrich, St. Louis, MO).
4. Stirring plate (30-40 rpm).
5. Cell culture incubator with CO_2, temperature, and humidity control.
6. New Brunswick Bioflo® 110 bioreactor (1.3 L) equipped with a three-blade pitched impeller (blades pitched 45° to vertical), dissolved oxygen (DO), pH and temperature probes and thermal jacket.
7. Schott bottle and screw cap GL45 with two ports (DURAN Group GmbH, Mainz, Germany).
8. Peristaltic pump (Ecoline- volume range: 1.6–5,000 mL/min, Ismatec, Germany).

2.4 Monitoring of Cell Culture in the Spinner Flask and Stirred Bioreactor

2.4.1 Cell Count and Viability

1. PBS solution.
2. TrypLE™ Select CTS™ (1×).
3. Expansion medium.
4. Thermomixer comfort.
5. Cell Strainer (100 μm, BD Biosciences).
6. Trypan blue stain 0.4 %.
7. Hemocytometer.

2.4.2 Metabolite Analysis

1. Automatic analyzer YSI 7100 MBS (YSI Life Sciences, Yellow Springs, OH).
2. Eppendorf tubes (1.5 mL).

2.4.3 Cell Distribution on Microcarriers

1. PBS solution.
2. 2 % Paraformaldehyde (PFA) solution: Dissolve 2 g of PFA (Sigma-Aldrich) in 100 mL of PBS (*see* **Note 3**). Filter (0.22 μm) before use and maintain at 4 °C.
3. 1.5 μg/mL 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) solution (Sigma-Aldrich) in PBS, store at 4 °C. Prepare from 1 mg/mL stock solution in deionized (DI) water stored at −20 °C.

2.5 MSC Characterization After Expansion Under Stirred Conditions

2.5.1 Immunophenotypic Analysis

1. PBS (1×) solution.
2. FACS tubes (BD Biosciences).
3. Mouse anti-human monoclonal antibodies PE-conjugated: CD31 (Biolegend, San Diego, CA), CD73 (Biolegend), CD80 (Biolegend), CD90 (Biolegend), CD105 (BD Biosciences), human leukocyte antigen (HLA)-DR (Biolegend), appropriate isotype controls: mouse IgG2a PE for HLA-DR and mouse IgG1 PE for the remaining (*see* **Note 4**).
4. 2 % PFA solution.
5. FACSCalibur flow cytometer (BD Biosciences).
6. FACSFlow™ sheath fluid (BD Biosciencies).

2.5.2 Multilineage Differentiation Ability

1. DMEM-10%FBS: Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10 % MSC-Qualified Fetal Bovine Serum (FBS, Thermo Fisher Scientific Inc., Asheville, NC), 1 % of Penicillin-Streptomycin (10,000 units/mL Penicillin + 10,000 μg/mL Streptomycin, Life Technologies) and 0.1 % of Fungizone (250 μg/mL (1,000×), Life Technologies).
2. StemPro® Adipogenesis Differentiation Kit (Life Technologies).
3. StemPro® Osteogenesis Differentiation Kit (Life Technologies).
4. StemPro® Chondrogenesis Differentiation Kit (Life Technologies).
5. PBS (1×) solution.
6. 2 % PFA solution.
7. 0.3 % Oil Red O (Sigma-Aldrich) solution in isopropanol.
8. Alkaline Phosphatase (ALP) Staining:

   Fast Violet B Salt (Sigma-Aldrich), store at 4 °C. Dissolve one capsule in 48 mL of Milli-Q water. Aliquot and store at −20 °C.

   0.25 % Naphthol AS-MX Phosphate Alkaline Solution (Sigma-Aldrich), store at 4 °C.

Prepare Reagent X: Add 4 % (v/v) Naphthol AS-MX Phosphate Alkaline Solution to a pre-thawed aliquot of Fast Violet Solution. Protect from light and use immediately.

9. 2.5 % silver nitrate solution (Sigma-Aldrich).
10. 1 % Alcian Blue (Sigma-Aldrich) solution in 0.1 N HCl.

3 Methods

3.1 MSC Thawing and Expansion Under Static Conditions

1. Retrieve a cryogenic vial of MSC (approximately 1 mL) from the liquid nitrogen tank and quickly thaw in a 37 °C water bath.
2. Dilute the content of the cryogenic vial 1:4 in thawing medium (warmed to 37 °C).
3. Centrifuge at 250 × *g* for 7 min, discard the supernatant, and resuspend the pellet in expansion medium.
4. Plate thawed cells on T-75 (10 mL of expansion medium) or T-175 (20 mL of expansion medium) flasks within a cell density range of 3–6 × 10^3 cells/cm^2.
5. Incubate cells at 37 °C, 5 % CO_2 in a humidified atmosphere.
6. Refresh culture medium every 3 days.
7. Passage cells at 70–80 % cell confluence. Remove the exhausted culture medium from the flasks and add PBS (same volume as culture medium) to wash the cell layer. Remove PBS and TrypLE™ Select CTS™ (1×) (4 and 7 mL for T-25 and T-75 flasks, respectively). Incubate at 37 °C for 7 min.
8. After complete cell detachment, recover the cell suspension to a polypropylene tube and dilute with twice the volume of expansion medium. Wash the flasks once with expansion medium. Centrifuge at 250 × *g* for 7 min.
9. Discard the supernatant and resuspend the pellet in culture medium. Determine cell number and viability using the trypan blue exclusion method. Mix cell suspension with 0.4 % trypan blue stain (1:1). Viable (unstained cells) and dead cells (blue-stained cells) are identified and counted using a hemacytometer under the optical microscope.
10. Replate the cells on T-flasks within a cell density range of 3–6 × 10^3 cells/cm^2 until reaching the required cell number to inoculate stirred culture (*see* **Note 5**).

3.2 Microcarriers Preparation

1. Weight 4 g of SoloHill plastic microcarriers in a 50 mL polypropylene tube and sterilize them by autoclaving (121 °C, 20 min) in DI water.
2. Let the beads settle and wash once with PBS.

3. Prepare 10 mL of CELLstart™ CTS™ solution and add to the microcarriers (*see* **Note 6**).
4. Place the tube in the Thermomixer for 1 h at 37 °C, with a cycle of 2 min agitated followed by 10 min non-agitated.
5. Wash the coated microcarriers once with PBS and expansion medium.

3.3 MSC Expansion Under Stirred Conditions

1. In order to prevent the microcarriers from sticking to the glass, the vessels (spinner flask and bioreactor) must be siliconized using Sigmacote (*see* **Note 7**). Add the necessary quantity of Sigmacote onto the vessels in order to wet the inner glass surfaces. Remove the excess of Sigmacote by pipetting and allow the vessels to air dry inside the laminar flow hood. Pipet DI water to rinse the inner surfaces of the vessels and repeat this procedure three times. Autoclave the vessels with DI water (121 °C, 20 min) until further use.
2. To start the spinner flask culture, autoclave vessel with DI water (121 °C, 20 min), remove the excess water inside the spinner and wash it once with expansion medium.
3. Resuspend the coated microcarriers in 50 mL of expansion medium (*see* **Note 8**) and add them to the spinner flask. Use more 20 mL of expansion medium to wash the conical tube containing the microcarriers and collect any remains.
4. Resuspend a total of 1×10^7 cells, previously expanded under static conditions in culture flasks, in 30 mL of expansion medium and gently add them to the microcarriers inside the spinner flask. This yields a culture with an initial MSC concentration around 1×10^5 cells/mL and 40 g/L of coated plastic microcarriers.
5. Place the spinner flask inside the incubator with the lids slightly unscrewed to allow gas transfer and for the first day set the agitation to 30 rpm for 18 h followed by 6 h non-agitated (*see* **Note 9**). Then, set the agitation continuously at 40 rpm. The culture is ready to transfer to the bioreactor when it reaches around 2×10^5 cells/mL (2–3 days).
6. After 2 days of culture in the spinner flask, start preparing the stirred tank bioreactor for operation: calibrate pH probe according to manufacturer's instructions, prepare all the connections and sterilize the bioreactor by autoclaving (121 °C, 20 min).
7. Connect the DO probe and allow it to polarize overnight.
8. Before inoculating the bioreactor, remove the water using a peristaltic pump and add 600 mL of expansion medium. In order to maintain the process aseptic, all the additions and removals of medium should be performed using a Schott bottle with a tubing connection system and all the connections to the bioreactor should be performed over a flame.

9. Calibrate the DO probe by sparging the expansion medium with N_2 (0 % DO) and compressed air (100 % DO).
10. Set the culture parameters in the bioreactor controller at pH 7.2, temperature at 37 °C, agitation at 60 rpm, DO at 20 % and aeration at 50 cm^3 per minute (ccm) (*see* **Note 10**).
11. Collect the microcarrier-cell suspension from the spinner flask to a Schott bottle and add fresh expansion medium to reach 200 mL (the working volume in the bioreactor is 800 mL).
12. Seal the bottle using a screw cap with a tubing connection system and transfer the microcarrier cell-suspension to the bioreactor vessel using the peristaltic pump.
13. Monitor bioreactor operation until set-points established for all culture parameters are reached.
14. After day 2, replace 25 % of the medium every 2 days (*see* **Note 11**). Stop the agitation and perform the medium renewal immediately after sedimentation of the microcarrier culture.

3.4 Monitoring the Cell Culture in the Spinner Flask and Stirred Bioreactor

3.4.1 Cell Count and Viability

1. Take daily duplicate samples from the homogeneous culture suspension.

   For the spinner flask culture take 0.5 mL samples (*see* **Note 12**), whereas for the bioreactor culture take 2 mL samples.
2. Allow microcarriers to settle down, collect the supernatant (which will be used for metabolite analysis—see Section 3.4.2) and wash with PBS.
3. Remove PBS, add 1 mL of TrypLE™ Select CTS™ (1×) to each sample and incubate in the Thermomixer for 7–8 min at 37 °C and 750–800 rpm (*see* **Note 13**).
4. Stop the enzymatic action by adding expansion medium in a proportion of 1:3.
5. Separate the cells from the microcarriers through filtration using a Cell Strainer.
6. Centrifuge at 250 × *g* for 7 min, discard the supernatant and resuspend the pellet in PBS (0.5–1 mL).
7. Determine cell number and viability using the trypan blue exclusion method.

3.4.2 Metabolite Analysis

1. Collect supernatant samples everyday from the spinner flask and bioreactor cultures to Eppendorf tubes.
2. For the spinner flask culture allow the microcarriers to settle down and collect 1 mL of supernatant.
3. For the bioreactor culture, collect 1 mL of supernatant from a 2 mL sample collected for cell counting (see Section 3.4.1). When medium is exchanged (every 2 days) make sure to collect a supernatant sample also after medium renewal. For

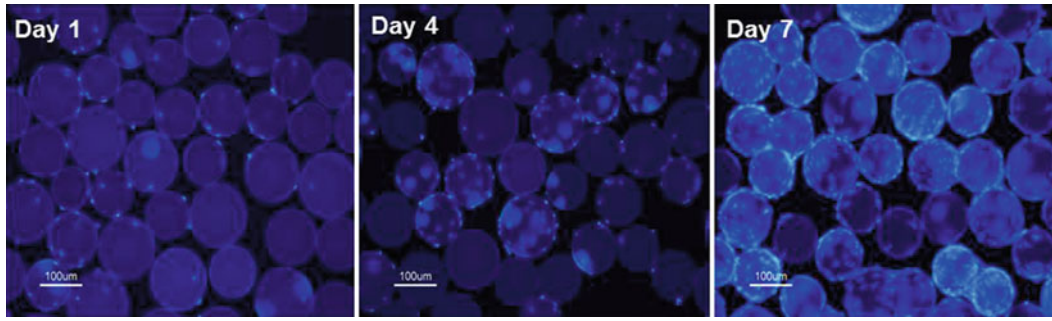

Fig. 1 DAPI images at different days of stirred culture of BM-MSC on SoloHill Plastic microcarriers precoated with CELLstart in StemPro MSC SFM XenoFree medium. Images attest homogeneous proliferation of MSC on the microcarriers

that, homogenize the culture after medium addition, stop the agitation and retrieve a 1 mL sample immediately after sedimentation of the microcarrier culture.

4. Centrifuge the samples for 10 min at 200 × *g* and then transfer to another Eppendorf tubes. Store at −20 °C until analysis.
5. Analyze the samples in an automatic analyzer YSI 7100 MBS to determine the concentration of glucose (nutrient, *see* **Note 14**), lactate and ammonia (metabolites) throughout culture.

3.4.3 Cell Distribution on Microcarriers

1. Every 2 days take a sample from the homogeneous culture suspension to a 24-well plate. For the spinner flask culture take a 0.4 mL sample, whereas for the bioreactor culture take a 1 mL sample.
2. Let microcarriers to settle down, remove the supernatant, and wash twice with PBS.
3. Fix cells with 0.5 mL of 2 % PFA solution for 20 min at room temperature.
4. Wash twice with PBS. Add 0.5 mL of 1.5 μg/mL DAPI solution and incubate in the dark at room temperature for 5 min (*see* **Note 15**).
5. Wash three times with PBS and keep it protected from light at 4 °C until observation (maximum 7 days). Observe using a microscope under UV light (Fig. 1).

3.5 MSC Characterization After Expansion Under Stirred Conditions

3.5.1 Immunophenotypic Analysis

1. Retrieve MSC from a culture sample (*see* **Note 16**) according to the method previously described in Section 3.4.1.
2. Centrifuge at 250 × *g* for 7 min, discard the supernatant, and resuspend the pellet in 800 μL of PBS.
3. Split the cell suspension into seven FACS tubes (100 μL each).
4. Add the respective antibody (5 μL) to each FACS tube and incubate for 15 min at room temperature in the dark.

5. Add 2 mL of PBS to remove the excess of antibody and centrifuge for 5 min at 160 × *g*.
6. Resuspend the cells, fix in 2 % PFA and store at 4 °C until analysis is performed.
7. Analyze the cells by flow cytometry to quantitatively determine the expression of each surface marker. Collect a minimum of 10,000 events for each sample and use an appropriate software for data acquisition and analysis.

3.5.2 Multilineage Differentiation Ability

Osteogenic/Adipogenic Differentiation

1. Plate MSC retrieved from microcarriers (see Section 3.4.1) on a 24-well plate at a cell density range of 3–6 × 10^3 cells/cm^2 using DMEM-10 % FBS.
2. Incubate cells at 37 °C, 5 % CO_2 in a humidified atmosphere.
3. Upon reaching 80 % cell confluence, replace the culture medium with the respective differentiation medium (osteogenesis or adipogenesis differentiation medium).
4. Refresh differentiation media every 3–4 days.

ALP/von Kossa Staining (Osteogenesis)

1. After 14 days of osteogenic differentiation, remove the differentiation medium and wash cells with PBS.
2. Fix cells with 2 % PFA for 20 min at room temperature.
3. Wash once with PBS and keep cells for 15 min in DI water at room temperature.
4. Incubate cells with 400 μL of Reagent X for 45 min at room temperature in the dark.
5. Wash three times with DI water and observe under an optical microscope.
6. Remove DI water and incubate cells with 400 μL of 2.5 % silver nitrate solution for 30 min at room temperature.
7. Wash three times with DI water and observe under an optical microscope.

Oil Red O Staining (Adipogenesis)

1. After 14 days of adipogenic differentiation, remove the differentiation medium and wash cells with PBS.
2. Fix cells with 2 % PFA for 20 min at room temperature.
3. Wash twice with PBS and add 400 μL of 0.3 % Oil Red O solution for 60 min at room temperature.
4. Wash three times with PBS, add DI water, and observe under an optical microscope.

Chondrogenic Differentiation

1. Retrieve MSC from microcarriers (see Section 3.4.1) and after centrifuging cells leave the pellet intact and remove as much supernatant as possible.

2. Seed 5 μL droplets of high density cell suspension on an ultra-low attachment 24-well plate (Corning Inc, Corning, NY). Incubate for 2 h at 37 °C, 5 % CO_2 in a humidified atmosphere to let the culture dry. Then add chondrogenesis differentiation medium and incubate.
3. Refresh differentiation medium every 2–3 days.

Alcian Blue Staining (Chondrogenesis)

1. After 14 days of chondrogenic differentiation, remove the differentiation medium and wash cells with PBS.
2. Fix the cells with 2 % PFA for 20 min at room temperature.
3. Wash twice with PBS and add 400 μL of 1 % Alcian Blue solution for 30 min at room temperature.
4. Wash three times with PBS, add DI water, and observe under an optical microscope.

4 Notes

1. MSC thawing has been routinely performed in our laboratory using FBS containing medium. This is the only step of the current protocol where a xenogeneic reagent is used and is mostly due to the fact that BM MSC and ASC available in the laboratory were originally isolated using FBS (MSC qualified) containing medium.
2. Prior to the bioreactor inoculation, MSC are cultured in spinner flasks with a working volume of 100 mL to perform the adhesion step.
3. PFA powder should be initially dissolved in a low volume of water at a high temperature (lower than 70 °C) in order to facilitate dissolution. The pH should be set to 7.3 and the final volume completed with 10× PBS.
4. The phenotypic markers analyzed are part of the criteria suggested by the International Society for Cellular Therapy to define MSC for both scientific research and preclinical studies (21).
5. To initiate the stirred culture there should be enough cells to inoculate the 100 mL spinner flask culture (1×10^7 cells) and to perform the immunophenotypic analysis of the cells prior to dynamic expansion according to the method described in Section 3.5.1.
6. Other coating substrates can be used in this step namely a human platelet lysate supplement (22) or human fibronectin.
7. Once glassware has been siliconized, it is not necessary to treat prior to each use. From our knowledge vessels should be siliconized every 20 cultures.

8. In this step the beads should be resuspended in a larger volume to minimize the amount of beads attached to the inner walls of the conical tube and pipette.
9. From our recent optimization studies this seeding protocol prevents cell-carrier aggregation when compared to intermittent agitation (22).
10. The addition of CO_2 is made through gentle sparging from the base of the reactor and 1.0 M of $NaHCO_3$ is used to maintain culture pH. Aeration is achieved through gentle sparging from the base of the bioreactor with a mixture of N_2, air, and CO_2 gas bubbles, and temperature was kept at 37 °C by an electric heating jacket.
11. The feeding regimen can be adjusted if nutrient depletion is verified. According to our previous study in stirred tank bioreactors, other feeding regime can be successfully used, namely a fed-batch regimen with concentrated feeds (20).
12. A homogeneous sampling is essential for accurate cell number determination. Before taking 0.5 mL samples, it is important to assure an evenly mixed culture inside the spinner flask. For that purpose, a stirring plate may be used inside the laminar flow chamber to homogenize culture inside the spinner flask before sampling.
13. In the final days of culture, larger cell-carrier aggregates are formed that require longer incubation times and higher agitation rates with TrypLE to be completely dissociated.
14. In these cultures, GlutaMAX (alanyl-L-glutamine dipeptide) is used as a glutamine substitute since it does not spontaneously break down to form ammonia. Cells cleave the dipeptide bond to release L-glutamine as needed and therefore it is not possible to determine glutamine consumption during culture.
15. The well-plate should be protected from light. It is also advised to turn off the laminar flow chamber light while preparing the sample.
16. The sample volume should allow collecting enough cells to perform flow cytometry analysis. Estimate the necessary volume according to cell concentration in culture.

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Methods in Molecular Biology (2015) 1283: 161–169
DOI 10.1007/7651_2014_104

Published online: 06 August 2014

Media Fill for Validation of a Good Manufacturing Practice-Compliant Cell Production Process

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Abstract

According to the European Regulation EC 1394/2007, the clinical use of Advanced Therapy Medicinal Products, such as Human Bone Marrow Mesenchymal Stem Cells expanded for the regeneration of bone tissue or Chondrocytes for Autologous Implantation, requires the development of a process in compliance with the Good Manufacturing Practices. The Media Fill test, consisting of a simulation of the expansion process by using a microbial growth medium instead of the cells, is considered one of the most effective ways to validate a cell production process. Such simulation, in fact, allows to identify any weakness in production that can lead to microbiological contamination of the final cell product as well as qualifying operators. Here, we report the critical aspects concerning the design of a Media Fill test to be used as a tool for the further validation of the sterility of a cell-based Good Manufacturing Practice-compliant production process.

Keywords: Media fill, Good manufacturing practice, Aseptic validation, Advanced therapy medicinal products, Tryptic soy broth, Thioglycollate medium, Quality control

1 Introduction

Human expanded cells for clinical applications, defined as Advanced Therapy Medicinal Products (ATMPs) by current European regulations (EC 1394/2007) (1, 2), must be produced in accordance with specific pharmaceuticals rules named Good Manufacturing Practices (GMPs). Although GMP compliance allows to minimize contamination risks trough a rigorous control of cell manipulation processes, absolute sterility cannot be guaranteed. Therefore, to adequately monitor the aseptic cell expansion processes, Annex 1 of Volume 4 of European (EU) Guidelines to GMP and PIC/S Recommendation on the Validation of Aseptic Processes require a specific additional validation, named Media Fill (3, 4). This test is the performance of an aseptic manufacturing procedure, using a sterile microbiological growth medium in place of the drug solution, during media filling. Translated to the ATMP world, a Media Fill test is a simulation of an aseptic process, by using a microbial growth medium instead of the cells, performed in order to evaluate the sterility confidence of the process itself. In

fact, such validation test ensures that the cell manipulation process repeatedly and reliably produces products with the required microbiological features. It is clear that the methods for simulating an aseptic process change according to the different processes and products.

This chapter describes the design of a Media Fill protocol that mimics a multistep GMP-compliant production of expanded cells suitable for autologous implantation in humans, such as Bone Marrow-derived Mesenchymal Stem Cells for bone tissue regeneration or Chondrocytes for cartilage repair (5, 6).

2 Materials

2.1 Raw Materials

When choosing of the microbial growth medium to be used to replace the cells in Media Fill tests, the first key element to be taken into account is its low selectivity. In fact, the selected medium should be capable of supporting a wide range of microorganisms and must pass a "growth promotion test" before starting process simulation. This test should demonstrate that the medium supports recovery and growth of low numbers of each type of microorganisms, i.e. 10–100 CFU/unit or less. The microorganisms to be tested are identified by the European Pharmacopoeia (Current Edition) (7) and should include also one or two of bacterial strains of the in house flora. Any microbial growth must be detected within 3 days for bacteria and up to 5 days for yeasts and moulds (4).

Guidance recommends the use of Tryptic Soy Broth (TSB) for aerobic bacteria, yeasts, and moulds and of an anaerobic medium such as fluid Thioglycollate (THIO) if the product is being filled in anaerobic conditions like a nitrogen environment (Fig. 1).

It is important that medium suppliers are able to provide the certification confirming that materials are sourced from Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy (BSE/TSE) free countries (8, 9).

In general, the used medium should be clear enough to allow observing turbidity and filterable if the cell manufacturing process requires a filtration step. In this case the medium should be capable of being filtered through the same grade (porosity) used in production.

Concerning plastic raw materials, they should be the same as those used for the routine process, meaning that they must be certified to be sterile, pyrogen free, latex free (where necessary), and tested for cell culture. Double or triple packages should be preferred.

Even materials for Quality Control (QC) sampling, such as contact and settle plates for environmental microbiological control (see below), should be the same utilized in routine production.

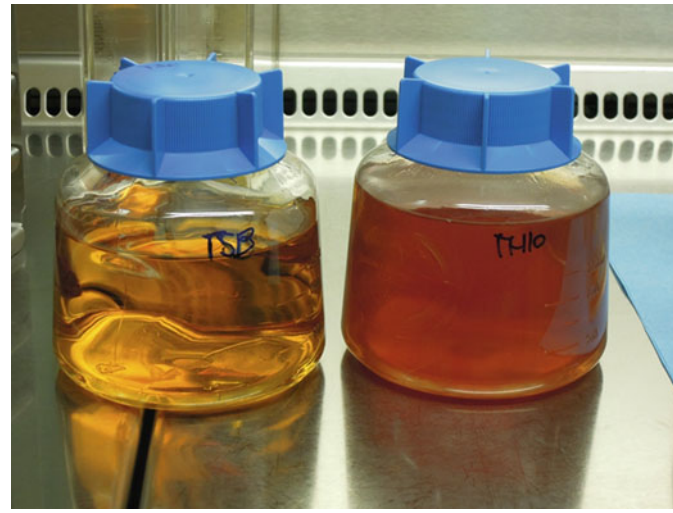

Fig. 1 Media Fill bacterial growth media. Tryptic Soy Broth and Thioglycollate Medium are the fluid bacterial growth media recommended by Guidance respectively for aerobic bacteria, yeasts, and moulds and for anaerobic microorganisms

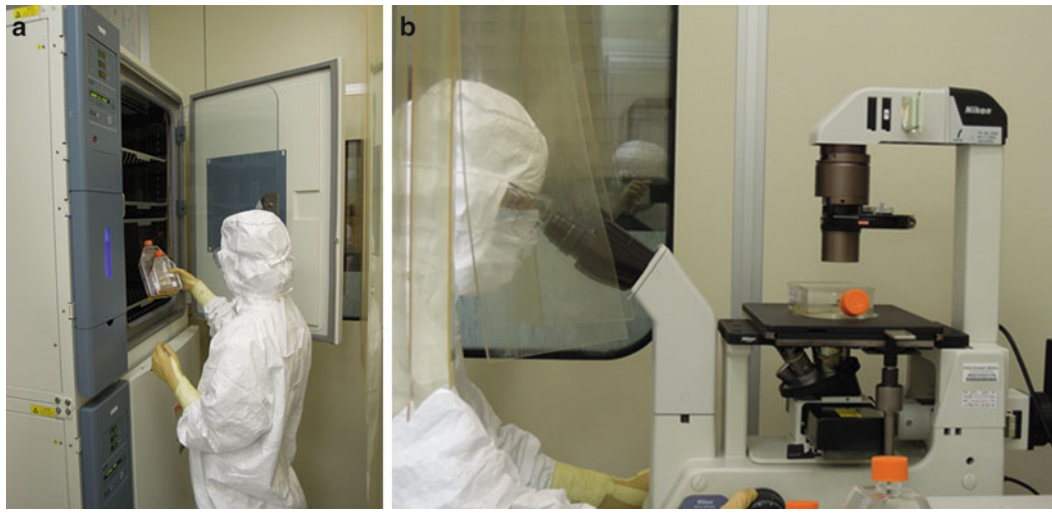

Fig. 2 Media Fill Equipment. Equipment used in Media Fill runs, like CO_2 incubators (**a**), Phase contrast microscope (**b**) etc., are the same as in cell manipulation production process

2.2 Equipments

Equipments should be the same used in the cell expansion process (Fig. 2a, b): Biohazard hoods, CO_2 Incubators (set at 37 °C temperature, 5 % CO_2 and 95 % relative humidity), Centrifuges, Phase contrast microscopes etc.

All equipments should be qualified and undergoing programmed maintenance, as required by EU-GMPs.

2.3 Infrastructure

In Europe, a GMP-compliant cell manipulation must be performed in an authorized GMP-production facility. This structure consists of clean rooms of different classification up to A (the local zone for high-risk operations i.e. Biohazard hood) in B work places (the background environment for the grade A zone). High-risk operations include manipulations where the cells are exposed to environment such as trypsinization and seeding. For lower risk operations, such as centrifugation, grade B areas are sufficient. As required by EU-GMPs, various factors, including air-borne contaminants, temperature, relative humidity, and differential pressure are kept under strict control by a real time measuring system. Only trained and equipped personnel are admitted in the structure. Operator's equipment includes protective and disposable clothes to wear. To minimize contamination raw materials are introduced separately from personnel, through a clean pass box (3).

Media Fill performance should be carried out in the GMP facility where routine production activities take place and by personnel usually involved in cell manipulation.

Similarly, all the QC analysis, such as growth promotion test, Media Fill samples incubation and all microbial tests performed during Media Fill performance, should be carried out in an authorized QC laboratory.

3 Method

3.1 Media Fill Protocol

1. Design the Media Fill protocol using as a template the Standard Operating Procedure (SOP) that describes in detail the cell production activities.
2. Describe in a specific SOP all the steps of the Media Fill protocol that should follow as closely as possible the routine aseptic manufacturing process (*see* **Note 1**).
3. Record all the phases to be performed in the simulation tests in a batch record, like for the GMP cell production.

When designing a Media Fill protocol several key elements have to be taken into account, including number and frequency of runs of Media Fill, duration, worst case conditions, environmental and personnel monitoring, incubation conditions, interpretation of data, and acceptance criteria.

3.2 Media Fill Frequency

Media Fill should be distinguished between "start-up" and "on-going" tests (4). A "start-up" performance consists of at least three consecutive separate successful runs, while an "on-going" one is just of one satisfactory simulation test per shift.

1. Carry out a "start-up" simulation for a new process validation and therefore before the routine manufacturing can start.

2. Perform "start-up" tests also with new critical equipment or after any change evaluated as a potential danger in the process itself, in equipment, environment, personnel, and in the clean-room HVAC (heating, ventilation, and air conditioning) system (*see* **Note 2**).
3. Perform "on-going" tests at least twice per year for the periodic monitoring of aseptic conditions during routine manufacturing or after less critical changes of processes, equipment, or environment (*see* **Note 3**).

3.3 Duration of a Single Media Fill Test (See Note 4)

1. Media Fill should be long enough to include all of the required interventions and stoppage and should reflect the potential operator fatigue (4, 8).
2. All the steps of the manipulation process (such as culture medium preparations, medium changes and trypsinizations, seeding onto a biomaterial/scaffold, handling on the initial raw material—bone marrow sample, cartilage biopsy, etc.—and on the final product) or all the transitions between inside and outside the biohazard hood should be performed, but shortening the incubation times in which the product is not opened or manipulated.

3.4 Worst Case

During Media Fill performance, it is usual, and this is recommended, to include the so-called worst case conditions (3, 4), namely several unfavorable situations that can occur in cell production runs and represent an additional risk for the sterility of the process. For example, it is possible to:

1. Simulate a technical intervention on an instrument or the environment (i.e. switching off the biohazard hood or the CO_2 incubator during process simulation);
2. Open and close the door during the process of aseptic handling;
3. Outsource the production simulation to a single operator when two should be required, or to an operator still in training and thus more susceptible to make mistakes.

3.5 Environmental and Personnel Monitoring

1. Microbial tests should be the same carried out during the routine cell manipulation process in order to verify the maintenance of aseptic conditions (*see* **Note 5**):
   - perform air sampling through either active and passive methods using settle plates.
   - carry out surface sampling at the end of aseptic process using contact plates.
   - perform personnel monitoring through glove prints.

2. Nonviable particle monitoring by the real time measuring system should be the same carried out during the routine cell manipulation (*see* **Note 5**).

3.6 Incubation Condition

1. At the end of the process simulation, Media Fill samples undergo a microbiological control in accordance to Validation of aseptic processes PI 007-6 1, January 2011 and European Pharmacopoeia, Current Edition:
   - Before incubation invert or otherwise manipulate the containers to ensure that all surfaces, including the internal one of the closure, are thoroughly wetted by the medium. In general, containers should not be completely filled with medium in order to provide sufficient oxygen for the growth of obligate aerobes;
   - Generally, it is accepted to incubate Media Fill samples at 22.5 ± 2.5 °C for a minimum of 7 days followed by an incubation at 32.5 ± 2.5 °C, for a total minimum incubation time of 14 days;
   - Perform a daily visual observation of the samples and record the observations on specific worksheets;
   - When inspecting the containers these should be compared to known sterile ones (*see* **Note 6**).
2. Perform a growth promotion test (see Sect. 2.1) on the Media Fill samples to further demonstrate the maintenance of the ability of the media to sustain bacterial growth in case of contamination (4).

3.7 Interpretation of Data

In general, the number of containers used for Media Fills should be sufficient to allow a valid evaluation. For cellular products, which are typically made up of small batches (i.e. a single vial of a cell suspension or a biomaterial seeded with cells), the number of containers should at least equal the size of the production batch. Contaminated samples should be examined for evidence of container/closure damage which might compromise the integrity of the packaging system. Therefore, damaged containers should not be included as positives when evaluating results (4).

3.8 Acceptance Criteria

For small batches, that is when fewer than 5,000 U are filled, the target should be zero growth (3, 4). Therefore, the first acceptance criterion is the absence of contamination in each Media Fill sample. Furthermore, the results of environmental and operators monitoring should be in compliance with GMPs. Finally, the maintenance of growth promotion capacity of the microbial media used in the simulation tests should be confirmed.

3.9 Conclusions

If results outside of the acceptance criteria occur, namely in case of any contamination, excluding those due to damaged containers, an investigation should be performed. This means that, in "start-up" simulations (see above), the new process or the activities resumption after critical changes cannot not even begin. On the other and, in "on-going" simulations, routine cellular production should be suspended. The investigation should firstly clarify if the microbiological test has been correctly performed (false positive results) and then find any possible weakness in the process or in personnel activities. After the appropriate investigations and eventual corrective actions, Media Fill should be repeated. If the acceptance criteria are all satisfied, the ability to proceed with the routine activities of production can be restored and a new process can start (3).

4 Notes

1. As in general ATMP processes end with the production of cell preparations, similarly a Media Fill performance generates Media Fill samples containing microbial growth medium instead of the cells. For example, when the final product is a small volume Mesenchymal Stem Cells suspension suitable for a clinical use in the regeneration of bone damages, Media Fill samples consist of the same volume of microbial medium (Fig. 3).

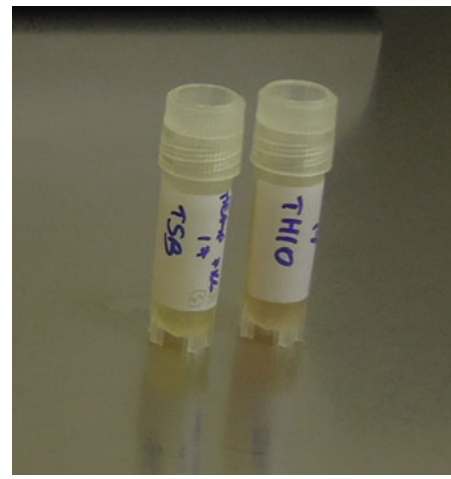

Fig. 3 Media Fill samples. Media Fill samples filled within the same final containers of the finished products of the cell manipulation process consist in the microbial growth medium instead of cells. In this case, the final product is a small volume Mesenchymal Stem Cells suspension suitable for a clinical use in the regeneration of bone damages and Media Fill samples consist of the same volume of microbial medium

2. In this case, routine activity should be stopped until the "start-up" test ends successfully.
3. In this case, it is not necessary to stop production activities because Media Fill can be carried on similarly to a production batch.
4. It is not mandatory that Media Fill simulations last for the same time required by the process. This is true especially for long-lasting, articulated, multistep cell expansion processes.
5. Regulatory and Pharmacopoeia references state the microbial condition at which Media Fill tests should be performed. Particularly, annex I of the EU/PIC/S Guide to GMP provides the basis for environmental and personnel monitoring requirements and recommendations. In general, microbial monitoring should be performed in and around areas of high operator activity. During Media Fill the number of sampling locations might be even increased with respect to the routine procedure to further confirm that critical locations have been identified. The same criteria should be applied for nonviable particle monitoring and the results should be in accordance with the specifications of annex 1 of the EU/PIC/S Guide to GMP.
6. The test is negative (absence of microbial growth) if no turbidity is detected within the established incubation times. The test is positive if a turbidity is detected and subcultures confirm and identify the microorganisms. These lasts, if detected, should be identified preferably to species level to aid determination of the possible source of contamination.
7. Detailed information and/or full texts about References are available at the following web pages:

   http://www.ema.europa.eu/ema/index.jsp?curl = pages/regulation/general/general_content_000294.jsp&mid = WC0b01ac05800241e0

   http://ec.europa.eu/health/documents/eudralex/vol-4/

   http://www.picscheme.org/publication.php?id = 8

   https://www.edqm.eu/en/european-pharmacopoeia-8th-edition-1563.html

Acknowledgements

This work was supported by the grant "Progetto di Medicina Rigenerativa" from "Regione Emilia Romagna" (Delibera di Giunta n. 2233/2008).

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Methods in Molecular Biology (2015) 1283: 171–186
DOI 10.1007/7651_2014_103

Published online: 05 August 2014

Standard Operating Procedure for the Good Manufacturing Practice-Compliant Production of Human Bone Marrow Mesenchymal Stem Cells

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Abstract

According to the European Regulation (EC 1394/2007), Mesenchymal Stem Cells expanded in culture for clinical use are considered as Advanced Therapy Medicinal Products. As a consequence, they must be produced in compliance with Good Manufacturing Practice in order to ensure safety, reproducibility, and efficacy. Here, we report a Standard Operating Procedure describing the Good Manufacturing Practice-compliant production of Bone Marrow-derived Mesenchymal Stem Cells suitable for autologous implantation in humans. This procedure can be considered as a template for the development of investigational medicinal Mesenchymal Stem Cells-based product protocols to be enclosed in the dossier required for a clinical trial approval. Possible clinical applications concern local uses in the regeneration of bone tissue in nonunion fractures or in orthopedic and maxillofacial diseases characterized by a bone loss.

Keywords: Bone marrow, Mesenchymal stem cells, Good manufacturing practice, Quality control, Advanced therapy medicinal products, Bone regeneration

1 Introduction

Mesenchymal Stem Cells (MSCs) display a low density in bone marrow, thus an in vitro expansion step is required in order to obtain a cell number suitable for clinical applications (1–3). European regulations define such expanded cells as Advanced Therapy Medicinal Products (ATMPs) that must be produced in accordance with the current Good Manufacturing Practice (GMP) (4, 5). Those rules, which have been already encoded for conventional drugs, allow for the manufacture of medicinal products in a standardized and controlled way minimizing, at the same time, contamination risks.

Here, we report a Standard Operating Procedure (SOP) describing the multistep GMP-compliant manual production of Bone Marrow-derived MSCs to be used for autologous implantation in humans (6–8). MSCs, isolated from an eparinated (1,000 I. U. of heparin) bone marrow sample harvested from the iliac crest of adult donors (9, 10) (about 10–20 ml), are expanded in monolayer

up to 3 weeks. The final product is a small volume MSC suspension suitable for the clinical use only after passing proper quality controls. In fact, the procedure involves several GMP's mandatory samplings for environmental and cell quality control analyses (3, 11) that must be carried out by specialized laboratories and will not be described in detail.

Previous validation studies have stated final product's shelf-life (properly named stability): we demonstrated that MSC's features (sterility, viability, and phenotype) are maintained at acceptable levels when the MSC suspension is stored at room temperature for a maximum of 72 h. After this time the product expires and can no longer be transplanted.

To reach full GMP-compliance, the process generates two types of reference samples collected from the final product—cells and supernatant—which should be both stored for at least 1 year after implantation and analyzed if issues arise (12).

If implantation is delayed for organizational reasons or patient's illness or if repeated doses at different times are required, cells are stored in liquid nitrogen as intermediate product.

This SOP can be utilized as a template for the development of investigational medicinal Mesenchymal Stem Cells-based product protocols to be enclosed in the dossier required for a clinical trial approval. Possible clinical applications concern local uses (cells alone or in combination with biomaterials and/or growth factors) for the regeneration of bone traumatic or degenerative damages, pseudoarthrosis and defects of consolidation, congenital disorders or maxillofacial injuries (13–15).

2 Materials

2.1 Raw Materials

Cell culture reagents suitable for cell therapy applications should be purchased from Companies that guarantee their GMP compliance production. Sterility should be certified by specific analyses, preformed in compliance with the requirements of the European Pharmacopoeia, current edition (16).

All reagents used in this protocols are: Dulbecco's Modified Eagle Medium (DMEM) low glucose (1 g/l) (basal medium); Dulbecco's Phosphate Buffered Saline (PBS) (1×); Fetal Bovine Serum (FBS) Pharma Grade, Australian Origin; L-Glutamine 200 mM (recombinant origin) and Trypsin-Ethylenediaminetetraacetic acid (EDTA) (1:250) 1× (porcine source).

Trypsin-EDTA and FBS must be also certified to be free from porcine and bovine mycoplasmas and viruses, respectively. In addition, FBS must be attested to be produced in a Bovine Spongiform Encephalopathy/Transmissible Spongiform Encephalopathy

(BSE/TSE) free Country, such as Australia or New Zealand, and screened for prion absence (17).

L-Glutamine's recombinant origin must be clearly indicated and certified too.

CryoSure-Dimethyl Sulfoxide (DMSO) is purchased from WAK–Chemie Medical GmbH, Germany.

Plastic material must be certified to be sterile, pyrogen free (latex free whenever necessary) and tested for cell culture (or stem cell tested). Vented culture flasks are recommended.

To manage high cell numbers we use a multilayer flasks system (Hyperflask vessels, Corning Life Sciences). Double or triple packages should be preferred to eliminate/reduce the need for alcohol wipe downs and similar procedures required in clean room production facilities.

Quality control sampling:

- BacT/ALERT® Aerobic Culture Bottles (SA) (BioMerieux Inc., Marcy L'Etoile, Craponne, France) (18).
- BacT/ALERT® Anaerobic Culture Bottles (SN) (BioMerieux Inc., (Biomerieux Industry, Marcy L'Etoile, Craponne, France) (18).
- Apirogenic Bottles for endotoxin testing (Charles River Laboratoires, l'Arbresle, France).
- PCR tubes for Real time PCR detection of Mycoplasma contamination (Venor®GeM-qDual Mycoplasma Detection Kit for Real-Time PCR, Minerva Biolabs GmbH, Berlin, Germany).
- Settle and contact plates for environmental microbiological control and glove prints (Heipha Dr Müller GmbH, Eppelnheim, Germany).
- Particle counter for environmental particle control.

2.2 Location

In Europe, a GMP-facility (Cell Factory) allowed to ATMP production by the competent authority is required (5). A GMP-facility consists in environments where specific parameters such as air filtration and ventilation, temperature, relative humidity, differential pressure, number of air particles, and bacterial colony forming units are standardized and continuously monitored. The structure includes clean rooms of different classification up to A (the local zone for high-risk operations i.e. biohazard hood) in B work places (the background environment for the grade A zone), according to European current GMP (5). High-risk operations include manipulations where the cells are exposed to environment such as trypsinization and medium change. For lower-risk operations, such as medium warming or centrifugation, grade B areas are appropriate. Only trained and equipped personnel are admitted to the facility.

Fig. 1 Disposable sterile garments for cell manipulation. Cell manipulating operators as well as personnel must wear disposable garments that ensure biological products protection. Only trained and equipped operators can enter the GMP-facility

Operator's equipment includes protective and disposable clothes to wear (Fig. 1). Raw materials must be introduced separately from personnel, through a clean pass box.

2.3 Equipment

Biohazard hood; CO_2 incubator (set at 37 °C temperature, 5 % CO_2 and 95 % relative humidity); centrifuge; phase contrast microscope; incubator (work chamber temperature of 37 °C); vortex; chilling plate/refrigerated racks; cell counter; refrigerator/freezer; liquid nitrogen tank.

3 Method

3.1 Cell Isolation and Seeding (See Notes 1–4)

1. Warm/thaw basal medium, L-glutamine and FBS in the incubator.
2. Under the biohazard hood prepare complete medium (basal medium supplemented with 10 % FBS and 4 mM L-glutamine): discard by aspiration 60 ml from a 500 ml bottle of basal medium, add 10 ml L-glutamine and 50 ml FBS; resuspend with a pipette.

Fig. 2 Quality control sampling on Mesenchymal Stem Cells. To harvest samples for microbiological control during critical steps, 1 ml of supernatant is inoculated into a BacT/ALERT® Aerobic Culture Bottle (SA) and 1 in a BacT/ALERT® Anaerobic Culture Bottle (SN) with a sterile syringe (*see* **Note 5**). The bottles are then sent to a GMP-compliant Quality Control Laboratory for the analysis

3. Aspirate the eparinated bone marrow with a 18-G needle inserted in a syringe and transfer in sterile tube/s.
4. Resuspend and measure bone marrow amount with a pipette.
5. Dilute the bone marrow samples 1:4 with complete medium and resuspend.
6. Harvest samples for microbiological control:
   - 1 ml with a sterile syringe to inoculate in a BacT/ALERT® Aerobic Culture Bottle (SA) (Fig. 2);
   - 1 ml with a sterile syringe to inoculate in a BacT/ALERT® Anaerobic Culture Bottle (SN) (Fig. 2);
   - 2 ml to collect in PCR tube for mycoplasma detection.

   Collected samples are then to be sent to the proper specialized quality control laboratories that will perform the analyses (*see* **Note 5**).
7. Seed cells in culture flasks, typically T75 (7 ml/T25; 14 ml/T75; 28 ml/T150; 560 ml/multilayer flasks).
8. Place the flasks in the CO_2 incubator.
9. Store the complete medium at 4 °C.

3.2 First Medium Change (See Notes 1 and 2)

1. After 3 days, warm complete medium and PBS in the incubator.
2. Pull out the flasks from the CO_2 incubator and observe the macroscopic appearance of the culture medium:
   - if the medium is opaque or dense and yellow there is the suspect of a contamination. In this case the procedure is stopped and sampling for microbiological control is needed (see Section 3.1);
   - if the appearance is clear and the color red or dark red go on with this procedure.
3. Under the biohazard hood filter the amount of complete medium required for processing with a vacuum filtration systems (or with a filter and a syringe).
4. Aspirate medium from the flasks and place it in sterile tubes.
5. Centrifuge the medium at 500 × *g* for 7 min, at room temperature.
6. In the meantime add to the flasks the PBS solution (5 ml for T25 flasks, 10 ml for T75 flasks, 20 ml for T150 flasks, 100 ml for multilayer flasks) in order to wash out stromal matrix debris and red blood cells (*see* **Note 6** and Fig. 3a).
7. Close the flasks and placed them horizontally for 1 min, gently shaking.
8. Aspirate and discard the PBS.
9. Run a second wash (only steps 7 and 8).
10. Evaluate the cultures under a phase-contrast microscope (Fig. 3b), noting the following parameters: cell adhesion (absent, present, partial, poor); cell density (confluence, non-confluence, next to the confluence); cells in the supernatant (presence, absence); matrix/red blood cells (presence, absence); unusual morphology.
11. Transfer the flasks under the biohazard hood and discard the PBS.
12. Transfer the centrifuged tubes under the biohazard hood, aspirate half supernatant volume of that normally used to each flask (3.5 ml for the T25 flasks, 7 ml for T75 flasks, 14 ml for T150 flasks, 280 ml for multilayer flasks) (*see* **Note 6**).
13. Add to the flasks the same amount of complete fresh medium and resuspend.
14. Place the flasks in the CO_2 incubator.
15. Store reagents at 4 °C.

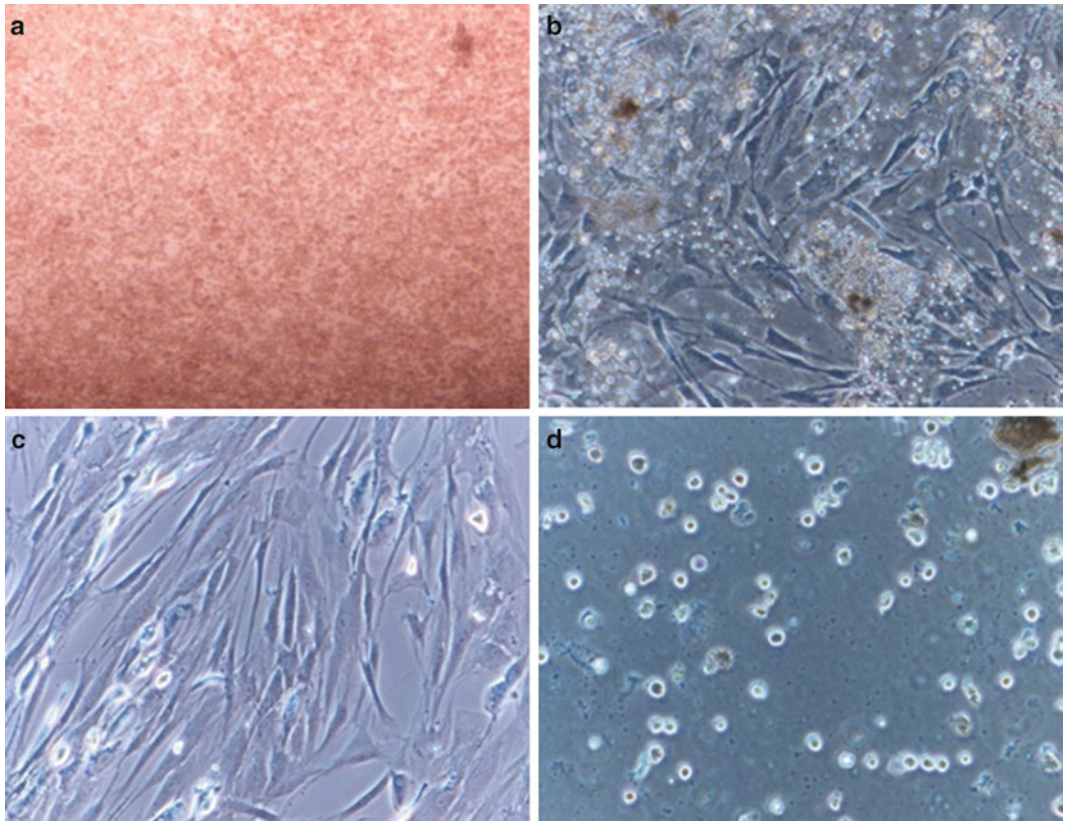

Fig. 3 Phase-contrast microscope morphological observation of Mesenchymal Stem Cells at different steps of the Standard Operating Procedure. (**a**) Three days after seeding (*First medium change*) red blood cells and matrix hamper observation. **(b)** Adherent cells become more visible only after two PBS washes**.** Matrix debris and/or red blood cells are still evident and should disappear in a few days. **(c)** Cells have reached 70–80 % confluence and trypsinization is recommended. **(d)** Full cell detachment occurs as a suspension of cells with round morphology after Trypsin-EDTA incubation. All images were taken with a digital camera (magnification $\times 4$)

3.3 Trypsinization and Intermediate Product Freezing (See Notes 1, 2, and 7)

1. After 3–4 days, macroscopically evaluate culture medium, noting the following parameters: clarity/turbidity, color (red orange, red violet, yellow).
2. Check the cells under a phase-contrast microscope noting the following parameters: cell adhesion (present, absent, poor); confluence (70–80 % confluent, nonconfluent, next to the confluence); cell presence or absence in the supernatant; matrix/red blood cells (presence, absence); unusual morphology.
3. Make a decision based on both observations (see steps 4, 5, 6, and 7).

4. If 70–80 % confluence is not reached, trypsinization should be delayed until reached and a medium change is needed.
5. In particular, change the culture medium with complete medium in the case of red medium color and/or adhesion; change the culture medium with a 20 % FBS supplement in the case of violet medium color and/or poor adhesion:
   - warm complete medium and FBS (if necessary);
   - prepare 10 or 20 % FBS medium;
   - transfer the cultures under the biohazard hood;
   - filter the needed amount of medium, depending on flasks size and number;
   - aspirate the medium from the flasks and add proper medium—complete or with 20 % FBS—(7 ml for T25 flasks, 14 ml for T75 flasks, 28 ml for T150 flasks; 560 ml for multilayer flasks);
   - close and place flasks in the CO_2 incubator.
6. Trypsinization and freezing can be performed in case of about 70–80 % confluence (Fig. 3c):
   - prepare complete medium if necessary;
   - warm complete medium and PBS in the incubator;
   - thaw Trypsin-EDTA in the incubator;
   - filter the amount of reagents required for processing;
   - transfer the cultures under the biohazard hood;
   - aspirate and discard the supernatants;
   - add the PBS wash solution (5 ml for T25 flasks, 10 ml for T75 flasks, 20 ml for T150 flasks, and 100 ml for multilayer flasks);
   - close and place horizontally the flasks, gently shaking for 30–60 s;
   - run a second wash;
   - add the Trypsin-EDTA solution (4 ml for T25 flasks, 7 ml for T75 flasks, 14 ml for T150 flasks, and 100 ml for multilayer flasks);
   - close and place the flasks in the CO_2 incubator for 15 min to allow cell detachment;
   - pick the flasks and check the cells under the phase-contrast microscope to verify cell detachment (which occurs as a suspension of cells with round morphology) (Fig. 3d);
   - if some cells are still adherent to the substrate, put the flasks again in the CO_2 incubator for 3–5 min to allow full detachment;

- if detachment is completed, inactivate the Trypsin-EDTA solution by adding complete medium to the flasks under the biohazard hood (8 ml for T25 flasks, 14 ml for T75 flasks, 28 ml for T150 flasks, and 200 ml for multilayer flasks);
- resuspend and transfer to 50 ml tubes;
- collect a 0.5 ml sample for cell counting;
- centrifuge 7 min at 500 × *g*, at room temperature;
- in the meantime count the cells;
- under the biohazard hood open the centrifuged tubes, carefully discard the supernatants and gently shake the pellets (*see* **Note 8**);
- set the number of cryovials to use for freezing: 1cryovial up to $3 \times 10^6 \pm 0.5 \times 10^6$ MSCs;
- place the empty cryovials in a refrigerated rack;
- prepare the cell freezing mixture in a tube: 0.9 ml of complete medium, 0.2 ml of DMSO and 0.9 ml of FBS (mixture for one cryovial) (*see* **Note 7**);
- resuspend, filter, and place the mixture in the refrigerated rack;
- carefully discard the supernatant of the centrifuged tubes and gently resuspend the cell pellet (*see* **Note 8**);
- add 1.6 ml of the freezing mix to each pellet, drop by drop, using a sterile pipette while gently shaking the tubes with the other hand;
- gently resuspend and transfer into the cryovials;
- close the cryovials and house them in a storage box that enables a slow decrease of the temperature (about 1 °C per minute);
- exit the cleanroom and place the container into a freezer set at a ≤75 °C temperature;
- after about 24 h place the cryovials in the vapor phase of a liquid nitrogen tank (*see* **Note 7**).

7. Collect samples for microbiological tests in case of suspected contamination (yellow color, unusual morphology) as in Section 3.1 and stop the procedure until results (*see* **Note 5**).

3.4 Intermediate Product Thawing and Seeding (See Notes 1, 2, 5, and 7)

1. Open the liquid nitrogen tank and harvest the cryovials, checking their integrity and identity.
2. Get samples in the cleanroom (through the pass box) within a refrigerated rack.
3. Prepare and warm a 20 % FBS supplemented medium.
4. Place the cryovials in the incubator.

5. After 3–4 min check if the cell suspension appears thawed.
6. If small ice chunks are still evident put the samples again in the incubator for 30–60 s.
7. When no more ice residues are visible, place the cryovials under the biohazard hood and transfer each cell suspension in a tube containing 18 ml of the 20 % FBS medium.
8. Close the tubes and vortex the solution for about 10 s.
9. Centrifuge at 500 × *g* for 7 min, at room temperature.
10. Transfer the supernatant into sterile tubes for quality control sampling as in Section 3.1.
11. Gently shake the pellets, add 20 % FBS medium (7 ml for T25 flasks, 14 ml for T75 flasks, 28 ml for T150 flasks, 560 ml for multilayer flasks) and resuspend (*see* **Note 8**).
12. Seed at low density in an adequate number of culture flasks (*see* **Note 9**).
13. Check cell presence under the phase-contrast microscope.
14. Place the flasks in the CO_2 incubator.

3.5 Expansion (See Notes 1, 2, and 10)

1. Pull out flasks from the CO_2 incubator.
2. Macroscopically evaluate culture medium, noting the following parameters: clarity/turbidity, color (red orange, red violet, yellow).
3. Check the cells under a phase-contrast microscope noting the following parameters: cell adhesion (present, absent, poor); confluence (confluent, nonconfluent, next to the confluence); cell presence or absence in the supernatant; matrix/red blood cells (presence, absence); unusual morphology.
4. Make a decision based on both observations as detailed below.
5. Change the culture medium with complete medium in the case of red medium color and/or cell adhesion; change the culture medium with a 20 % FBS supplement in the case of violet medium color and/or poor adhesion, as described in Section 3.3.
6. Trypsinization in case of about 70–80 % confluence (*see* **Note 10**) (Fig. 3c) or if matrix debris and/or red blood cells are still evident (*see* **Note 6**) (Fig. 3a, b):
   - trypsinize (until centrifugation step) and count the cells, as detailed in Section 3.3
   - add to the pellets the necessary amount of complete medium in order to allow a low density seeding, resuspend and place in culture flasks (14 ml for T75 flasks, 28 ml for T150 flasks, 560 for the multilayer flasks) (*see* **Note 9**);

- check cell presence in the flasks under the phase-contrast microscope;
- place the flasks in the CO_2 incubator.

7. Collect samples for microbiological tests in case of suspected contamination (yellow color, unusual morphology) as in Section 3.1 and stop the procedure until results (*see* **Note 5**).

3.6 Final Product Packaging and Reference Samples Generation (See Notes 1 and 2)

1. Warm reagents and prepare final medium: basal medium supplemented with L-glutamine (no FBS) (*see* **Note 11**).
2. Trypsinize and count the cells as detailed in Section 3.3, until centrifugation step.
3. Gently shake the pellets and add 40 m final medium (*see* **Note 8**).
4. Centrifuge 7 min at 500 × *g*, at room temperature.
5. Repeat steps 3 and 4 (*see* **Note 11**).
6. Under the biohazard hood transfer the supernatant into sterile tubes from which to collect the following samples that are then to be sent to the proper specialized laboratories of quality control:
   - 1 ml with a sterile syringe to inoculate in a BacT/ALERT® Aerobic Culture Bottle (SA);
   - 1 ml with a sterile syringe to inoculate in a BacT/ALERT® Anaerobic Culture Bottle (SN) (*see* **Note 5**);
   - 2 ml with a sterile pipette to be placed in a proper tube for PCR mycoplasma detection;
   - 0.5 ml with a sterile pipette to inoculate in an a-pyrogenic Bottle for endotoxin testing;
   - 0.5 ml with a sterile pipette to be placed in a tube and frozen as counter sample at −80 °C.
7. Gently shake the pellets, add final medium in order to reach a 1–2 × 10^6 cell concentration.

   Resuspend and collect the following samples which are then to be sent to the proper specialized laboratories of quality control (*see* **Notes 5** and **12**):
   - 1.2 × 10^6 cells in micro-tubes for the phenotypical analysis (immuno-phenotype and tri-lineage ability);
   - 2.0 × 10^6 cells for karyotyping;
   - 1.0 × 10^6 cells in a tube as a counter sample to be frozen as in Section 3.3.
8. Transfer cell suspension into proper, identified tubes (primary container) (Fig. 4a)

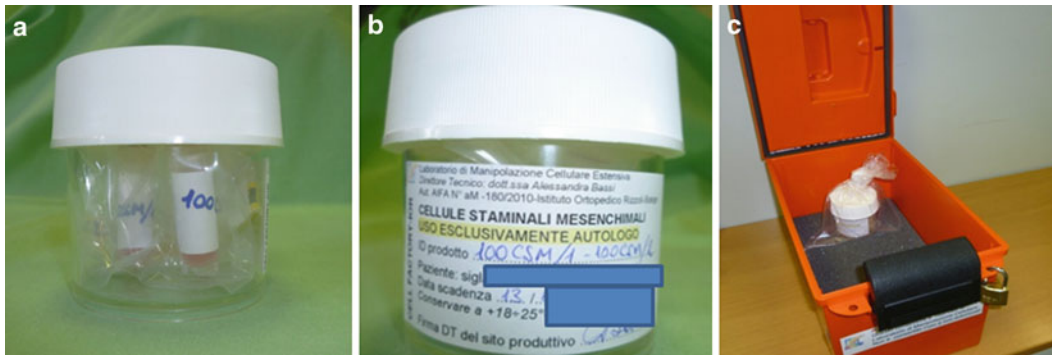

Fig. 4 Final product packaging. The final cellular products is transferred into proper tubes (primary container, **a**), then put it into secondary sterile bags (secondary container, **a**). The product is then inserted in a plastic jar, properly labeled (**a** and **b**). For the shipment a container with lock is utilized (**c**)

9. Close each tube and put it in a secondary plastic sterile bag (secondary container) (Fig. 4a).
10. Close and insert in outer jar container (Fig. 4b).
11. Put a label on the outer container, which must contain (Fig. 4b):
    - identification of the Manufacturer;
    - identification of the product;
    - indication of the use (i. e. autologous use only);
    - batch number;
    - identification code of the patient;
    - expiration date;
    - signature of the person in charge.
12. Exit the Cleanroom and store the final product at room temperature for a maximum of 72 h.
13. For the shipment use a container with lock (Fig. 4c).

4 Notes

1. To avoid the risk of cross-contamination during manipulation it is required to use disposable materials and not to share reagents between cultures. Moreover, it is not possible to simultaneously process cultures from different patient, but only sequentially and after decontamination of the biohazard hood and relative equipment.
2. Sampling for environmental (microbiological and particles) control is required during each phase of the process. At the

end of cell manipulation, each operator should take glove prints on agar plates. Recommended limits for contamination are indicated in EudraLex-Volume 4 GMP Guidelines (5).

3. A peripheral blood sample from each patient is needed in order to evaluate the presence of transmissible pathologies (Acquired Immune Deficiency Syndrome, Hepatitis B and C, and syphilis) (12). If a GMP-facility does not have the possibility to manipulate the infected cells in separated areas, positive patients must be excluded. Therefore, manipulation can start only after negativity is stated. This usually takes 1 or 2 days. In the meantime the bone marrow samples can be stored at 5 ± 3 °C.
4. Density-gradient separation method is considered a standard step for MSC isolation from bone marrow. However, such a procedure is time consuming and reagents are not often approved for clinical use. Therefore many GMP-facilities are trying to develop alternative methods, such as direct or diluted bone marrow seeding (9).
5. Microbiological control and Mycoplasma analysis are performed in the critical steps of isolation, thawing, and final product packaging. Isolation and thawing criticism have been established by our GMP-facility on the basis that cell material is newly introduced or reintroduced in the cleanroom, respectively. Being "final product packaging" the most critical step since cells are then implanted in the patient, additional sampling for quality control analyses are required, such as for endotoxin detection, viability, phenotype, and genotype stability evaluation (4, 5, 7). If a cell contamination is detected, the process must be stopped. Since the here described orthopedic applications are not lifesaving, contaminated cells cannot be sterilized by irradiation or other treatments and must be discarded.
6. In the "first medium change step" matrix and red blood cells presence (Fig. 3a) should be reduced by two PBS washes. This makes also possible to check cell presence under the phase-contrast microscope (Fig. 3b). Usually, debris gradually reduces until disappearance in 7-8 days. However, if residues still persist in the cultures, a trypsinization, which in general dissolves all of them, is recommended. The first medium change is then performed using half of the centrifuged supernatants of the isolation step and half fresh complete medium. The supernatants, centrifuged in order to remove matrix debris and red blood cells, are utilized since rich of soluble factor produced by the cells themselves that can be useful for adhesion and growth. On the other hand, since it can carry also catabolic factors, fresh medium is added to enhance cell metabolism.

7. If the intermediate product is not to be frozen, it is possible to switch directly out 3.2 Section to 3.5 one. Freezing procedure should be slow in order to lead to the deposition of ice in the extracellular environment. In fact, the rapid freezing leads to the formation of ice crystals within the cells, leading to the rupture of the plasma membrane at the time of thawing. Since the DMSO makes cell membranes permeable, the freezing mixture encloses also FBS at high concentration in order to maintain cell integrity. To avoid contamination (for example, mycoplasma), and for safety reasons, it is recommended to store cell cryovials in the upper gas phase and not to soak them in liquid nitrogen. Moreover, if the cryovials are stored by immersion in liquid nitrogen, the nitrogen may penetrate the samples. As a consequence, during defrosting, the vaporized nitrogen can generate a high pressure resulting in an explosion and release of material. During handling of cryogenic frozen vials, adequate protection measures should always be followed by wearing safety glasses and gloves and working on a suitable work surface.
8. After each centrifugation the supernatants are discarded and a first cell resuspension is performed by gently shaking the pellets (or giving little shots to the tube). In fact small volumes allow the resuspension to occur without forming aggregates. The proper volume of medium can be then added and cells will be more easily resuspended with a pipette.
9. Increasing evidence in the literature suggests that MSC seeding density affects their proliferation rate and required functions (6). Some clinical trials have involved high cell density, but some studies highlighted that lower densities have faster proliferation than those cultured at higher densities. However, low or very low densities requires wide culture surface and can be difficult to manage. A plating density of 1,000 cells/cm^2 has been suggested by Sensebè et al. (6) for a high number of harvested cells. However, it is important for each GMP-facility to investigate and identify the "optimal" cell density condition, taking into account cell type and target, clinical protocol, application (local o systemic), and equipment/technology.
10. In the expansion phase, cells are checked twice a week and, depending on their density status, a medium change or a trypsinization occurs. Based on our experience and given cell's biological variability, the number of trypsinization may vary between 1 and 2.
11. FBS, besides animal origin related problems, may imply immune responses in patients. We justify our choice to use FBS in monolayer conditions because it allowed a better cell growth standardization. However, since its potentially dangerous action could not be ignored, we decided to avoid FBS

presence in the final products. Therefore, in order to reduce/minimize FBS presence, cells are centrifuged twice and the final product is resuspended in a serum free medium. However, it is possible that FBS residues remain in the suspension and this must be highlighted in the accompanying documentation.

12. The problem with living cells products is that they need to be implanted before most quality control results are completed. Therefore, a robust process validation must be performed before starting the clinical trial and a careful risk analysis must be carried out in order to assess the corrective actions to be taken if the results are out of specification (e.g., the administration of antibiotics to a patient in case of microbial contamination). As a precaution we decided to routinely give the permission to implantation only after 24 h occur from product preparation, time that allows at least endotoxin results to be already available.

Acknowledgments

This work was supported by the grant "Progetto di Medicina Rigenerativa" from "Regione Emilia Romagna" (Delibera di Giunta n. 2233/2008).

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Methods in Molecular Biology (2015) 1283: 187–198
DOI 10.1007/7651_2014_102

Published online: 26 July 2014

Culture of Human Limbal Epithelial Stem Cells on Tenon's Fibroblast Feeder-Layers: A Translational Approach

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Abstract

The coculture technique is the standard method to expand ex vivo limbal stem cells (LSCs) by using inactivated embryonic murine feeder layers (3T3). Although alternative techniques such as amniotic membranes or scaffolds have been proposed, feeder layers are still considered to be the best method, due to their ability to preserve some critical properties of LSCs such as cell growth and viability, stemness phenotype, and clonogenic potential.

Furthermore, clinical applications of LSCs cultured on 3T3 have taken place. Nevertheless, for an improved Good Manufacturing Practice (GMP) compliance, the use of human feeder-layers as well as a fine standardization of the process is strictly encouraged.

Here, we describe a translational approach in accordance with GMP regulations to culture LSCs onto human Tenon's fibroblasts (TFs). In this chapter, based on our experience we identify and analyze issues that often are encountered by researchers and discuss solutions to common problems.

Keywords: Limbal stem cells, Feeder layers 3T3, Human Tenon's fibroblasts, Cell therapy, Good manufacturing practices (GMPs)

1 Introduction

The discovery of Limbal stem cells (LSCs) (1–3) has opened novel frontiers for regenerative medicine in ophthalmology. To date, the majority of articles in this area have mainly focused on specific challenging issues concerning the correct identification, isolation, and expansion of LSCs and even though a large body of work on the subject is available, these topics are still far from solved.

The standard method to culture LSCs implies feeder layers of murine embryonic 3T3 cells. Although the employment of this technique has led to successful clinical results (4) with no reports of adverse effects thus far (5), the use of a murine embryonic cell line combined with some relevant cell culture supplements such as fetal bovine serum (FBS) in the cocultures, still poses potential xenogeneic and infectious risks.

The 3T3/LSC system has proved to be an efficient and tested methodology; however, further improvements are required but any replacements must preserve specific essential features of cocultures

such as gene expression of stem and corneal differentiation-related markers, appropriate cell growth and viability, clonogenic ability and possibly in vivo regenerative properties.

Various alternative methods to culture LSCs are under evaluation. Some of which are still limited to animal models (6), others range from improved cytofluorometry to identify LSCs (7) to the simulation of 3D-models/sandwiches aiming to recreate niche microenvironments by hydrogels, to separate LSCs from feeder layers or even to reprogram limbal fibroblasts directly into the corneal epithelial lineage (8–11). Even the development of a line of labelled immortalized eliminable human dermal fibroblast cells has been recently described (12).

Before clinical trials can take place, a consolidated research methodology needs to be adapted to a clinical/translational approach. Consequently, the setting up of Good Manufacturing Practice (GMP)-compliant procedures is mandatory. Accordingly, the researcher is required to acquire a broader mentality that makes a research-grade protocol an Investigational Medicine Product (IMP. See Directive 2001/20/EC, Article 2 (d) for IMP's definition).

Firstly, to comply with GMP regulations (international and national guidelines; specific European Directives are designed for European member states. See www.ema.europa.eu), each single step throughout the procedure needs to be standardized in order to assure safety, reproducibility, and efficacy of the IMP at issue. In combination with the careful scientific and practical assessment of the process, guidelines are set, in order to help to harmonize the whole system.

In the present chapter, we describe step by step an alternative procedure to culture LSCs on human origin feeder layers such as Tenon's fibroblasts (TFs). We recently demonstrated that TFs are eligible candidates as GMP-grade replacement in compliance with European directives and standards (13, 14). Herein we propose a double scenario where autologous or heterologous TFs can sustain LSC growth in vitro. However, it is not unconceivable that the development of a fully autologous coculture system could potentially overcome the infectious and xenogeneic risks even compared with the heterologous system. Autologous fibroblasts as autologous serum have a major disadvantage, as they hold an intrinsic genetic variability (15). Nevertheless, autologous cell culture systems represent a better option than murine systems (3T3) to overcome not only safety and regulatory critical issues, but also to provide the best in vitro microenvironments, in order to more accurately mimic the physiological scenario (14). Besides, we cannot rule out the possibility that cocultures of murine embryonic 3T3 and human LSCs could lead to formation of hybrid cell lines.

This chapter focuses on providing more than simple suggestions such as reagent concentrations and techniques acceptable for GMP-grade protocols. We have attempted to highlight the

criticalities of the described process (which are indicated as Notes) in order to walk the reader through the though process required to scale up to a clinical/translational approach.

Finally, based on our experience we also discuss effective alternatives and/or precautions that arise to implement the process where possible.

2 Materials

All surgery procedures should be carried out only by trained medical staff. Written informed consent from patients before surgical procedure is mandatory. Serological test results for HIV, Hepatitis B and C, showing no sign of infections should be also obtained.

The protocol has to be conducted in compliance with the tenets of the Declaration of Helsinki for experiments involving human tissues. Procedures must always be performed in accordance with the ethical standards of the Ethic Committee of the hospital.

All cell culture material should be handled in a class II biological hood using aseptic techniques and following GMP regulations. To date, many cell culture reagents are available as GMP-grade, and therefore the use of such products is encouraged. Lastly, follow all waste disposal regulations.

2.1 Preparation of Autologous Serum (AS)

1. Use Vacutainer® Blood Collection tubes containing clotting agents (B&D, Franklin Lakes, NY, USA) in order to obtain AS.
2. The transport of the sample should be at room temperature (20–25 °C).

2.2 Preparation of Feeder Layers

1. Obtain Human TFs as previously described (13) (*see* **Note 1**).
2. Prepare culturing medium; DMEM complete medium composed of DMEM high glucose, 10 % AS or FBS, 1 % nonessential amino acids (1×), 2 mM L-glutamine, and 100 IU/ml Pen/Strep. Store at 3–4 °C up to 3 weeks. If the medium turns fuchsia, fresh medium must be prepared.

2.3 Mitomycin C Inactivation of Feeder Layers

1. Preparation of Mitomycin C (MMC, M4287, Sigma-Aldrich, St. Louis, MO, USA): perform this step using a class II biological hood, without direct light as MMC is light sensitive. A face mask is also recommended. Prepare a stock (1 mg/ml), weighting 1 mg of MMC. Carefully dissolve the powder in 1 ml of DMEM (sterile PBS can be also used). Protect MMC from the light by wrapping the reagent in aluminum foil. Store at 3–4 °C for maximum of 7 days (*see* **Note 2**).
2. DMEM complete medium w/o AS or FBS.
3. Keratinocytes growth medium (KGM, Lonza).
4. Incubator (5 % CO_2 in air).

2.4 Cocultures of LSCs onto TF Feeder Layers

1. Balanced Salt solution (BSS, Alcon® Laboratories, Inc. Fort Worth, Texas 76134, USA).
2. No. 21 scalpel.
3. Collagenase type I (Gibco®, Life Technologies, Italy). Prepare a stock (1 mg/ml), weighting 1 mg of Collagenase and resuspending in 1 ml of Hank's Balanced Salt Solution (HBSS). Filter the solution through a 0.22 μm sterile filter using a syringe. Then store the reagent at −20 °C until use.
4. On the day of the experiment, prepare fresh enzymatic solution composed of: 0.05 % Trypsin–0.02% EDTA and 1 μg/ml Collagenase type I.
5. Keratinocytes growth medium (KGM, Lonza) complete: add 0.2 % AS or FBS and 100 IU/ml Pen/Strep to KGM medium and protect from light.
6. EGF (Miltenyi Biotec, Calderara di Reno, Bologna, Italy).
7. Optical microscope.
8. PBS–EDTA 0.02 %.

2.5 Immunofluorescence

1. Round glass coverslips.
2. 4 % paraformaldehyde.
3. 0.5 % Triton X-100.
4. PBS–0.25 % Gelatin.
5. Blocking buffer composed of PBS–0.2 % Gelatin.
6. PBS.
7. Incubation buffer composed of: PBS–0.25 % Gelatin–0.1 % Triton X-100.
8. p63α antibody (Cat. N. 4892S Cell Signaling, MA, USA).
9. Vimentin antibody (Santa Cruz, CA, USA, clone V9).
10. KRT3 antibody (Millipore Merck, MA, USA, Clone AE5).
11. Secondary antibodies (Alexa Fluor 488 and Alexa Fluor 594, Invitrogen, Life Technology, Italy).
12. DAPI (4′-6′-Diamidino-2-phenylindole, powder ≥98 %; Sigma, St. Louis, MO, USA). Prepare the stock solution according to the manufacturer's instructions.
13. Mounting medium.
14. Fluorescent microscope.

2.6 Colony-Forming Efficiency Assay (CFE Assay)

1. 60 mm petri dishes.
2. 4 % paraformaldehyde.

3. Rhodamine B (Sigma, St. Louis, MO, USA). Prepare a 1 % Rhodamine B solution with PBS. Use a face mask when weighting and protect from the light.
4. Optical microscope.

3 Methods

3.1 Preparation of Autologous Serum (AS)

1. Obtain 5–10 ml of blood in Vacutainer® tubes from the patient before surgery. Process the sample within 3 h (*see* **Note 3**).
2. Carefully transfer the blood from each Vacutainer® into a sterile 15 ml Falcon tube using a sterile pipette, then centrifuge the blood at 2,095 rcf (×*g*) for 20 min at room temperature.
3. After centrifugation, aspirate the supernatant (which contains the serum) and transfer it into a clean 15 ml tube. Spin again at 335 rcf (×*g*) for 10 min at room temperature. Aspirate the supernatant (*see* **Note 3**) and transfer it into a new clean 15 ml tube.
4. Filter the serum through a 0.22μn sterile filter using a syringe. Then store the sample at −20 °C until use (*see* **Note 4**).

3.2 Preparation of Feeder Layers

1. Culture TFs with DMEM complete medium as already described (13) (*see* **Notes 1** and **Note 5**).

3.3 Mitomycin C Inactivation of Feeder Layers

1. Seed TFs (2.4×10^4 cells/cm^2) in DMEM complete medium. The following day, remove the medium, supplement with fresh complete DMEM w/o AS/FBS, and add MMC (2.5 μg/ml), then incubate for 2 h at 37 °C (*see* **Note 6**).
2. At the end of the incubation period, aspirate off the medium and wash TFs 3× to remove the MMC, then incubate in KGM medium until LSCs are ready to be seeded.

3.4 Cocultures of LSCs onto Feeder Layers

1. Keep the limbal biopsy in BSS until use (*see* **Note 7**).
2. Wash the explant extensively with 1 % Pen/Strep for 20 min, then chop the tissue into pieces of ~0.5–1 mm using a sterile no. 21 scalpel.
3. Collect the small tissue fragments into a sterile 2 ml eppendorf and add 1 ml of the enzymatic solution (*see* **Note 8**).
4. Incubate for 90 min using a shaking water bath at 37 °C. Vortex the sample every 30 min.
5. Afterwards remove and discard the undigested tissue using forceps, then add 1 ml of complete KGM to the homogenate, to inactivate the enzymatic solution.

6. Centrifuge at 335 rcf (×*g*) for 10 min. Discard the supernatant, resuspend the cells in 1 ml of complete KGM, and perform the cell count by Trypan Blue (*see* **Note 9**).
7. Seed the cells (2.5×10^4 cells/cm^2) in KGM complete medium (*see* **Note 10**) onto confluent TFs (previously treated with MMC, *see* Section 3.3).
8. After 72 h add 10 ng/ml EGF to the culture, replacing half of the medium with fresh complete KGM (*see* **Note 11**).
9. Incubate the coculture for 10 days, repeating step 7 every 3 days (*see* **Notes 11** and **12**).
10. At the end of the incubation time, in order to separate LSCs from TFs, remove the medium, add PBS–EDTA 0.02 % and gently tap the plate or the petri dish, using the palm of your hands to release TFs (*see* **Note 13**). Then, remove the medium and trypsinize the remaining LSCs.

3.5 Immunofluorescence

1. Place one or more round glass coverslips on the bottom of the plate before plating TFs (*see* Section 3.3), then coat the glass coverslip/s with PBS–0.25 % Gelatin and incubate for 30 min. Wash 2× with PBS and proceed as described in Section 3.3 (*see* **Note 14**).
2. When the coculture is ready to be arrested, remove the round glass coverslip/s using sterile forceps and place each slide onto a new microscope slide. Wash 2× with PBS, then fixed with 4 % paraformaldehyde for 10 min.
3. Afterwards, permeabilize the coverslip/s with PBS–0.5 % Triton X-100 for 10 min, incubate in blocking buffer for 1 h at room temperature then washed 2× in PBS.
4. Prepare the primary antibodies p63α (1:200), Vimentin (1 μg/ml), or KRT3 (10 μg/ml) in the incubation buffer and incubate overnight at 4 °C.
5. The following day, wash 3× with PBS, then incubate with the appropriate secondary antibody (1:1,000 dilution) at room temperature for 1 h (*see* **Note 15**).
6. Wash 3× with PBS, then perform a staining for DAPI to identify the nuclei. Wash again 3× with PBS, then mount the slides using the mounting medium, before acquiring images by fluorescent microscope (*see* Fig. 1).

3.6 Colony-Forming Efficiency Assay (CFE Assay)

1. After obtaining the single cell suspension from the biopsy, seed LSCs (1×10^3/cm^2) onto MMC-inactivated TFs in 60 mm petri dishes (*see* **Note 16**).
2. After 14 days of incubation, fix the colonies with 4 % paraformaldehyde. Stain with 1 % Rhodamine B for up to 1 h. Wash 3× with PBS. Protect petri dishes from the light.

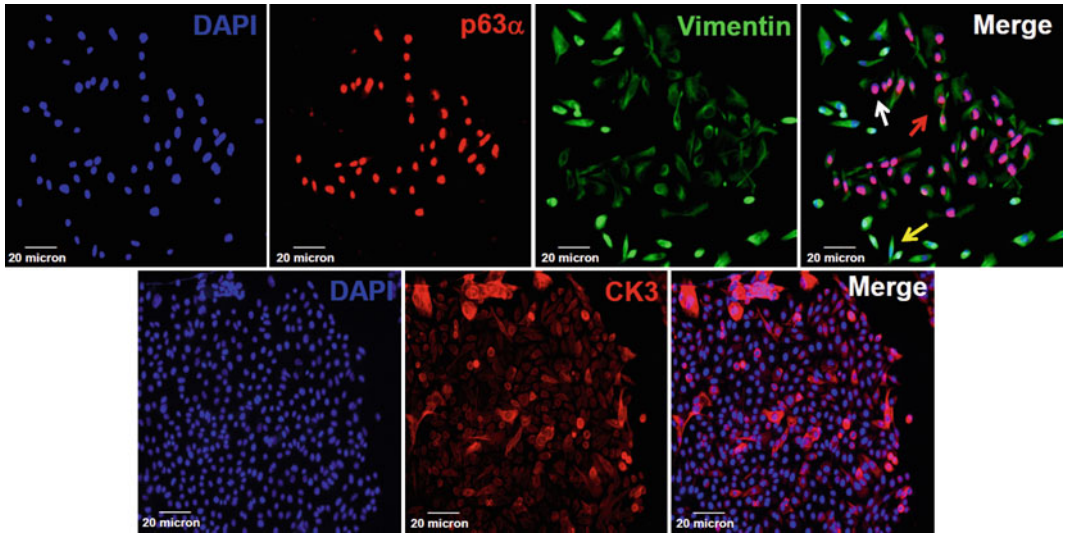

Fig. 1 Immunofluorescence of LSC cocultures onto TFs. LSCs can be identified by positive expression of p63α. In particular, in our system we can describe the following phenotypes: $DAPI^{+}/p63\alpha^{high}/vimentin^{-}$ LSCs (*white arrow*), $DAPI^{+}/p63\alpha^{high}/vimentin^{low}$ LSCs (*red arrow*), and $DAPI^{+}/vimentin^{high}$ TFs (*yellow arrow*). In addition, cocultures are positive for KRT3, which is specifically found in the corneal epithelium. *Blue*, DAPI; *Red*, p63α and KRT3; *Green*, vimentin

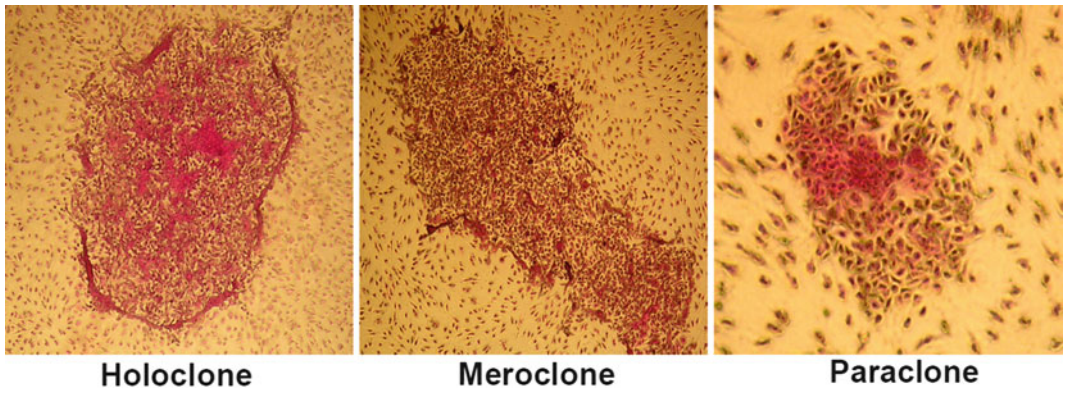

Fig. 2 CFE Assay. Optical images (Rhodamine-B staining) of LSCs isolated from patients and immediately seeded onto TFs. LSCs are able to give rise to holoclones, meroclones, and paraclones, which can be identified according to their morphology (*round*, *irregular*, and *square shape*, respectively). Magnification 5×

3. Wash 3× with tap water.
4. Using an optical microscope, enumerate and classify the clones (holoclones, meroclones, and paraclones). Then calculate the CFE, according to the following formula: CFE (%) = (Colonies formed at the end of growth period/Total number of viable cells seeded) × 100, as previously described (14, 16, 17) (*see* Fig. 2).

4 Notes

1. It is essential that autologous or heterologous TFs are obtained 2–3 months before scheduling the withdrawal of limbal biopsy. Alternatively, a TF cell bank can be set up by expanding TFs for cryopreservation. In this case, freezing and defrosting procedures as well as detection of viruses and other microorganisms in vitro and/or in vivo (*see* ICH guidelines at www.ich.org) must be validated in advance. In addition, the use of TFs should be limited to passages 3–6 in order to avoid cell senescence (18, 19) and more importantly potential tumorigenic and/or genetic changes in TFs cultures. Regarding the use of FBS, *see* **Note 4**.
2. If precipitates are observed in the MMC solution, it is recommended to dispose of the sample and prepare a fresh stock.
3. Blood sample can also be obtained the day before surgery. In this case, it can be stored at 4 °C overnight, to spontaneously allow the separation between serum components and erythrocytes. In addition, obtaining hemolyzed blood samples frequently occurs. In this case, it is recommended to proceed with a different alternative (*see* **Note 4**), instead of using erythrocytes-enriched serum, which is expected to interfere with the cell culture yield.
4. When heterologous cocultures (TFs and LSCs derived from different patients) are performed, AS has to be obtained from the limbus donor. If AS is not available or the sample is hemolyzed (*see* **Note 3**), it is required to consider safe and effective alternatives but from a GMP-standpoint. If this is not possible, it is essential to justify that the reagent cannot be excluded from the coculture, unless substantial changes in the coculture efficiency are introduced. In this specific case, the researcher should consider GMP-grade FBS (pathogen inactivated South American, New Zealand or Australian origin. Also *see* at http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/06/WC500143930.pdf "Guideline on the use of bovine serum in the manufacture of human biological medicinal products), by assessing its performance in advance. We suggest to test products from three different companies and to evaluate at least three different lot numbers to identify the best product. In our experience, we have demonstrated that in TFs cultures AS and FBS exhibit comparable proliferative properties (13). In addition, considering that the percentage of AS in the coculture media is extremely low (0.2 %), the replacement with FBS should not compromise the cell-system and it should contain the minimum risk. It seems that the advantage of using AS is more related to a safety issue than a beneficial effect on cells.

5. Routinely quality/safety control tests (at each passage, before freezing or after defrosting) on TFs cultures have to be performed. Among them, cell viability (by Trypan Blue) and sterility checks (for instance blood agar microbiological plates) are essential and critical, because they assure that from the starting material to the final product, there is no risk of infection. Other quality tests such as cell growth assessment or immunofluorescence for Vimentin (the most frequently found intermediate filament in fibroblasts) can also be performed in order to assess the quality of the culture.
6. TFs can be seeded into 12-well plates, 60 or 100 petri dish according to your needs. We have already validated the effective concentration of MMC (2.5 μg/ml MMC) to arrest TFs (14). Although feeder layers such as 3T3-J2 can be lethally irradiated as elsewhere described (20), MMC represents a valid alternative to irradiation to inhibit feeder layer growth with equal efficacy (21). Besides, from a practical standpoint, the irradiation could require additional facilities, whereas MMC can be directly used in a cell factory.
7. The dimension of a limbal biopsy normally ranges between 1 and 2 mm^2. The processing of the tissue should be performed within 3 h, in order to avoid loss of cell viability. The limbal tissue should always be derived from the superior limbus, as it is well demonstrated that this region represents the most LSC-rich zone (3).
8. To avoid products of animal origin, including trypsin (normally of porcine origin), it is recommended to use alternatives such as the bacterial or plant derived recombinant trypsin, provided that they are properly validated. As stated in a recent document by the European Medicine Agency: "The use of bacterial or plant derived recombinant trypsin minimises in principle the risk for animal virus contamination and the application of such alternatives is therefore encouraged." However, no general recommendation to replace porcine trypsin can currently be given considering that these alternatives need a careful assessment of suitability, quality, sterility, and performance characteristics (*see* at http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139532.pdf).
9. Cell lamps at this stage are likely to form when counting. Therefore, it can be useful to pass the solution through a 21 G syringe before counting.
10. In our experience, cocultures should be left undisturbed for the first 72 h of incubation. Besides, it is recommended to add only a thin layer of KGM complete medium, in order to optimize the contact between LSCs and TFs.

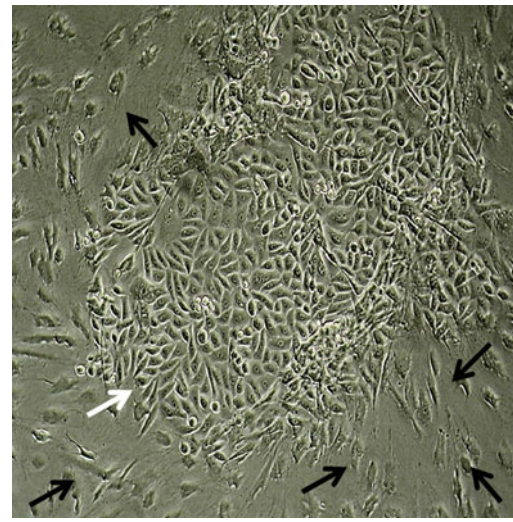

Fig. 3 Optical image of an LSC coculture onto TFs. Note the keratinocyte-like epithelial area of LSCs (*white arrow*) surrounded by TFs (*black arrows*) after 10 days of culture. Magnification 5×

11. The medium should be not completely removed, due to the presence of important growth factors and/or cytokines that enhance cell growth. A proper expansion of LSCs should assure a great number of LSCs combined with a consistent level of progenitor cells throughout passages in the coculture. However, repeated passages in vitro could cause depletion of the isolated progenitor stem cell pool and senescence, thus we suggest to limit the serial cultivation to a defined number of passages in vitro.
12. Morphological changes can be monitored by optical microscope. A well performed coculture should show LSCs with distinctive keratinocyte-like morphology, forming an epithelial layer surrounded by TFs (*see* Fig. 3). Moreover, at this stage it is possible to enhance LSC expansion using cholera toxin (30 ng/ml). Even though GMP compliant cell cultures should be both free of animal derived products and exogenous growth factors, cholera toxin could be suitable to enhance cell proliferation, particularly when a low number of LSC is obtained after isolation.
13. The use of PBS–EDTA 0.02 % allows to remove only TFs, whereas LSCs can be obtained by trypsinization and used for quality control tests.
14. Cytospin centrifuge (4 rcf (×*g*) × 4 min) can be used instead of round glass coverslips. However, it is important to highlight that the use of the Cytospin in this case does not preserve the cell morphology well. Consequently, TFs and LSCs become morphologically indistinguishable, unless a double staining (p63α and vimentin) is performed.

15. The putative stem cell marker p63α is a critical quality control test to evaluate the percentage of LSCs present in the final cocultures. Vimentin is equally essential, because it allows to both detect and exclude contaminating fibroblasts that normally express high levels of vimentin, but also to quantify the number of LSCs $p63\alpha^{+}$/$vimentin^{low}$, as we and others have previously described (14, 16, 17, 22, 23). The use of KRT3 is useful to confirm that the cocultures are specifically differentiated into corneal epithelial cells.

    In addition, the three markers (p63α, Vimentin, KRT3) can be also detected by Real Time PCR as we previously described (14); however, immunofluorescence is preferable as it detects the protein and it allow for direct visualization and discrimination of both LSC and TF populations in the coculture.
16. The clonogenic potential is strictly patient dependent. This test can be used to both evaluate and quantify the percentage of CFE of patients just after LSC isolation, but also to evaluate the quality of the cocultures with regard to the number of LSC progenitors throughout cell culture passages. Clones can be enumerated and characterized according to their unique morphology (holoclones, meroclones, paraclones), as previously described (14, 24, 25).

Acknowledgement

We thank Fondazione Roma and Colin Murdoch for their generous help. The corresponding author personally dedicates this chapter to all goodwill Italian researchers, whose contribution is still vital for the country in such difficult times. This manuscript was financially supported by University of Rome "Sapienza," Grant "Ateneo 2011 Prot. C26A11MP7J," Department of Science and Medical-Surgical Biotechnologies, University of Rome "Sapienza," Polo Pontino, Latina, Italy.

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Methods in Molecular Biology (2015) 1283: 199–218
DOI 10.1007/7651_2014_117

Published online: 25 December 2014

Bioreactor Expansion of Human Mesenchymal Stem Cells According to GMP Requirements

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Abstract

In cell therapy, the use of autologous and allogenic human mesenchymal stem cells is rising. Accordingly, the supply of cells for clinical applications in highest quality is required. As hMSCs are considered as an advanced therapy medicinal products (ATMP), they underlie the requirements of GMP and PAT according to the authorities (FDA and EMA). The production process of these cells must therefore be documented according to GMP, which is usually performed via a GMP protocol based on standard operating procedures. This chapter provides an example of such a GMP protocol for hMSC, here a genetically modified allogenic cell line, based on a production process in a microcarrier-based stirred tank reactor including process monitoring according to PAT and final product quality assurance.

Keywords: hMSC, GMP protocol, SOP, PAT, Authorities

1 Introduction

As described in several publications, the potential and importance of autologous and allogenic human mesenchymal stem cell application in cell therapy is rising (1–3). To allow clinical trials and medical treatment of patients, the production of allogenic or autologous cells is a challenge, as the cells are treated as an advanced therapy medicinal product (ATMP) underlying the guidelines of good manufacturing practice (GMP) given by the authorities of the European Medicines Agency (EMA) and the Food and Drug Administration (FDA). This should assure medical grade cells with defined quality characteristics that are safe for the patient. Special characteristics of hMSCs include "their high differentiation potential, their ability to migrate to sides of injured tissue after implantation or intravenous injection in order to control the inflammation process, their use among cell death and tissue regeneration due to the release of cytokines, and also their specific growth factors, antioxidants and other bioactive molecules that are present in an effective therapeutic dose" (4).

As clinical trials show, to achieve a cell dose-dependent efficacy of the treatment, a minimum of 1.5–6 × 10^7 cells per single dose, depending on the type of indication, is required (5, 6). Therefore, large production processes for hMSC, fulfilling GMP requirements, are needed. The success of a commercial product therefore depends on the robustness, reproducibility, and efficiency of the GMP process assuring final product quality (7–9).

In good manufacturing practices, quality cannot be determined by testing the finished product; the process must underlie close control systems to assure quality, safety, and efficacy of the final product and must be established throughout the production process (10, 4).

A good overview on the regulatory responsibilities is given by Unger et al. (11) as well as by Oppermann et al. (4). For Europe, the EU Regulation 1394/2007, guideline EMEA/CHMP/410869/2006, directive 2004/23/EC as well as commission directives 2006/17/EC and 2006/86/EC by the EMA are of major importance and commissions are continuously improving and enhancing these. In the United States, the code of Federal Regulation (CFR) by the FDA provides a set of regulations, being divided in different sections: investigational new drug applications (INDs: 21 CFR 312), biological regulations (21 CFR 600), and cGMP (21 CFR 211).

To follow these regulations, usually standard operating procedures are applied. Such SOPs must be available for every process step that may have an impact on the quality of the product. To remain document control, the SOPs underlie a standardized numbering system, format, and content (10).

As described in a literature summary, several processes for the expansion of autologous and allogenic human mesenchymal stem cells have been described (4). The challenge of a GMP-conform process limits the possibilities as the process demands are very high. In contrast to a standard production process of proteins, a living organism is the product, which is related to advanced process and product requirements. Processes are generally provided in pre-expansion of the cells, inoculation, expansion, harvesting (12–18), processing (19, 20), and quality assurance of the final product (21). Going along with the process requirements, there is a variety of methods applied to assure process monitoring and quality assurance throughout the whole process, each following GMP requirements (22).

The specific demands of the product are given or suggested by various institutions, such as the International Society for Cell Therapy (ISCT). The major focus is based on the cell characteristic.

They include the identity of cells including the growth and proliferation characteristics as well as the proof of various surface antigens (23). The purity of product, especially regarding contaminations of bacterial, viral, or mycoplasm origin, is an issue. Finally the potency of the cells must be remained by analysis of adipogenic, chondrogenic, and osteogenic differentiation.

In the following, a GMP protocol based on SOPs is presented serving as an example for the production of allogenic genetically modified human mesenchymal stem cells (hMSC-TERT) within a microcarrier-based 3 l stirred tank reactor (21).

Within this protocol, general standard methods and protocols as well as methods for monitoring and control, such as analytical methods (glucose and lactate concentration, cell thawing, cell passaging, and the application of dielectric spectroscopy (24, 25) as well as offline cell concentration determination of adherent cultures (24)), will be referred to SOPs and not considered in detail. In that case, only the final results are to be inserted into this protocol, but the detailed protocol must be attached to the document. The applied SOPs are ordered according to a code giving information on the type of the SOP and a letter coding (please *see* Chapter 4) for the single procedure step.

Please consider the following information: Any italic written letters and words should serve as an example and must not be taken as granted. Any grey highlighted field must be filled with information during the production process and is specific for each batch. If changes/corrections are made, they must be clearly indicated and signed. The operator usually has a shortcut of two to three letters, which are used for signature.

2 Manufacturing Protocol for Human Mesenchymal Stem Cell Expansion

2.1 Pre-expansion Protocol

The pre-expansion protocol includes the thawing, the pre-expansion of the cells in T75 and T300 flasks, and the preparation of the cell suspension for the inoculation process.

Name of the cell line: *hMSC-TERT*

2.1.1 Thawing and Inoculation Process (Day 0)

Date: _____

Thawing of the cryovial from the MCB (master cell bank) and seeding into one T75 flask according to ***SOP-CP-A-01:*** The procedure will be recorded in the following section.

Used solutions and equipment

Solution	Supplier	Cat. #	Lot:	Signature
89% EMEM (hMSC-culture medium)	*Manufacturer 1*			
2mM Glutamin (Stock 200mM)	*Manufacturer 2*			
10% Fetal Bovine Serum	*Manufacturer 3*			
T75 cell culture tissue flask				
T175 cell culture tissue flask				
T300 cell culture tissue flask				
Serological pipettes (2-50 µl)				
Incubator				
Hemocytometer				
Water bath				
Clean bench				
Microscope				
Phosphate buffer saline	*Manufacturer 1*			
Trypsin/EDTA 1x	*Manufacturer 2*			

Preparation

	Signature
Fill one T-75 flaks with 25± 5 ml medium and pre-equilibrate it in a CO_2-incubator _____(room and incubator number) at 5 % CO_2, 37°C and 95 ± 5 % humidity › 30 min Media *hMSC full medium*_____(name) was prepared at _____(date) by _____(operator) according to ***SOP-MP-A-01.***	

Thawing and Inoculation of the Cells

	Signature
Remove the cryovial from the _____(tank-Nr., location) time:_____ Time of thawing in the water bath at 37°C is ≤5min. Before opening, disinfect the cryovial and dry it under the clean bench. Opening of the cryovial–Nr: _____ Number of cells: _____cells passage:_____ Date, when the cryovial was produced:_____ Time, when the cryovial was opened: _____ Resuspend the content of the cryovial carefully using a 1-ml-pipet and transfer it into the prepared T75 flask. Take a sample of 100 µl and determine the cell concentration and viability of the cell suspension according to ***SOP-TM-A-01:***	

	nominal value by GMP-Protocol-Nr.:_____	actual value
Total Number of Cells	≥$3*10^6$ *cells*	
Viability	≥90%	

Close the T-flask, gently rotate it and incubate it in the CO_2-incubator _____(room and incubator number) at 5 % CO_2, 37°C and 95 ± 5 % humidity.

Replace the media the day after the thawing process: ***(day 1)***	Signature
Date of replacement: _____	
Volume replaced: _____ ml	
Media used: *_hMSC full medium* ____(name) was prepared at _____(date) by _____(operator) according to ***SOP-MP-A-01***	

2.1.2 Passaging of the Cells

The cells are evaluated for confluency and morphological abnormalities. From the thawed cells in one T75 flask, three times passaging is required to achieve eight confluent T300 flasks. The passaging procedure is performed according to ***SOP-CP-B-01***, which also contains the way of calculation for the suspension volume to be transferred. Cell concentration and viability determination were done according to ***SOP-TM-A-01.***

The incubation for cell growth is performed in the CO_2 incubator _____(room and incubator number) at 5 % CO_2, 37 °C and 95 ± 5 % humidity.

Passaging step 1: Passage 1× T75 flask onto 2× T175 flasks (*day 2*): Date: _____

Before passaging

Passage number before		Signature
Microscopic Analysis: □ cells attached □ cells spread □ fibroblastic phenotype % confluence: ___*85%*___ % confluence expected: 80-85%		

Passaging

	Value	Unit	Expected value	Signature
Cell concentration (volumetric)		cells/ml		
Cell concentration (per area)		cells/cm^2	*5-7·10^4*	
Viability		%	*>95%*	
Total cell number		cells	*3.75-5.25·10^6*	
Transferred cell volume		ml		
Inoculation density (volumetric)		cells/cm^2	*5·10^3*	
New Passage number				

Media used: *_hMSC-production medium*_____(name) was prepared at _____(date) by _____(operator) according to ***SOP-MP-B-01***

Passaging step 2: Passage 2× T175 onto 4× T300 ***(day 5), date:*** _____

Before passaging

Passage number before		Signature
Microscopic Analysis: □ cells attached □ cells spread □ fibroblastic phenotype % confluence: _____% confluence expected: 80-85%		

Passaging

	Value	Unit	Expected value	Signature
Cell concentration (volumetric)		cells/ml		
Cell concentration (per area)		cells/cm^2	*5-7·10^4*	
Viability		%	*>95%*	
Total cell number (both flasks)		cells	*1.75-2.5·10^7*	
Transferred cell volume		ml		
Inoculation density (volumetric)		cells/cm^2	*5·10^3*	
New Passage number				

Passaging step 3: Passage 4× T300 onto 8× T300 ***(day 9): date:*** _____

Before passaging

Passage number before		Signature
Microscopic Analysis: □ cells attached □ cells spread □ fibroblastic phenotype % confluence: _____% confluence expected: 80-85%		

Passing

	Value	Unit	Expected value	Signature
Cell concentration (volumetric)		cells/ml		
Cell concentration (per area)		cells/cm^2	*5-7·10^4*	
Viability		%	>95%	
Total cell number		cells	*7-8.4·10^7*	
Transferred cell volume		ml		
Inoculation density (volumetric)		cells/cm^2	*5·10^3*	
New Passage number				

Media used: _*hMSC-production medium*_____(name) was prepared at _____(date) by _____(operator) according to ***SOP-MP-B-01.***

2.2 Preparation of the Bioreactor and the Required Units (Day 10 and 11)

2.2.1 Lists of Chemicals, Solutions, and Equipment that Will Be Used

Solution/ Chemical	Supplier	Cat. #	Lot:	Signature
Microcarrier Name:*Solohill glass-coated* Size:…*125-212 µm* Density: *1.022-1.030 g/cm^3* Surface: *high silica glass*	*Solohill Engineering Inc.*	*G102-1521*		
NaOH 1M				
hMSC-production media ***SOP-MP-B-01***				
2mM Glutamin (Stock 200mM)				
10% Fetal Bovine Serum				
Carbon dioxide gas				
Oxygen gas				

Used Sensors

Sensor	Type	Supplier	Signature
pH			
temperature	*Pt-100*		
Oxygen	*optical*		
dielectric spectroscopy	*12 mm Probe*		

Bioreactor

System	Supplier	Total Volume	impeller	d/D	Reactor material	Signature
3L-bioreactor	*Manufacturer 4*	*3L*	*marine*		*Glass*	
Control unit						

Details on the bioreactor are given in a separate specification document: ***SD-STR-001***.

Single use and other equipment

Material/ Equipment	Supplier	Cat. #	Lot:	Signature
1000 ml glass bottles with olive				
Centrifuge tube 1.5 ml (sterile)				
Aluminum foil				
Serological pipettes (2,5,10,25,50 ml) (sterile)				
Silicon tubes 4 mm inner diameter				
10 ml luer-lock syringes single use				
20 ml luer-lock syringes single use				
50 ml luer-lock syringes single use				
Sterile filters				

2.2.2 Preparation and Sterilization of Equipment (Day 11):

_____*(Date)*

Calibration of the sensors

Sensor	According to SOP-Nr.	Calibration value	Calibration value	Slope	expected range	Date	Signature
pH	***SOP-EQ-A-01***	pH7:_____	pH4: _____				
oxygen	***SOP-EQ-B-01***	0%:_____	100%:_____				

The pH and dielectric spectroscopy should be calibrated by the manufacturer and must be tested frequently.

Sensor	Last testing (date)	Test interval	Next test date	Sensor range	Signature
Temperature					
Dielectric spectroscopy					

Preparation of the bioreactor, further equipment and integration of sensors and units:

The bioreactor will be equipped with all required elements according to ***SOP-EQ-C-01*** and prepared for the sterilization process.

Elements to be connected to/in the bioreactor	Signature
Sparger with a tube and external connection to oxygen and carbon dioxide supply from the control unit	
Sterile filter between gas supply and bioreactor	
Unit for the temperature probe insertion	
Air-off unit with a sterile filter	
Sampling tube reaching close to the bioreactor bottom; 3-way connection with a sterile filter, Luer-lock-connection, clamps	
Silicon tube for the media and carrier addition, with luer-lock connector	
Sensors: Temperature, pH, oxygen, dielectric spectroscopy	

Further units, that must be prepared and must be sterilized

Equipment	Volume	To be connected to the equipment	Signature
1x inoculation bottle with olive at the bottom side	1000 ml	- Air exchange filter - Connection from the olive to the top of the bottle with microcarrier - Clamp to the silicon tube	
1x bottle with microcarrier	1000 ml, 42,15 g Microcarrier with 100 ml PBS	- Air exchange filter - Short tube from the olive to be connected the bioreactor (long silicon tube at the bioreactor) - Clamp to the tube close to the olive - Luer-lock connection unit	
beaker covered with aluminum foil	100 ml		
1x bottle with 1M NaOH	100 ml	- Air exchange filter - Silicon tube reaching from the inside of the bottle over the pump to the reactor - Clamp (close for the sterilization process)	

Sterilization process according to ***SOP-EQ-C-01***:	Signature
Steam-Sterilize all required equipment at 121°C, 1bar 20 min.	
Autoclave used: _____(autoclave number, location)	
Sterilization process successful?:_____(yes/no)	
The sterilization protocol is attached to this document.	

2.2.3 *After the Sterilization Process*

After the sterilization process, the following steps must be performed

	Signature
Close all clamps	
Remove all parts from the autoclave	
Are all units in the condition they must have	
Set the bioreactor and all units to the place of production	
Connect all sensors with the control unit	
Attach the heating jacket to the reactor	
Cover the bottom of the bioreactor with aluminum foil	
Insert the tube of the NaOH-bottle to the pump of the control unit, assure to have the correct direction	

2.2.4 Finishing of the Preparation (Day 11)

_____*(Date)*

Process	Signature
Preheat the prepared culture medium in the waterbath at 37°C for at least one hour.	
Defrost 40 ml trypsin-EDTA-solution and preheat it to 37°C.	
Start the program for process control: _____ (name and version of the software).	
Let the carrier in the autoclaved bottle settle and remove the PBS gently under sterile condition. Add the amount of media to achieve a final volume of ___1.64 l____ using the bottle with the microcarrier and the media bottle attached to it.	
Connect the media+ carrier-bottles to the media tube at the bioreactor under sterile condition. Open the clamp and let all carrier and media run into the bioreactor. The bottle must be gently moved to have the carrier in suspension (to avoid clocking the tube). Detach the tube from the glass bottle and press 10 ml sterile air (syringe and sterile filter) through the tube. Close all clamps.	
Take a sample according to ***SOP-TM-D-01*** with sample number_____ (eg. *S1*)	
Measure the fluorescence intensity of the "blank sample" for the fluorescence assay according to **SOP-TM-B-01**. Average of the measured blank value: _____	
Measure the glucose and lactate concentration of the "blank sample" according to **SOP-TM-E-01**: Average of the glucose concentration: _____ g/l Average of the lactate concentration: _____g/l Are the standard-deviations within the range?: _____(yes/no)	
Measure the glutamine and ammonia concentration of the "blank sample" according to ***SOP-TM-F-01*** and ***SOP-TM-G-01.*** Average of the glutamine concentration: _____g/l Average of the ammonia concentration: _____g/l Are the standard-deviations within the range?: _____(yes/no)	
Start the stirrer and the heating control (*120 upm, 37°C*)	
As the temperature is reached, start the aeration, wait for saturation and control the calibration value for 100% pO_2 according to ***SOP-EQ-D-01 and SOP-EQ-B-01***. Calibration value prior to sterilization:_____ Value after saturation process: _____ Value-overwrite necessary: _____(yes/no)	
Start pH control (*pH7.4*) according to ***SOP-EQ-D-01***	
Prepare the dielectric spectroscopy according to ***SOP-EQ-E-01***: - Critical frequency: 300kHz - Fc-scan: apply - Cleaning: off - Measurement interval: 30s	

2.3 Expansion Process (Starting Day 11) _____ *(Start Date)* _____ *(Start Time)*

Harvest the cells from all eight T300 flasks in parallel. Harvest the cells according to the harvest procedure in ***SOP-CP-C-01***.

Pool the harvest volume of each two T300 flask into one centrifuge tube (250 ml) and determine the cell concentration and viability according to ***SOP-CP-D-01.***

2.3.1 Inoculation

Suspension volume		Cell concentration		Cell viability		Signature
	ml		cells/ml		%	

Centrifuge the cells for *5 min at 250 × g*. Remove the medium and resuspend the cells in an overall volume of minimum 10 ml hMSC-production media to have *9.6 × 10^7 cells/10 ml* in one vial. This allows an inoculation density of *7,500 cells/cm^2*.

	Cell concentration/ number/ viability		Unit	Signature
Vial 1			cells/ml / %	
Vial 2			cells/ml / %	
Vial 3			cells/ml / %	
Vial 4			cells/ml / %	
Total cell number			cells	
Volume to use for resuspension			ml	
Volume to be used for inoculation			ml	
Growth surface in the bioreactor			cm^2	
Final inoculation cell density			cells/ cm^2	

	Signature
Soak up the volume to be used for inoculation with a syringe under sterile conditions and close it with a cap. Soak up *20 ml hMSC-production media* in a separate syringe and close it with a cap. Connect the syringe with the cell suspension to the bioreactor inoculation port and completely insert the suspension. Start the dielectric spectroscopy at a measurement interval of *30 s*. Replace the syringe with the medium-filled syringe and completely insert the medium though the same port to rinse it.	

Note the time: _____ (eg.: 08:30 am) Have the stirrer rotate at 100 rpm for 2 min according to ***SOP-PP-A-01***, take a sample according to ***SOP-TM-D-01***with sample number _____ (eg. S2). Stop the stirrer for 45 min. Repeat the rotation/ stop-phase 4 times: 3. Cycle: sample number:_____ 4. Cycle: sample number:_____ 5. Cycle: sample number:_____	**Signature**

With each sample, perform analysis of the cell density and microscopic analysis according to ***SOP-TM-B-01*** and ***SOP-TM-C-01***. Attach representative pictures from the microscopic analysis of each sample to this document.

Sample number	Fluorescence intensity average netto value	Standard deviation of the netto value	Calculated cell density [cells/cm^2]	Anomaly in the microscopic analysis (yes/no)	Signature
2					
3					
4					
5					

2.3.2 Expansion Process

To start the expansion process, set the rotation speed to 120 rpm. Then remain the process parameters as listed in the following:

Parameter	Value	Signature
Rotation speed	*Start with 120 rpm, increase each by 10 rpm after 72h, 96h, and 120h*	
Oxygen partial pressure	*>60%*	
Aeration rate, when pulsed aeration is active	*0.02 vvm*	
Temperature	*37°C*	
pH	*7.4*	
Volume	*1.68 l*	
Medium	*hMSC-production media*	
Microcarrier	*Solohill glass-coated*	
Carrier concentration	*25 g/l*	
Growth surface	*13600 cm^2*	
Cell density aimed at harvest	*5.5·10^4 cells/cm^2*	

2.3.3 Sampling

During the expansion process, a sample is taken according to ***SOP-TM-D-01*** twice daily with a constant time difference and the following analysis is performed for each sample and the values are inserted into the table on the next page:

Analysis	According to SOP
Microscopy	***SOP-TM-C-01***
Cell density	***SOP-TM-B-01***
Glucose concentration	***SOP-TM-E-01***
Lactate concentration	***SOP-TM-E-01***
Glutamine concentration	***SOP-TM-F-01***
Ammonia concentration	***SOP-TM-G-01***

The materials and equipment required to perform the sample analysis are each listed in the SOP of the method.

The cells are evaluated to confluence and morphological abnormalities. Decisive for the culturing period (3–4 days) is a confluence of 90–98 %.

	Date	**Time**	**Confluence**	**Morphological Abnormalities**	**Signature**
Cell Seed(day0)			----------	----------	
Cell Harvesting (day5or 6)			%		

From each sample, a part of the supernatant is stored as a backup at −20 °C in the following freezer: _____(room, freezer number, box number). Transfer the available values from the blank sample and the last sample of the inoculation process in the table.

sample nr.	date	time	cultivation time	Reaction volume	cell concentration	pO_2	pH	temperature	glucose concentration	lactate concentration	glutamine concentration	ammonia concentration	Ok?	signature
			h	ml	cells/cm²	%		°C	g/l	g/l	g/l	g/l		
0														
5														

2.4 Cell Harvest (ca. Day 16)

_____(Date)
_____
(Start Time)

Prepare the harvest unit according to ***SOP-PP-C-01*** and steam-sterilize it at 120 °C for 20 min using the autoclave _____(room, autoclave number, protocol number).

Attach the sterilization protocol to this document.

2.4.1 Preparation

Used solutions for the harvesting process

Solution	Required Quantity	Storage Temperature	Signature
Medium by ***SOP-MP-B-01***	*250ml*	*5±3°C*	
PBS without Mg^{2+},Ca^{2+}	*1000ml*	*Room Temperature*	
Trypsin/EDTA-Solution	*100ml*	*-20±3°C; remove from the freezer prior to use and remain at room temperature*	

2.4.2 Harvest

_____(Date)
_____(Start Time)

Determined cell concentration prior to harvest: _____ cells/cm^2 according to ***SOP-TM-B-01.***

Process (SOP-PP-C-01)		Signature and comments
Release the media and carrier into the harvest unit	Duration: _____min	
Drain the media (carrier with cells remain)		
Wash the carrier gently with PBS without Mg^{2+},Ca^{2+}	Volume PBS used: _____ml	
Add trypsin and incubate for 15 min at RT	RT:_____°C	
Stop the enzymatic reaction with culture Medium	Volume medium used:	
Transfer the cells to sterile centrifuge tubes		

Suspension volume: _____ml
Cell concentration of the cell suspension: _____cells/ml
Vitality of the suspension: _____%

Centrifuge the suspension at 250g for 10 min.
Remove the supernatant and add it with fresh hMSC-full medium.
Resuspend to achieve *1*10^{6}cells/ml*.

New suspension volume: _____ml

Take a sample of 25 ml cell suspension into a sterile tube for quality control.

Signature

2.4.3 Processing

The cell suspension is processed according to ***SOP-PP-D-01,*** which contains all detailed information.
Final storage information

		Signature
Product form	*Cell suspension* ; concentration: _____cells/ml	
Storage building		
Storage room		
Storage temperature		
Storage unit number		

3 Quality Control (Day 16–36)

Quality analysis is required according to GMP. This is done according to SOPs, here specifically for hMSC.

Prepare the solution required for all quality assurance methods as demanded by EMA/CAT/CPWP/571134/2011.

The methods applied serve to gain the suggested quality information according to the ISCT.

3.1 Identity

The proof of the product identification here is performed by the analysis of the growth characteristics, the proliferation, and cell-specific surface antigens.

The *growth characteristics* were performed according to ***SOP-QA-A-01***, where cells are seeded into 24-well plates and each day, 2 wells are harvested. The cell concentration and viability are determined according to ***SOP-TM-A-01***. The growth rate is calculated according to ***SOP-TM-H-01***. Additionally, the glucose and lactate concentrations are determined daily according to ***SOP-TM-E-01***.
The following results were obtained

Sample	date	time	cell concentration [cells/cm^2]		glucose concentration [g/l]		lactate concentration [g/l]		Signature
			measured	Expected range	measured	Expected range	measured	Expected range	
start									
1									
2									
3									
4									

The *proliferation assay* is performed according to ***SOP-QA-A-02*** based on a WST-1 assay.

The following results were obtained

Sample	date	time	Average blank value	Average netto sample value	Expected range of the netto value	Signature
start						
1						
2						
3						
4						

The cell-specific surface antigen analysis is to be tested using quantitative real-time reverse transcriptase PCR (qRT-PCR). The procedure is to be performed according to ***SOP-QA-A-03***. Details on the procedure and the used solutions are listed with the SOP attached to this document.
The following results were gained:

Surface antigen	Value	Expected range	Signature
CD90			
CD73			
CD105			
CD45	negative		

3.2 Purity

The purity of the product is tested by analysis of bacterial and yeast, viral, as well as mycoplasm-based contamination. This is done according to the SOPs ***SOP-QA-B-01***, ***SOP-QA-B-02***, and ***SOP-QA-B-03***. Details on the procedure and the used solutions are listed within the SOP attached to this document.

Results of the analysis

		Date of Analysis	Signature
Analysis for microbial and yeast contamination (positive/ negative)	*negative*		
Analysis for viral contamination (positive/ negative)	*negative*		
Analysis for mycoplasm contamination (positive/ negative)	*negative*		

3.3 Potency

The potency of the cells is proved by cell differentiation into three different lineages: adipogenic, osteogenic, and chondrogenic according to the specific SOP:

The *adipogenic differentiation* was performed according to ***SOP-QA-C-01***. Details on the procedure and the used solutions are listed with the SOP attached to this document. Pictures of the adipogenic differentiation analysis are attached to this protocol.

Results of the analysis

		Signature
Date of the analysis		
Microscopic settings		
Adipogenic differentiation (positive/negative?)		

The **osteogenic differentiation** was performed according to ***SOP-QA-C-02***. Details on the procedure and the used solutions are listed with the SOP attached to this document. Pictures of the osteogenic differentiation analysis are attached to this protocol.

Results of the analysis

		Signature
Date of the analysis		
Microscopic settings		
Osteogenic differentiation (positive/negative?)		

The *chondrogenic differentiation* was performed according to ***SOP-QA-C-03***. Details on the procedure and the used solutions are listed with the SOP attached to this document. Pictures of the chondrogenic differentiation analysis are attached to this protocol.

		Signature
Date of the analysis		
Microscopic settings		
chrondrogenic differentiation (positive/negative?)		

4 Attached SOPs

According to the GMP protocol, various SOPs are used, which are listed in the following to remain documentation control:

4.1 For Cell Preparation: SOP-CP-. . ..

		Signature
SOP-CP-A-01	From the cryovial	
SOP-CP-B-01	Cell passaging	
SOP-CP-C-01	Cell harvest from the pre-culture	
SOP-CP-D-01	Cell preparation for the bioreactor inoculation	

4.2 For Media Preparation: SOP-MP-. . ..

		Signature
SOP-MP-A-01	hMSC full medium	
SOP-MP-B-01	hMSC production medium	

4.3 For Equipment-Related Policies and Preparation: SOP-EQ-. . .

		Signature
SOP-EQ-A-01	pH probe calibration	
SOP-EQ-B-01	pO_2-probe calibration	
SOP-EQ-C-01	Bioreactor Equipment and sterilization	
SOP-EQ-D-01	Process control preparation and settings	
SOP-EQ-E-01	Dielectric spectroscopy handling and settings	

4.4 For the Production Procedure: SOP-PP-. . .

		Signature
SOP-PP-A-01	Inoculation of the bioreactor	
SOP-PP-B-01	Cell expansion in the bioreactor	
SOP-PP-C-01	Cell harvest from the bioreactor	
SOP-PP-D-01	Cell processing	

4.5 For the Test Methods/Analytical Methods: SOP-TM-. . .

		Signature
SOP-TM-A-01	Cell concentration determination of suspended cells	
SOP-TM-B-01	Cell concentration of hMSC-TERT on microcarrier using the fluorescence assay	
SOP-TM-C-01	Optic cell analysis by fluorescence microscopy	
SOP-TM-D-01	Sampling from the bioreactor	
SOP-TM-E-01	Glucose and lactate concentration determination	
SOP-TM-F-01	Glutamine concentration determination	
SOP-TM-G-01	Ammonia concentration determination	
SOP-TM-H-01	Growth rate calculation	

4.6 For the Quality Assurance Methods: SOP-QA-...

		Signature
SOP-QA-A-01	Cell identity- cell growth	
SOP-QA-A-02	Cell identity – cell proliferation	
SOP-QA-A-03	Cell identity – cell surface antigens	
SOP-QA-B-01	Product purity – bacterial contamination	
SOP-QA-B-02	Product purity – viral contamination	
SOP-QA-B-03	Product purity – mycoplasm contamination	
SOP-QA-C-01	Cell potency – adipogenic differentiation	
SOP-QA-C-02	Cell potency – osteogenic differentiation	
SOP-QA-C-03	Cell potency - chrondrogenic differentiation	

Furthermore, the specification data sheet for the bioreactor is attached: SD-STR-01.

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Methods in Molecular Biology (2015) 1283: 219–220
DOI 10.1007/7651_2015

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