INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online atwww.ijpcbs.com

Research Article

ISOLATION OF HUMAN UMBILICAL CORD MESENCHYMAL STEM/STROMAL CELLS FROM A STEM CELL BANK PERSPECTIVE: AN INTEGRATED OVERVIEW

Pedro Silva Couto*

Biological Engineering (IST), Lisbon, Portugal.

ABSTRACT

The discussion around stem cell therapies has been arousing the attention of scientific and medical community. In the particular case of mesenchymal stem/stromal cells their immunomodulatory properties as well as multilineage differentiation ability might be important to target conditions such as graft-versus-host disease, Chron's disease, bone and cartilage regeneration and even myocardial regeneration. Recognizing that potential, stem cells banks from all over the world are storing MSC from umbilical cord which has several advantages over other sources of MSC namely adult ones such as bone marrow or adipose tissue. The aim of this review is to focus the MSC isolation processes from UCM from a stem cell bank perspective. Since the isolation is only the first step of a cell therapy, this review also offers an integrated perspective focusing also expansion, quality control, clinical trials and actual legal framework around MSC isolation in stem cell bank's context.

Keywords: Mesenchymal stem/stromal cells, umbilical cord matrix, regenerative medicine.

INTRODUCTION

During the last decades stem cells have been arousing the scientific community interest worldwide(Figure 1). The rationale behind this intensive study is related with their impressive abilities of prolonged self-renewal, maintaining unimpaired their wide differentiation potential[1,2].Mesenchymal stem cells (MSC) are a particular subset of multipotent stem cells that were isolated for the first time from bone marrow (BM) [3].Additionally to BM, MSC can be isolated from other adult sources such as adipose tissue (AT)[4], [5] or dental pulp (DP)[6–8]. Immunomodulatory properties and trophic activity[9–13] as well as multilineage differentiation ability[14]have been the most explored features of MSC. These characteristics make them an attractive tool for tissue engineering and regenerative medicine applications[15, 16].Genetic disorders have also been studied using MSC[17].Due to their potential MSC are currently under evaluation for several types of disorders including liver cirrhosis, graft versus of host disease (GvHD), bone and cartilage repair, liver among others (clinical trials section).In order to standardize the MSC phenotype around the world,the International Society for Cellular Therapy (ISCCT)[18]proposed a group of criteria to define MSC. Additionally to plastic adherence properties, the cells have also to show a defined subset of surface markers(Table 1) as well as multilineage differentiation ability into osteoblasts, chondrocytes and adipocytes.Since the quantity of MSC in human body decreases exponentially with age[12] alternative sources have been studied to replace adult ones. Thus neo-natal sources of MSC such as umbilical cord matrix (UCM)[19, 20], umbilical cord blood (UCB)[21, 22], placenta[23, 24] and even amniotic fluid[25, 26]can be seen as possible alternatives. The main advantages of MSC

isolation from neo natal sources includethe non-invasive collection procedure, high isolation success rates, and higher proliferation ability when compared with MSC extracted from adult sources[27,28]. Additionally to these differences, since neo-natal MSC are collected after child birth, aging phenomena[29], and diseases accumulation are much less expected than if adult sources are chosen[30].Erstwhile seen as a medical waste, umbilical cord (UC) has been stored by many stem cells banks around the world since it contains bothhematopoietic stem cells (HSC)present in blood[31] and MSC in UCM[19, 20]. Umbilical cord,that during foetal development is responsible for blood maintenance between the mother and fetus, is mainly composed by collagen[32]. Some of the physical characteristics of UC[33], [34] are summarized in Table 2.UCM-MSCare being explored by stem cell banks that are recognizing their wide potential in clinical field. Towards clinical use, after isolation MSC have to be expanded in order to reach the high quantities of MSC required for clinical applications (typically 2 to 5 millions of cells per kg of patient's body weight)[35, 36]. The role of isolation processes is extremely important since if they were efficienttheyare responsible for maximizing the cell yield starting with an unrefined source maintaining also the functional identity of the isolated cells. Thus the present review aims to identify the current methods of MSC isolation from UCM as well as to identify their critical steps. Several stem cell banks can benefit from this review, since most of them adopt isolation processes followed by cryopreservation as core business. An integrated perspective with expansion, quality control, clinical applications is also provided remembering that isolation process is the beginning step of every stem cell therapy. Since in the next years it is expected an increase in the advanced therapy medicinal products (ATMP) based on MSC, this review also provides an overviewsince isolation until clinical applications.

ISOLATION OF MSC FROM UCM

When the goal is to isolate MSC from UCM two different strategies can be adopted: explants method *versus* digestion method(Figure 2).The explants isolation method is based on the adherence and migration properties of MSC. This approach[20, 33, 37–47]is not exclusive of UCM (nor MSC) being used also to isolate cells from other types of tissues[48, 49].In a research lab context, this isolation method starts with UC mincing in several pieces (called explants) that are then plated in culture dishes and covered by medium. After waiting several days it is possible to collect the MSC which migrated from the explants towards surface's culture dish. On the other hand, digestion method[19, 27, 33, 39–42, 47, 50–67] involves the degradation of the collagen matrix and microfibrils of UC[32]. After enzymatic digestion, the resultant suspension is submitted to several separation steps such as filtrations, centrifugations and washing steps in order to concentrate the isolated cells in a small volume.It is possible to assert that even before isolation these two methods share some unit operations, such as washing steps. Right after childbirth, the UC is collected and after arriving to the lab isusually immersed within a stable solution carrying antibiotic solutions such as penicillin-streptomycin (pen-strep). The rationale behind this step is to reduce the eventual microbial charge inherent to a birth scenario. After this preparation step the cords are usually dissected and minced. The mincing step is far from consensus within research groups. Most research groups opt to process only the UCM completely free of vessels[27, 38–42, 50, 51, 55, 57–60, 64, 65, 67].Other researchers opt to include also the umbilical cord vein (UCV) as well claiming that it also contains MSC[68–70]. In an attempt to maximize the cell yield during the isolation, all cord processing approaches can be also found in literature[52, 56]. After dissection step the cord is minced in small explants which vary from 2-3 mm[41]up to 4-5 cm[27]. After these common steps the isolation methods are carried out independently. In the digestion method the minced cord is then digested using enzymatic solutions such as collagenase[27, 33, 39, 47, 51, 59, 64, 65], trypsin[40, 46,] or even combinations of several enzymes [19, 33, 37, 39–42, 46, 50, 52–54, 56, 58, 60–63,66](the most frequent combinations involves collagenase, trypsin and/or hyaluronidase). Regarding enzymatic solutions used despite in low frequency, dispase[33]is also used to digested the UCM.It is important to highlight here that each enzyme has its own way to break the collagen matrix of UCM. Thus, enzymatic solutions as well as incubation times should be carefully chosenin order to avoid cell death. In fact, enzymatic digestion is one of the most critical steps regarding process global cell yield since excessive and prolonged digestion might lead to cell's death[39, 71].Centrifugations and washing steps typically performed after digestion are also associated with cellloss (Figure 2). In digestion method, after enzymatic action the cells need to be concentrated in a smaller volume which is performed using centrifugation, filtration and also

washing steps. Regarding stem cell banks, after processing the isolated cells are cryopreserved.Back to explants method, after the mincing step the small UC fragments are usually transferred to stable solutions such as autologous plasma[38] or human albumin[41]. After this short processing the explants can be used for MSC isolation using adherence steps or cryopreserved, as in stem cell bank context. It is important to stress out that the obtained product of each method has different properties: in the digestion method a cellular suspension is obtained in contrast with the explants method which is characterized by explants carrying entrapped MSC. Therefore, in order to understand the differences (Table 3) between each isolation method it is also useful to identify the advantages and disadvantages of each method (Table 4).Starting the comparison by the complexity of each process, digestion one is associated with higher complexity levels due to the several steps required. For this reason digestion method is also considered as a more prolonged option than explants method[37, 43, 55].This might be seen as a disadvantage from stem cell bank's point of view, where many UC are processed every day. Thus explants method is a faster and technically less difficult alternative to digestion one when the process time and labour intensity are compared[37, 43, 55].If the comparison target is the manipulation degree, differences can also be identified. Based on the guidelines established by EMA for ATMP[72],some steps of MSC isolation process are associated with "substantial" degree of manipulation. Considering the simplified explants and digestion isolation methoddiagram (Figure 2), it is possible to assert that digestion methodhave a higher degree of manipulation than explants method. Thus an eventual ATMP based on MSC from UCM having digestion method as initial isolation step, might face additional difficulties of getting approval.As every bioprocess, stem cell isolation requires the establishment of a tight quality control strategy (further developed in quality control section), especially important towards the clinical use of these cells. Remembering that in the end of processing, explants method ends up in a solution carrying UCM fragments and digestion method ends is a single cell suspension, the quality control strategies have some different characteristics.Thus in order to address the quality control parameters often analysed in stem cells banks (e.g. viability, phenotype, stability[73]])an additional step of adherence isnecessary to access cell's features, if explants method is chosen. This can be seen as adisadvantage associated with extra operational costs (e.g.: reagents, medium exchanges) as well as undesired additional human handling.Since the cells are completely isolated in the end of digestion method it is easier to address cell's features making easier the quality control of digestion method when compared with explants one.When the process cost is compared, digestion method seems to be a more expensive approach than explants method due to the expensive enzymatic solutions and several solutions used. These differences are reflected in the final price of the isolation/cryopreservation services offered by several companies around the world[74]. After isolation, expansion steps have to be carried out in order to reach the high amounts of cells required for clinical applications. Thus expansion strategies are also influenced by the isolation method selected(detailed in expansion section). Usually adherence steps have to be performed in order to promote the MSC migration form UCM. It should be noted that even digestion method having a more prolonged process time, expansion can be performed directly after processing, which is not possible when the explants method is chosen. This suggests that despites of amore prolonged processtime associated with digestion method, it is possible to obtain completely isolated MSC in a shorter period of timethan whether explants method is used[42, 55].All of the discussed process features are summarized in Table 4 (adapted from[75]**)**.Additional to the isolation method characteristics, it is also important to compare the characteristics of the isolated cells using each method. Starting with the isolation rate, no differences were reported regarding explants and digestion isolation method[19, 37, 57]. Comparing with other neo-natal sources, the isolation of MSC from UCM can been seen as an more effective processthan the MSC collection from UCB which is associated with low isolation success rates (ranging from 10 % to 60% under optimized conditions)[76–78]. This somehow explains why stem cell banks around the world opt to store HSC from blood and MSC exclusively from UCM. Regarding cellular passaging time, literature suggests that the first passage is shorter in digestion method when compared with explants methods[42, 47] due to the MSC migration step required.Since the high quantities of MSC needed for therapeutic applications often implies an expansion step, explants and digestion-isolated MSC proliferation ability must be compared. In Table 3 it is

summarized the proliferation ability of MSC from UCM, isolated using different protocols. The difference observed inproliferation ability, might reflect not only donor variability but also different culture conditions. Nevertheless no differences regarding proliferation ability were found, when digestion and explants method were compared under the same conditions[47]. This data seems suggest that, in this particular case, the MSC's proliferation ability it is not affected by the isolation method.Another important process outcome representative of the process efficiency is cell yield. In the specific case of cell yield, the published results are pointing in different directions. It was possible to identify cases that showed higher cell yields associated with digestion method[33, 47,] but other reports indicated the opposite tendency[51]. Thus regarding cell yield it is difficult to identify the best method, due to the lack of consensual data. Still regarding cell yield, from a stem cell bank perspective, it is important to evaluate the transport time impact on cell yield. Data from Iftimia-Mander*et al*[47] suggests that transport time has an crucial impact in cell yield, only when digestion method is considered. On the contrary cell yield associated with explants method did not differ significantly when the transport time increases. This data suggests that explants method might be a more robust and suitable option to perform especially if the UC sample is being processed several hours after their collection time (e.g. delay in transportation).

AN IDENTIFICATION PROBLEM

The lack of an univocal surface markeronly associated with MSC (e.g. such as CD34 for hematopoietic cells)makes more difficult their identification by flow cytometry[79].Towards MSC identification,a list of criteria has been established. Since that list defined by ISCT[18]is considered toominimalist due to the lack of information about potency, new criteria of identification have been widely discussed[80]. ISCT's list of criteria[18] include three different features: plastic adherence, surface markersexpression and differentiation ability. Currently associated with a "fibroblast-like" morphology, to be considered as MSC the cells must be plastic-adherent. The analysed cells must also express several positive (CD90, CD75, CD105) and negative (CD45, CD34, CD14, CD79 and HLA-DR) surface markers. Differentiation ability into osteoblasts, adipocytes and chondrocytes is also needed towards MSC identity confirmation. An additional problem is related with the set ofsurface markers used. Since they are not exclusive for MSC it is difficult to identify them clearly. Towards the MSC identification using flow cytometry several markers have been used. Using some of the MSC from UCM isolation protocols available in literature, it was here identified some of the most used surface markers to target MSC. Starting with positive markers CD73[20, 33, 37, 38, 40, 42, 45, 46, 50, 54, 58, 59, 62, 63, 65, 66,] CD90[19, 20, 27, 33, 37, 39–41, 43, 45–47, 52–54,56, 58, 60, 62–66]and CD105[19, 20, 33, 37, 38, 40, 41, 45–47, 50, 53, 54, 56, 58–60, 62–66]are some of the mostcommonly used. Additionally,several research groups also chooseto include CD29[19, 38, 41, 46, 50, 54, 56, 59, 60]and CD44[19, 20, 33, 38–40, 43, 45, 46, 50, 52–54, 56, 60, 62–65.] The rationale behind of these two last surface markers is related with their function: CD44 is a transmembrane protein that act as receptor for hyaluron[81]whichhas an important role in cell-matrix interactions [82, 83]and CD29is an integrin surface marker[50].Remembering heterogeneous cellular composition of UC, negative surface markers must also be included.The isolation protocols analysed revealed that CD14[19, 38, 46, 54, 60, 65, 66,] CD31[27, 37,39, 42, 44, 45, 54, 59, 60, 65, 66,] CD34[19, 33, 37, 38, 40, 44–47, 50, 52–54, 58–60, 62–66,] CD45[27, 37–41, 43–47, 50, 52–54, 58–60, 62, 64–66]and HLA-DR[37, 38, 46, 53, 54, 58, 60, 65, 66] are the most usednegative surface markers to target MSC.Typically the hematopoietic cells presence is detected by CD34 and CD45. Consideringeventual endothelial cell contaminations, they are usually identified using CD31[84].Recalling the adherence properties of MSC, it is important to exclude the contamination of other adherent cells, namely macrophages,positive for CD14[85].Regarding HLA-DR, which is known as an MHC-II cell surface receptor, the main reason for being included in negative surface markers list, is related with the "immunoprivileged" status of MSC. This status is characterized by low levels of MHC-I and negligible levels of MHC-II. Many issues arise from the poor identification of MSC such as potency (the ability to differentiate into several types of cells), and plasticity. Despite of this extensive list of surface markers, the MSC identification is far from consensus throughout scientific community.The lack of criteria to identify exclusively MSC might be associated with unusual plasticity events (the cell's ability of giving rise to cells functionally different from their own origin[86]). In literature there are some examples where MSC were *in vitro* differentiatedinto neurons, astrocytes, hepatocytes and pancreatic islets-like cells[87–91]. Therefore MSC's plasticity topic is under intense debate over scientific community not only because of the poor criteria to

define MSC but also due to the absence of suitable animal models to use. In order to include potency in MSC definition criteria, Stro-1 and CD106 expression have been associated with donor and/or culture age. If confirmed this hypothesis, this two markers should be included in the surface markers list to address potency[92].For these reasons many research groups are involved in the development of a consensual list of criteria to identify exclusively MSC including also cell's potency.

INTEGRATION OF PROCESSES

After MSC isolation further steps are needed until the cells are released for clinical administration. To increase the number of the MSC, expansion has to be carried out. In the next section are present some of the current strategies of MSC expansion together with an integrated perspective over the UCM-MSC isolation methods.

Expansion

Due to their importance in clinical field, not only as support cells for HSC expansion[93] but also for scenarios of graft versus-host disease (GvHD)[35], Chron's[94], cartilage and bone defects[95] and even myocardic regeneration[96], there has been a growing interest in MSC expansion. The decrease of MSC's number in human body with aging[12]and the high quantity of cells required for cellular therapies[97, 98],increase the need of efficient expansion methods development. If the goal is to expand MSC (independently on the origin source) two main strategies have been applied: (1) static 2-D using monolayer cultures and (2) bioreactors (using microcarriersor spheroids).Based on their intrinsic ability to growth attached to surfaces, MSC expanded using cultures dishes is the most used strategy. Alternatively, T-flasks have been also used due to a better surface per unit of volume than culture dishes. Both of these 2D expansion systems have several drawbacks associated. Since static systems do not include mixing devices, the culture medium is highly heterogeneous and may lead to toxic metabolites accumulation. This can be critical remembering that high concentrations of lactate, arising from the degradation of glucose by mammalian cells (and also ammonia from glutamine degradation), leads to a decrease in pH inhibiting MSC growth[99-101].Relatively to the physicochemical parameters that needs to be monitored during MSC expansion (e.g. pH, temperature, osmolarity, dissolved oxygen, hydrodynamic shear stress) static cultures does not include robust systems to control them. Looking to static cultures in a scale up approach, other drawbacks must also be referred such as excessive human handlingrequired, which is undesired due to the potential risk of contaminationand also high operational costs. Due to these limitations static cultures are usually associated with scale-out rather than scale-up approaches.In order to overcome some static cultures limitations, always with large scale expansion as main goal, bioreactors have been used. Despite the wide variety of reactors available, spinner flaks [102, 103]mimicking stirred tank reactor(STR)[104] have been the main explored strategy. Due to their unique properties and knowledge accumulated with mammalian cell's expansion, these reactors have been used to study MSC expansion using microcarriers and spheroids (3D aggregates). Microcarriers can be defined as small porous spherical beads with diameters ranging from 100 to 400 μm[105]. Microcarriers offer several options regarding their chemical composition (e.g. dextran, polystyrene, cellulose, gelatine and collagen) charge and porosity. One of the main advantages of microcarriers use over 2D systems is their high surface to volume ratio. Another important advantage of microcarriers is that they enable the increase of culture periods through the addition of fresh ones avoiding overload phenomena[106]. Besides these advantages, the recovery of cells from microcarriers might be an issue. According to Malda*et a*[105]the recovery efficiency is function of chemical composition as well as degree of porosity. Regarding spheroids,they have been described as three dimensional cell aggregates with diameters up to1 m[107]. According to Bartosh*et al*[108] the major advantage of MSC expansion using spheroids is the enhancement of their anti-inflammatory properties. In addition to expansion, since spheroids offer a three dimensional interaction between the cells,they have been also used for models of differentiation[109] and cancer developmen[107]. Remembering that *in vivo* the MSC are interacting with other cells, spheroids approach offers a "simulation" of the 3D environment which can be also important for stem cell biology studies. Such importance was demonstrated by Genever[110] showing that a higher degree of cell-cell interaction provided by spheroids can also modify gene and protein expression. Despite these advantages, spheroids are not as used as microcarriers due to the mass transference problems between culture medium and spheroid's

core [102, 109, 111]. Often in larger spheroids, the nutrients exchange between medium and spheroid's core is so difficult that can end up in necrosis[108]. Besides stirred tanks other bioreactors (e.g. rotating wall vessels [112, 113], parallel plates [114], hollow fiber-like[115] and rotating bed[116] have been also tested to expand MSC(Table 5). Since the intrinsic features and operation mode of each bioreactor are out of the scope of this article it will not be further covered in this review. Integrating the isolation of MSC from UCM and the expansion options it is important to note that for expansion purposes the MSCneed to be isolated i.e. completely separated from their tissue/fluid of origin. In the particular case herein focused, after processing by the explants method an adherence step is mandatory. Regarding the digestion isolation method, since the cells are already isolated in a liquid suspension, the adherence step is not mandatory, but sometimes it is also performed.Looking at isolation and expansion in an integrated perspective since the MSC are completely available in the end digestion method, this might be a more advantageous strategyif the timeframe to perform the expansion is tight.

Quality Control and Good Manufacturing Practises

The increasing number of stem cell banks during the last decade throughout world, raised the discussion around the safety of using previously stored cells.In order to be available for medical applications, all the steps since collection passing by processing and storage must follow the Good Manufacturing Practises (GMPs) and a tight scheme of quality control (QC). Together with GMP there is an increase need for developing standard operating protocols (SOPs) that can be reproduced by several laboratories in order to reach the robustness required for clinical applications.The major issue in stem cell banks is related with GMPs and the nature of the reagents used. Robustness and reproducibility are more problematic regarding research labs rather than stem cell banks because the last ones have their own SOP completely established and approved.Concerning the methods used during isolation, the major concern arising must be to evaluate whether the processing steps do not change the intrinsic characteristics of the cells. As present in the Figure 2, several quality control samples must be collected after the isolation procedure with that goal in mind. Especial care must be taken in the unit operations susceptible to change cells intrinsic properties (e.g. prolonged digestions, cryopreservationsetc). Therefore there are a group of criteria that has to be fulfilled to ensure the quality of the cells stored:viability, identify, purity and stability cells [73]. Regarding viability,usually dye exclusion methods are used (such as trypan-blue). Additionally, it is important to include also other viability markers, for instance early markers of apoptosis (such as annexin V).The viability measurement it is fundamental to evaluate whether the isolation process is harmful to the cells or not.Concerning identity, flow cytometry has been widely used. Although due to the lack of a univocal group of markers (section "An identification problem"), the flow cytometry information must be complemented with other assays. Always with the clinical application goal in mind, purity (defined here as the absence of microbiological contamination) is an important target of quality control.It is important to note that the undesired microbial contamination can occur from different ways such as type of birth delivery, type of collector, time between collection and processing among others[117]. In order to avoid contamination during the sample's processing all the processes must follow the GMP guidelines which also includes environmental control in every lab station where cells are processed[118]. The need of microbial control is reinforced since it is now proved that microbes can survive to extremely low temperature of cryopreservation[119, 120]. In order to test cell's stability, the isolated MSC should be expanded for several passages to confirm whether their characteristics remain unchanged.Another relevant issue in stem cell banks is the use of reagents that can damage the cells or can be potential dangerous for humans. A major concern transversal to the stem cells field is the use of serum. Starting from the ethical concerns arising from the collection method[121]patient's safety is also compromised since serum might carry prions and virus[122]. Patient's safety is not the only drawback of serum use. High cost, high variability between batches are other important disadvantages that hinder serum use in cell therapy applications. Thus scientists should explore alternatives to replace serum role such as, xenofree medium with no need of serum supplementation[123]or platelets lysate[124,125]. Regarding safety for clinical applications, cryoprotectant agents (CPA) have been also a concern in stem cell banks. Some CPAthat play a crucial role in cryopreservation,have also been referred as being potential dangerous to the cells. For instance the

most commonly used, dimethyl sulfoxide (DMSO) is toxic at room temperature[126, 127]. The cytotoxicity raises other constrains, namely the need of several prolonged and complex steps of washing to remove the DMSO before further steps such as expansion or transplantation. Due to these concerns some research groups are trying to replace DMSO by other CPA such as glycerol, hydroxyethyl starch[128]or trehalose and sucrose[129].

Clinical Trials

Demonstrating the clinical potential of MSC-related discoveries during the last years, several clinical trials have been conducted. Regarding the MSC source used in those trials, it was possible to identify that most of them uses BM (Figure 3). Othersubstantialsources of MSC used in clinical trials are the UC and UCB, legitimating and emphasizing the stem cell banks-related business.Imunomodulatory properties[13], multiple differentiation ability[1], and homing capacity to damaged or injured areas [130]are the main properties explored in these clinical trials. In accordance with the MSC abilities is the large scope of diseases that are being studied(Figure 4 and Table 6). First it should be highlighted the tremendous variety of conditions studied.Differentiation into cardiac-like cells[131], pancreatic-like cells[132]is explored in some clinical trials in diseases such as dilated cardiomyopathy and liver cirrhosis. The immnomodulatory properties and paracrine action are other characteristics explored in wound healing [133, 134] and immune system diseases such as GvH[135]. In these last two cases, the MSC paracrine action plays a key role since they can induce a response in dendritic cells[136],T-cell[137], B-cell[138] and NK cells[139] through soluble molecules secretion. Since not only paracrine factors secreted by MSC but also cell-cell contact can enhance HSC expansion [140] this have also been explored in clinical trials that aims to expanded HSC for transplantation purposes. Regarding blood disorders evaluated, the immunosuppressive potential of MSC seems to be the main explored feature. Another interesting and relevant group of disorders studied using MSC are neurological ones. According to Momim*et al*[141]neurological diseases might benefit from migration , immunosuppressive abilities and even differentiation potential. In the same publication it is suggested that MSC can transdifferentiate into neural cells, which might be beneficial for diseases such as Parkinson's, Alzheimer's and also Amyotrophic Lateral Sclerosis. Although, this MSC transdifferentiation potential it is not accepted through all the scientific community (section "An identification problem"). Showing the growing interest in stem cell therapies business, demonstrating that the knowledge from several studies already crossed the bench-barrier Hearticellgram® AMI from FCB Pharmicell (the world's first approved stem cell product, approved by Korean FDA[142]) to target cardiac muscle repair and Prochymal® from Osiris Therapeutics™ to target GvHD are some of the current available MSC-based products in the market.

Legal Framework

The increase knowledge related with stem cells has been promoting the establishment of several companies with stem cell therapies as core business. In the last few years, scientists, doctors and physicians have been alerting for some dubious and non-ethical clinical practices[143–145]. Nowadays, several companies located in Europe, Asia or America claim to have novel stem cell therapies to target the most variable diseases. Taking advantage of the existents gaps in law, these companies are offering cell therapies not approved by local regulatory agencies, omitting important scientific information to the patient[146]. The marketing strategies used to motivate clients for all over the world have been also criticized. These practices raise many ethical and even health questions since some of those therapies were not even evaluated using clinical trials. This suggests that some of these companies are offering "alternative" medicine with no proved therapeutic success with profit as main goal. In order to legislate and regulate the growing market of cell therapies in India, theIndian Council of Medical Research (ICMR) launched in 2012 the Guidelines of Stem Cell Research[147]. Apart from this discussion are Indian stem cell banks that have been recognized by their high standards of quality and safety, both by local (ICMR) and international authorities (eg AABB, WHO). Almost of the 10 private stem cell banks in India [74]invested a lot of effort in standards and accreditation. Some of them opt to try international accreditation from AABB, WHO or Fact for instance. This indicates that, the market of cell banks is widely accredited and certified by internal and external entities that ensures the quality of offered services by Indian companies.

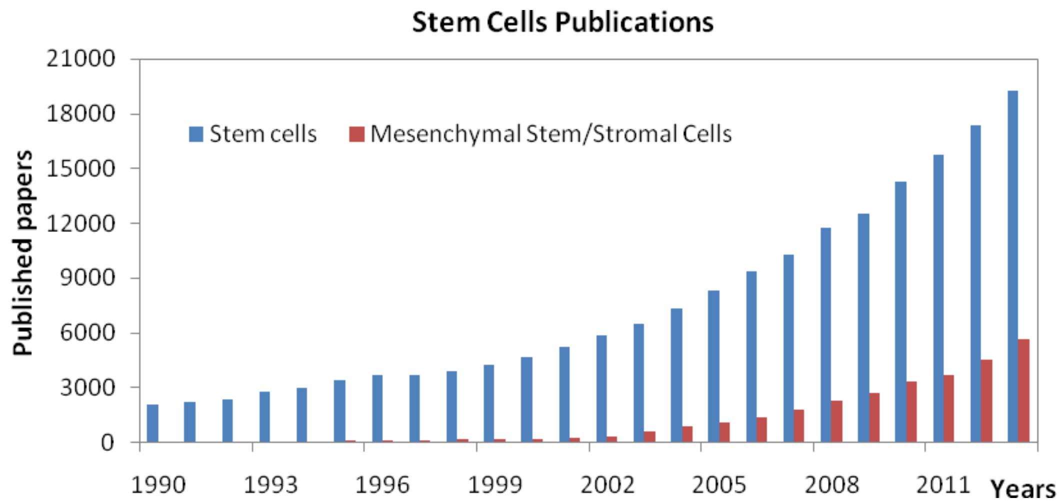

Fig. 1: Number of published articles per year regarding stem cells and mesenchymal stem/stromal cells (blue and red, respectively). Data collected from *Pubmed* database using the words "stem cells","mesenchymal stem cells"and "mesenchymal stem/stromal cells". Data collected during March, 2014.

Table 1: Description of surface markers required to identify MSC established by ISSC[18]

Positive	Negative
CD105	CD45
CD90	CD34
CD73	CD14 or CD11b
	CD79α or CD19
	HLA-DR

Table 2: Summary of some UC physical parameters

Characteristic	Value (units)	References
Length	30 to 60 cm	[33]
Weight	40 to 50 g	[33]
Diameter	14±1.5 mm	[34]
Surface area	139±24.6	[34]
Weight/length ratio	1.2±0.4 g/cm	[51]

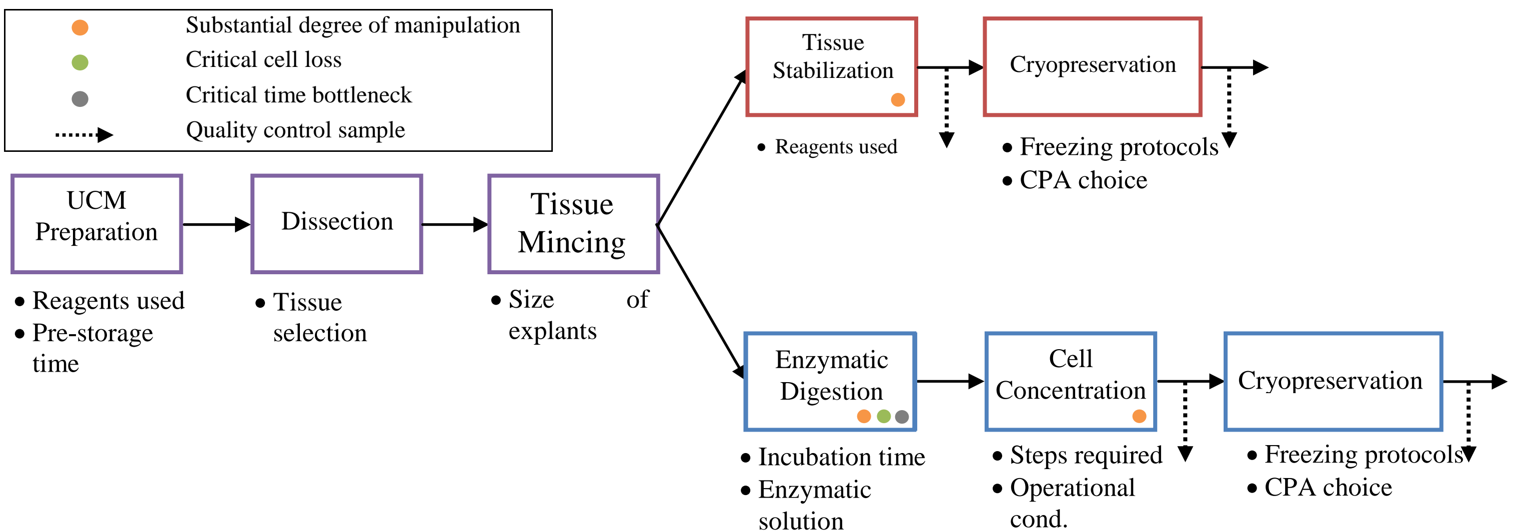

Fig. 2: Schematic representation of generic unit operations considering MSC isolation using explants method (red) or digestion method (blue). Common steps are represented in purple. After cryopreservation, the resultant products are usually transferred to nitrogen tanks and stored around -180 °C. Below each step (box) are identified some of the differences among protocols.

Table 3: Literature review of several protocols of digestion and explants methods of isolation. Type of isolation, enzymes used, differentiation ability, size of the tissue processed, incubation time, cell yield and population doubling time were analysed. NR, not reported.

Type of isolation	Enzymatic solution used	Differentiation ability	Tissue size	Incubation Time(37 °C)	Cell Yield	PD_{time}	Refs
Enzymatic	Collagenase I, IV andHyaluronidase	Neuronsand glia cells	1.5 cm	45 min	$(4.7 \pm 0.2) \times 10^6$ *	24.47±0.3 h; 26.25±0.5 h	[19]
Enzymatic	Collagenase I, IV and Hyaluronidase	Neurons	2.0 cm	45 min	NR	NR	[53]
2-Enzymatic (D) and explants(TE)	Collagenase II; IV and Trypsin	Osteocytesandadipocites	1 mm³	16.5-20.5 h	$(6.3 \pm 1.7) \times 10^5$ (TE) * $(12.1 \pm 2.0) \times 10^5$ (D) *	39.0±7.8h (TE); 41.3±7.5h (D)	[39]
3-Enzymatic and explants	Collagenase, hyaluronidase, and trypsin	Osteocytes and adipocytes	3-5 cm (E) 2 mm (TE)	2 to 3 h	1×10^3 to 1×10^4 * 0.5×10^4 to 1×10^5 **	NR	[40]
Enzymatic	Collagenase II, Trypsin	Osteocytes, adipocytes and neurons	1-2 mm³	30 min	1.0×10^6 *	≈20 h	[54]
Enzymatic and explants	Collagenase I, Hyaluronidase, Trypsin	NR	2-3 mm	1.5 h	NR	NR	[41]
Enzymatic and explants	CollagenaseI, Hyaluronidase and Trypsin	NR	1 cm³ (E) 3-5 mm (TE)	30 min	NR	NR	[42]

Enzymatic	Collagenase, Trypsin	Chondrocytes, adipocytesandcardio miocytes	NR	16.5 h	NR	NR	[50]
Explants	NA	NR	0.5-1 mm^2	NA	NR	NR	[43]
Enzymatic	CollagenaseI, Hyaluronidase	NR	3-5 cm	1.0 h	NR	NR	[55]
Enzymatic	CollagenaseI, Hyaluronidasean dTrypsin	Osteocytesandadipoc ytes	Allcord	3.5 h	6.5×10^5 * 7.4×10^5 (D)**	NR	[56]
Explants	NA	NR	1-2 mm^3	NA	NR	NR	[38]
Explants	NA	Osteocytesandadipoc ytes	1,5 cm	NA	NR	NR	[44]
Enzymatic	Collagenase	Chondrocytes, adypocytes, osteocytes and neurons	NR	4.0 h	2.6×10^4 *	85.0±7.2 h	[57]
Enzymatic	Collagenase	Osteocytes, adipocytes and chondrocytes	4-5 cm	18-24 h	NR	NR	[27]
Enzymatic	Collagenase I, Hyaluronidase and Trypsin	Osteocytes, adipocytes and endothelial cells	Smallpieces	1.5 h	NR	NR	[58]
Enzymatic	Collagenase I	Cardiomyocytes	1 mm^3	3.0 h	NR	NR	[59]
Enzymatic	Collagenase I, Hyaluronidase and Trypsin	NR	2-4 cm	45-60 min	NR	NR	[60]
Enzymatic and Explants	Collagenase, Dispase	Osteogenic, chondrogenic	Small sections	1.5 h	$(1.75) \times 10^5$ (TE)** $(5.3) \times 10^5$ (D)**	24±1.1 h	[33]
Enzymatic	Collagenase I, Hyaluronidase and Trypsin	Chondrogenic	3-5 cm	1.5 h	NR	NR	[61]
Explants	NA	NR	0.5 cm^3	NA	NR	19.7 h	[20]
Enzymatic	Collagenase I, Hyaluronidase	NA	Cord with vessels	3-5 h	NA	NA	[62]
Enzymatic	Collagenase	Osteocytes, adipocytes	2-3 cm	≈16 h	NR	35±22.5 h	[63]
Explants	NA	Osteocytes, adipocytes and chondrocytes	5-10 cm	NR	NR	NR	[37]
Explants	NA	Osteocytes, adipocytesandchondr ocytes	0.5 cm^3	NA	NR	24.5±1.2 h	[45]
Enzymatic	Collagenase I	Osteocytes	4–5 cm	18-24h	NR	≈20 h	[64]
Enzymatic	Collagenase II	Osteocytes, adipocytes and chondrocytes	NR	4 h	1.9×10^5 (TE)***	NR	[65]
Enzymatic	Collagenase, Hyaluronidase	Adipocytes, osteocytes	10 cm	19-21h	NR	NR	[66]
Explants	NA	Osteocytes, adipocytes, chondrocytesandhep atocytes	1.5-2.5 mm	NA	NR	NR	[46]
Enzymatic	Collagenase I, trypsin, dispase II	Osteocytes, adipocytes ,chondrocytes and neurons	All cord	1.3 h	NR	NR	[52]
Enzymatic	Collagenase I, trypsin	Neurons	0.5 cm^3	18.5 h	NR	NR	[67]
Enzymatic and explants	Collagenase	NR	2-3 mm	30 min	$(4.89 \pm 3.2) \times 10^5$ (TE)** $(1.75 \pm 2.2) \times 10^5$ (D)**	NR	[51]

Enzymatic and explants	Collagenase I	Osteocytes, adipocytes and chondrocytes	1-2 mm³	18 h	$1.8 \times 10^5 (TE)$*** $2.3 \times 10^5 (D)$***	NR	[47]

*cell per cm of cord; **cell per gram of cord; *** in cord slices with 200 to 400 mg

Table 4: Qualitative comparison between explants and digestion method regarding technical (time consuming, technical difficulty, quality control and integration of processes), regulatory (degree of manipulation) and economic aspects (process cost) based on Dong-Ru*et al*[75].

	Explants method		Digestion method	
	Processing	**Culture**	**Processing**	**Culture**
Time consuming	Lower	Higher	Higher	Lower
Technical difficulty	Lower	Higher	Higher	Lower
Degree of manipulation	Lower		Higher	
Process cost	Lower		Higher	
Quality control	Harder		Easier	
Integration of processes	Harder		Easier	

Table 5: Summary of several strategies used in dynamic expansion of MSC. NA, not available, NR not reported. Not available parameters are related with the bioreactor's own nature. Operational parameters of each bioreactor can be further explored in the corresponding reference.

Type of Reactor	Culture Method	Cellular outcome	References
Spinner flask	Spheroids	NR	[102]
Spinner flask	Microcarriers	1.7×10^6 cell/ml	[103]
Rotating wall vessel	Spheroids	NR	[102]
Rotating wall vessel	Suspension	8.9±0.4 (FI)	[113]
Parallel plates bioreactor	Fibrous matrices	4.22×10^7 cell/ml	[114]
Stirred bioreactor	Microcarriers	10.4±0.4 (FI)	[104]
Hollow fiber-like	NA	9.4 to 20 (FI)	[115]
Rotating bed bioreactor	NA	NR	[116]

Table 6: Diseases covered by clinical trials using MSC from UC.Data from *clinicaltrials.gov* using "mesenchymal stem cells" and "umbilical cord" as search criteria. Data searched during March, 2014

Group of Conditions	Clinical Applications
Auto-immune/Immune rejection	GvHD, rheumatoid arthritis, systemic lupserythematosus, lupus nephritis, graft failure, autoimmune hepatitis
Blood Related	Aplastic anemia,myelodysplastic syndromes, co-infusion with HSC, cord blood expansion
Cardiac	Dilated cardiomyopathy, cardiopathy, ischemic cardiomyopathy
Diabetes-related	Diabetes type I, diabetes type II, diabetic foot
Gastrointestinal	Ulcerative colitis
Genetic	Duchenne muscular dystrophy, epidermis bullosa
Liver-related	Liver cirrhosis, liver failure, HBV-related liver cirrhosis, decompensated liver cirrhosis, liver transplant tolerance, primary billiard cirrhosis
Lung-related	Bronchopulmonary dysplasia
Neurological	Hereditary ataxia, multiple sclerosis and neuromyelitisoptica, hypoxic ischemic encephalopathy, amyotrophic lateral sclerosis, Alzheimer's, autism, cerebral paralysis
Others	Ankylosing spondylitis, premature ovarian failure, chronic renal failure, osteoarthritis, articular cartilage defects, HIV reconstitution, tissue engineering applications
Spinal cord injury	Spincal cord injury
Wound healing	Acute burn

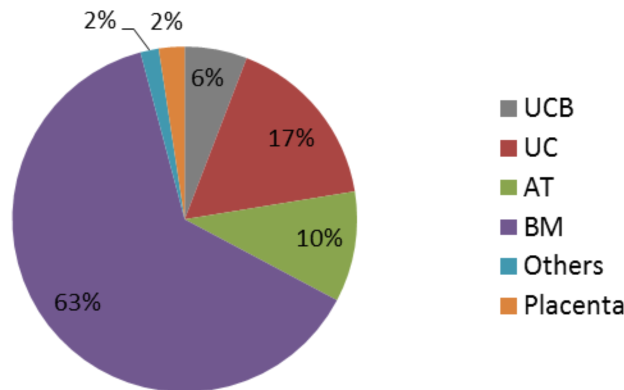

Fig. 3: Quantitative evaluation of MSC sources used in clinical trials. Search criteria used in *clinical trials.gov* were “umbilical cord blood”, “umbilical cord”, “adipose tissue”, “bone marrow”, “placenta” and “mesenchymal stem cells”. The class denominated “others” includes “endometrium”, “synovium” and “dental pulp” mesenchymal stem cells. Data searched during March, 2014.

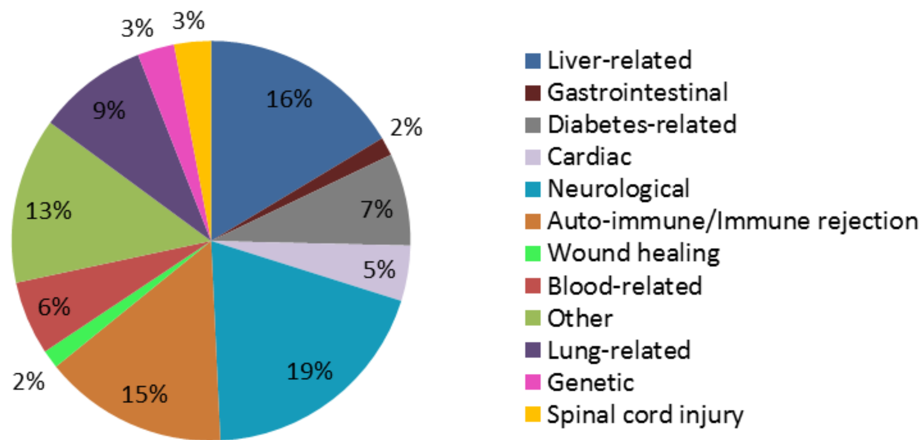

Fig. 4: Quantitative overview of the conditions covered by MSC clinical trials using UC. Data from *cliniclaltrials.gov* using as search criteria “umbilical cord” and “mesenchymal stem cells”. Data searched during March,2014.

REFERENCES

1. Caplan AI. Mesenchymal stem cells.J Orthop Res.1991;9(5):641–50.
2. Fortier LA. Stem cells: classifications, controversies, and clinical applications.Vet Surg.2005;34(5):415–23.
3. Friedenstein AJ, Chailakhjan RK and Lalykina KS. The development of

fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells.Cell Tissue Kinet.1970;3(4):393–403.
4. Francis MP, Sachs PC, Elmore LW and Holt SE. Isolating adipose-derived mesenchymal stem cells from lipoaspirate blood and saline fraction. Organogenesis.2010;6(1):11–4.
5. Pawitan JA. Prospect of Adipose Tissue Derived Mesenchymal Stem Cells in Regenerative Medicine.Cell Tissue Transplant Ther. 2009;2:7–9.
6. Karaöz E, Doğan BN, Aksoy A, Gacar G, Akyüz S, Ayhan S, Genç ZS, Yürüker S, Duruksu G, Demircan PC and Sariboyaci AE. Isolation and in vitro characterisation of dental pulp stem cells from natal teeth.Histochem Cell Biol. 2010;133(1):95–112.
7. Hilkens P, Gervois P, Fanton Y, Vanormelingen J, Martens W, Struys T, Politis C, Lambrichts I and Bronckaers A. Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells.Cell Tissue Res. 2013;353(1):65–78.
8. Perry BC, Zhou D, Wu X, Yang F, Byers MA, Chu TMG, Hockema JJ, Woods EJ and Goebel WS. Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use.Tissue Eng Part C. Methods. 2008;14(2):149–56.
9. Newman RE, Yoo D, LeRoux MA and Danilkovitch-Miagkova A. Treatment of inflammatory diseases with mesenchymal stem cells.Inflamm Allergy Drug Targets. 2009;8(2):110–23.
10. Nauta AJ and Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells.Blood. 2007;110(10):3499–506.
11. Le Blanc K and Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience.J Intern Med. 2007;509–525.
12. Caplan AI. Why are MSCs therapeutic? New data: new insight.J Pathol. 2009;217(2):318–24.
13. Caplan AI and Correa D. Perspective The MSC : An Injury Drugstore.Stem Cell. 2011;9(1):11–15.
14. Gimble JM, Guilak F, Nuttall ME, Sathishkumar S, Vidal M and Bunnell BA. In vitro Differentiation Potential of Mesenchymal Stem Cells. Transfus Med Hemother. 2008;35(3):228–238.
15. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine.J Cell Physiol. 2007;213(2):341–7.
16. Caplan AI. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng. 2005;11(7–8):1198–211.
17. Horwitz EM, Prockop DJ, Fitzpatrick L, Koo WW, Gordon PL, Neel M, Sussman M, Orchard P, Marx JC, Pyeritz RE and Brenner MK. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. Nat Med. 1999;5(3):309–13.
18. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D and Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement.Cytotherapy. 2006;8(4):315–7.
19. Fong CY, Subramanian A, Biswas A, Gauthaman K, Srikanth P, Hande MP and Bongso A. Derivation efficiency, cell proliferation, freeze-thaw survival, stem-cell properties and differentiation of human Wharton's jelly stem cells.Reprod Biomed Online. 2010;21(3):391–401.
20. Hatlapatka T, Moretti P, Lavrentieva A, Hass R, Marquardt N, Jacobs R and Kasper C. Optimization of culture conditions for the expansion of umbilical cord-derived mesenchymal stem or stromal cell-like cells using xeno-free culture conditions.Tissue Eng Part C Methods. 2011;17(4):485–93.
21. Lee OK, Kuo TK, Chen WM, Lee KD, Hsieh SL and Chen TH. Isolation of multipotent mesenchymal stem cells from umbilical cord blood.Blood.2004;103(5):669–75.
22. Sibov TT, Severino P, Marti LC,. Pavon LF, Oliveira DM, Tobo PR, Campos AH, Paes AT, Amaro E, Gamarra LF and Moreira-Filho CA. Mesenchymal stem cells from umbilical cord blood:

parameters for isolation, characterization and adipogenic differentiation.Cytotechnology. 2012;64(5): 511–21.
23. Semenov OV, Koestenbauer S, Riegel M, Zech N, Zimmermann R, Zisch AH and MalekA. Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation.Am J Obstet Gynecol. 2010;202(2):193.e1–193.e13.
24. Yen BL, Huang H, Chien C, Jui H, Ko B, Yao M, Shun C, Yen M, Lee M and Chen Y. Isolation of Multipotent Cells from Human Term Placenta. 2005;161:3–9.
25. In 't Anker PS, Scherjon SA, Kleijburg-van der Keur C, Noort WA, Claas FHJ, Willemze R, Fibbe WE and Kanhai HHH. Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. Blood. 2003;102(4):1548–9.
26. You Q, Cai L, Zheng J, Tong X, Zhang D and Zhang Y. Isolation of human mesenchymal stem cells from third-trimester amniotic fluid. Int J Gynaecol Obstet. 2008;103(2):149–52.
27. Baksh D, Yao R and Tuan RS. Comparison of proliferative and multilineage differentiation potential of human mesenchymal stem cells derived from umbilical cord and bone marrow. Stem Cells. 2007;25(6):1384–92.
28. Christodoulou I, Kolisis FN, Papaevangeliou D and Zoumpourlis V. Comparative Evaluation of Human Mesenchymal Stem Cells of Fetal (Wharton's Jelly) and Adult (Adipose Tissue) Origin during Prolonged In Vitro Expansion: Considerations for Cytotherapy.Stem Cells Int. 2013;246134.
29. Stenderup K, Justesen J, Clausen C and Kassem M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone. 2003;33(6):919–26.
30. Hass R, Kasper C, Böhm S and Jacobs R. Different populations and sources of human mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.Cell Commun Signal. 2011;9(1):12.
31. Smith AR and Wagner JE. Alternative haematopoietic stem cell sources for transplantation: place of umbilical cord blood. Br J Haematol. 2009;147(2):246–61.
32. Franc S, Rousseau JC, Garrone R, van der Rest M and Moradi-Améli M. Microfibrillar composition of umbilical cord matrix: characterization of fibrillin, collagen VI and intact collagen V. Placenta, 1998;19(1):95–104.
33. Schugar RC, Chirieleison SM, Wescoe KE, Schmidt BT, Askew Y, Nance JJ, Evron JM, Peault B and Deasy BM. High harvest yield, high expansion, and phenotype stability of CD146 mesenchymal stromal cells from whole primitive human umbilical cord tissue. J Biomed Biotechnol. 2009; 789526.
34. Di Naro E, Ghezzi F, Raio L, Franchi M and D'Addario V. Umbilical cord morphology and pregnancy outcome. Eur J Obstet Gynecol Reprod Biol. 2001;96(2):150–7.
35. Sato K, Ozaki K, Mori M, Muroi K and Ozawa K. Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes.," J Clin Exp Hematop. 2010;50(2):79–89.
36. Battiwalla M and Hematti P. Mesenchymal stem cells in hematopoietic stem cell transplantation. Cytotherapy. 2009;11(5):503–15.
37. De Bruyn C, Najar M, Raicevic G, Meuleman N, Pieters K, Stamatopoulos B, Delforge A, Bron D and Lagneaux L. A rapid, simple, and reproducible method for the isolation of mesenchymal stromal cells from Wharton's jelly without enzymatic treatment. Stem Cells Dev. 2011;20(3):547–57.
38. Friedman R, Betancur M, Boissel L, Tuncer H, Cetrulo C and Klingemann H. Umbilical cord mesenchymal stem cells: adjuvants for human cell transplantation. Biol Blood Marrow Transplant. 2007;13(12):1477–86.
39. Han YF, Tao R, Sun TJ, Chai JK, Xu G and Liu J. Optimization of human umbilical cord mesenchymal stem cell isolation and culture methods.," Cytotechnology. 2013;51.
40. Salehinejad P, Alitheen NB, Ali AM, Omar AR, Mohit M, Janzamin E, Samani FS, Torshizi Z and Nematollahi-Mahani SN.

Comparison of different methods for the isolation of mesenchymal stem cells from human umbilical cord Wharton's jelly. In Vitro Cell Dev Biol Anim. 2012;48(2):75–83.

41. Koliakos I, Tsagias N and Karagiannis V. Mesenchymal cells isolation from Wharton's jelly, in perspective to clinical applications. J Biol Res. 2011;16:194–201.
42. Azandeh S. Mixed enzymatic-explant protocol for isolation of mesenchymal stem cells from Wharton's jelly and encapsulation in 3D culture system. J Biomed Sci Eng. 2012;5(10):580–586.
43. Petsa A, Gargani S, Felesakis A, Grigoriadis N and Grigoriadis I. Effectiveness of protocol for the isolation of Wharton's Jelly stem cells in large-scale applications. In Vitro Cell Dev Biol Anim. 2009;45(10):573–6.
44. La Rocca G, Anzalone R, Corrao S, Magno F, Loria T, Lo Iacono M, Di Stefano A, Giannuzzi P, Marasà L, Cappello F, Zummo G and Farina F. Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. Histochem. Cell Biol. 2009;131(2):267–82.
45. Majore I, Moretti P, Stahl F, Hass R. and Kasper C. Growth and differentiation properties of mesenchymal stromal cell populations derived from whole human umbilical cord. Stem Cell Rev. 2011;7(1):17–31.
46. Zhang YN, Lie PC and Wei X. Differentiation of mesenchymal stromal cells derived from umbilical cord Wharton's jelly into hepatocyte-like cells. Cytotherapy. 2009;11(5):548–58.
47. Iftimia-Mander A, Hourd P, Dainty R. and Thomas RJ. Mesenchymal Stem Cell Isolation from Human Umbilical Cord Tissue: Understanding and Minimizing Variability in Cell Yield for Process Optimization. Biopreserv Biobank. 2013;11(5):291–298.
48. Jing W, Xiao J, Xiong Z, Yang X, Huang Y, Zhou M, Chen S, Lin Y and Tian W. Explant culture: an efficient method to isolate adipose-derived stromal cells for tissue engineering. Artif. Organs. 2011;35(2):105–12.
49. Priya N, Sarcar S, Sen Majumdar A, and Sundarraj S. Explant culture: a simple, reproducible, efficient and economic technique for isolation of mesenchymal stromal cells from human adipose tissue and lipoaspirate. Journal of tissue engineering and regenerative medicine. 2012;1-12.
50. Wang HS, Hung SC, Peng ST, Huang CC, Wei HM, Guo YJ, Fu YS, Lai MC and Chen CC. Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. Stem Cells. 2004; 22(7):1330–7.
51. Yoon JH, Roh EY, Shin S, Jung NH, Song EY, Chang JY, Kim BJ and Jeon HW. Comparison of explant-derived and enzymatic digestion-derived MSCs and the growth factors from Wharton's jelly. Biomed Res Int. 2013; 428726.
52. Kadam S, Tiwari S and Bhonde R. Simultaneous isolation of vascular endothelial cells and mesenchymal stem cells from the human umbilical cord. Vitr Cell Dev. 2009;23–27.
53. Fong C, Richards M, Manasi N, Biswas A and Bongso A. Comparative growth behaviour and characterization of stem cells from human Wharton's jelly. Reprod Biomed. 2007;15(6):708–718.
54. L. Lu, Y. Liu, S.-G. Yang, Q. Zhao, X. Wang, W. Gong, Z.-B. Han, Z. Xu, Y.-X. Lu, D. Liu, Z. Chen, and Z.-C. Han, "Isolation and characterization of human umbilical cord mesenchymal stem cells with hematopoiesis-supportive function and other potentials.," Haematologica, vol. 91, no. 8, pp. 1017–26, Aug. 2006.
55. Puranik SB, Nagesh A and Guttedar RS. Isolation of mesenchymal-like cells from Wharton's jelly of umbilical cord. Int J Pharm Chem Biol Sci. 2012;2(3):218–224.
56. Tsagias N, Koliakos I, Karagiannis V, Eleftheriadou M and Koliakos GG. Isolation of mesenchymal stem cells using the total length of umbilical cord for transplantation purposes. Transfus Med. 2011;21(4):253–61,
57. Karahuseyinoglu S, Cinar O, Kilic E, Kara F, Akay GG, Demiralp DO, Tukun A, Uckan D and Can A. Biology of stem cells in human umbilical cord stroma: in situ and in vitro surveys.," Stem Cells. 2007;25(2):319–31

58. Hartmann I, Hollweck T, Haffner S, Krebs M, Meiser B, Reichart B and Eissner G. Umbilical cord tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and maintain their phenotypic and functional properties. J Immunol Methods. 2010;363(1):80–89.
59. Pereira WC, Khushnooma I, Madkaikar M and Ghosh K. Reproducible methodology for the isolation of mesenchymal stem cells from human umbilical cord and its potential for cardiomyocyte generation. J Tissue Eng Regen Med. 2008;2(7):394–9.
60. Weiss ML, Medicetty S, Bledsoe AR, Rachakatla RS, Choi M, Merchav S, Luo Y, Rao MS, Velagaleti G and Troyer D. Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. Stem Cells. 2006; 24(3):781–92.
61. Wang L, Seshareddy K, Weiss ML, and Detamore MS. Effect of initial seeding density on human umbilical cord mesenchymal stromal cells for fibrocartilage tissue engineering. Tissue Eng Part A. 2009;15(5):1009–17.
62. Sarugaser R, Ennis J, Stanford WL, and Davies JE. Isolation, propagation, and characterization of human umbilical cord perivascular cells (HUCPVCs). Methods Mol Biol. 2009; 482:269–79.
63. Nekanti U, Rao VB, Bahirvani AG, Jan M, Totey S and Ta M. Long-term expansion and pluripotent marker array analysis of Wharton's jelly-derived mesenchymal stem cells. Stem Cells Dev. 2010;19(1):117–30.
64. Sarugaser R, Lickorish D, Baksh D, M. Hosseini M and Davies JE. Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. Stem Cells. 2005;23(2):220–9.
65. Simões IN, Boura JS, dos Santos F, Andrade PZ, Cardoso CMP, Gimble JM, da Silva CL and Cabral JMS. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. Biotechnol J. 2013;8(4):448–58.
66. Girdlestone J, Limbani VA, Cutler AJ, and Navarrete CV. Efficient expansion of mesenchymal stromal cells from umbilical cord under low serum conditions. Cytotherapy. 2009;11(6):738–48.
67. Fu YS, Cheng YC, ,Lin AMY, Cheng H, Chu PM, Chou SC, Shih YH, Ko MH and Sung MS. Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons in vitro: potential therapeutic application for Parkinsonism. Stem Cells. 2006;24(1):115–24.
68. [68] D. T. Covas, J. L. C. Siufi, a R. L. Silva, and M. D. Orellana, "Isolation and culture of umbilical vein mesenchymal stem cells.," Braz. J. Med. Biol. Res., vol. 36, no. 9, pp. 1179–83, Sep. 2003.
69. Romanov YA, Svintsitskaya YA and Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells. 2003;21(1):105–10.
70. Kim JW, Kim SY, Park SY, Kim YM, Kim JM, Lee MH and Ryu HM. Mesenchymal progenitor cells in the human umbilical cord. Ann Hematol. 2004;83(12):733–8.
71. Conconi MT, Burra P, Di Liddo R, Calore C, Turetta M, Bellini S, Bo P, Nussdorfer GG and Parnigotto PP. CD105(+) cells from Wharton's jelly show in vitro and in vivo myogenic differentiative potential. Int J Mol Med. 2006;18(6):1089–96.
72. Parliament THEE, Council THE, The OF and Union P. Regulation (EC) No.1394/2007 of the european parliament and of the coucil of 13 November. 2007;1394.
73. Healy L, Young L and Stacey GN. Stem cell banks: preserving cell lines, maintaining genetic integrity, and advancing research. Methods Mol Biol. 2011; 767:15–27.
74. Frances Verter. Parent's Guide to Cord Blood Foundation. http://parentsguidecordblood.org, 2006.
75. Li DR and Cai JH. Methods of isolation, expansion, differentiating induction and preservation of human umbilical cord mesenchymal stem cells. Chin Med J. (Engl). 2012;125(24):4504–10.

76. Secco M, Zucconi E, Vieira NM, Fogaça LLQ, Cerqueira A, Carvalho MDF, Jazedje T, Okamoto OK, Muotri AR and Zatz M. Multipotent stem cells from umbilical cord: cord is richer than blood. Stem Cells. 2008; 26(1):146–50.
77. Bieback K, Kern S, Klüter H and Eichler H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. Stem Cells. 2004;22(4):625–34.
78. Divya MS, Roshin GE, Divya TS, Rasheed AR, Santhoshkumar TR, Elizabeth KE, James J and Pillai RM. Umbilical cord blood-derived mesenchymal stem cells consist of a unique population of progenitors co-expressing mesenchymal stem cell and neuronal markers capable of instantaneous neuronal differentiation. Stem Cell Res Ther, 2012;3(6):57.
79. Bianco P, Robey PG and Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. Cell Stem Cell. 2008;2(4):313–9.
80. Shen H. Stricter standards sought to curb stem-cell confusion. Nature. 2013;499(7459):389.
81. Bajorath J. Molecular organization, structural features, and ligand binding characteristics of CD44, a highly variable cell surface glycoprotein with multiple functions. Proteins. 2000;39(2):103–11.
82. Schieker M, Pautke C, Haasters F, Schieker J, Docheva D, Böcker W, Guelkan H, Neth P, Jochum M and Mutschler W. Human mesenchymal stem cells at the single-cell level: simultaneous seven-colour immunofluorescence. J Anat. 2007;210(5):592–9.
83. Schieker M, Pautke C, Reitz K, Hemraj I, Neth P, Mutschler W and Milz S. The use of four-colour immunofluorescence techniques to identify mesenchymal stem cells. J Anat. 2004;24(2):133–9.
84. Khan SS, Solomon MA and McCoy JP. Detection of circulating endothelial cells and endothelial progenitor cells by flow cytometry. Cytometry B Clin Cytom. 2005;64(1):1–8.
85. Khazen W, M'bika JP, Tomkiewicz C, Benelli C, Chany C, Achour A and Forest C. Expression of macrophage-selective markers in human and rodent adipocytes. FEBS Lett. 2005;579(25):5631–4.
86. Chatterjee CT, Sarkar CRS, Dhot BPS, Kumar LCSC and Kumar CVK. Adult Stem Cell Plasticity : Dream or Reality . 2010;56–60.
87. Wang Y, Yang J, Li H, Wang X, Zhu L, Fan M and Wang X. Hypoxia promotes dopaminergic differentiation of mesenchymal stem cells and shows benefits for transplantation in a rat model of Parkinson's disease. PLoS One. 2013; 8(1):e54296.
88. Woodbury D, Schwarz EJ, Prockop DJ and Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 2000;61(4):364–70.
89. Kopen GC, Prockop DJ, and Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci. 1999;96(19):10711–10716.
90. Lin N, Lin J, Bo L, Weidong P, Chen S, and Xu R. Differentiation of bone marrow-derived mesenchymal stem cells into hepatocyte-like cells in an alginate scaffold. Cell Prolif. 2010;43(5):427–34.
91. Marappagounder D, Somasundaram I, Dorairaj S and Sankaran RJ. Differentiation of mesenchymal stem cells derived from human bone marrow and subcutaneous adipose tissue into pancreatic islet-like clusters in vitro. Cell Mol Biol Lett. 2013;18(1):75–88.
92. Boxall SA and Jones E. Markers for characterization of bone arrow multipotential stromal cells. Stem Cells Int. 2012;975871.
93. da Silva CL, Gonçalves R, Crapnell KB, Cabral JMS, Zanjani ED and Almeida-Porada G. A human stromal-based serum-free culture system supports the ex vivo expansion/maintenance of bone marrow and cord blood hematopoietic stem/progenitor cells. Exp Hematol. 2005;33(7):828–35.
94. Dalal J, Gandy K and Domen J. Role of mesenchymal stem cell therapy in Crohn's disease. Pediatr Res. 2012; 71(4):445–51.
95. Granero-Molto F, Weis JA, Longobardi L and Spagnoli A. Role of mesenchymal

stem cells in regenerative medicine: application to bone and cartilage repair. Expert Opin Biol Ther. 2008;8(3):255–68.
96. Gnecchi M, Danieli P and Cervio E. Mesenchymal stem cell therapy for heart disease. Vascul Pharmacol. 2012;57(1):48–55.
97. Klingemann H, Matzilevich D and Marchand J. Mesenchymal Stem Cells - Sources and Clinical Applications. Transfus Me. Hemother. 2008;35(4): 272–277.
98. Nekanti U, Mohanty L, Venugopal P, Balasubramanian S, Totey S and Ta M. Optimization and scale-up of Wharton's jelly-derived mesenchymal stem cells for clinical applications. Stem Cell Res. 2010; 5(3):244–54.
99. Schop D, Janssen FW, van Rijn LDS, Fernandes H, Bloem RM, de Bruijn J D and van Dijkhuizen-Radersma R. Growth, metabolism, and growth inhibitors of mesenchymal stem cells. Tissue Eng Part A. 2009;15(8): 1877–86.
100. Patel SD, Papoutsakis ET, Winter JN, and Miller WM. The lactate issue revisited: novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures. Biotechnol Prog. 2000;16(5):885–92.
101. Hassell T, Gleave S and Butler M. Growth inhibition in animal cell culture. The effect of lactate and ammonia. Appl Biochem Biotechnol. 1991;30(1):29–41.
102. Frith JE, Thomson B and Genever PG. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. Tissue Eng Part C. Methods. 2010;16(4):735–49.
103. Sart S, Schneider YJ and Agathos SN. Ear mesenchymal stem cells: an efficient adult multipotent cell population fit for rapid and scalable expansion. J Biotechnol. 2009;139(4): 291–9.
104. Zhou L, Kong J, Zhuang Y, Chu J, Zhang S and Guo M. Ex vivo expansion of bone marrow mesenchymal stem cells using microcarrier beads in a stirred bioreactor. Biotechnol Bioprocess Eng. 2013;18(1):173–184.
105. Malda J and Frondoza CG. Microcarriers in the engineering of cartilage and bone. Trends Biotechnol. 2006;24(7):299–304.
106. Schop D, Janssen FW, Borgart E, de Bruijn JD and van Dijkhuizen-Radersma R. Expansion of mesenchymal stem cells using a microcarrier-based cultivation system: growth and metabolism. J Tissue Eng Regen Med. 2008;2(2-3): 126–35.
107. Bates RC, Edwards NS and Yates JD. Spheroids and cell survival. Crit Rev Oncol Hematol. 2000;36(2-3):61–74.
108. Bartosh TJ, Ylöstalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, Lee RH, Choi H, and Prockop DJ. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. Proc Natl Acad Sci. U. S. A. 2010;107(31):13724–9.
109. Baraniak PR and McDevitt TC. Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. Cell Tissue Res. 2012;347(3):701–11,
110. Genever PG. The generation of three-dimensional tissue structures with mesenchymal stem cells. Altern Lab Anim. 2010;38(suppl1):31–4.
111. Cheng NC, Wang S and Young TH. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. Biomaterials. 2012;33(6):1748–58.
112. Chen X, Xu H, Wan C, McCaigue M, and Li G. Bioreactor expansion of human adult bone marrow-derived mesenchymal stem cells. Stem Cells. 2006;24(9):2052–9.
113. Dos Santos F, Andrade PZ, Abecasis MM, Gimble JM, Chase LG, Campbell AM, Boucher S, Vemuri MC, Da Silva CL and Cabral JMS. Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions. Tissue Eng Part C Methods. 2011;17(12):1201–10.
114. Zhao F and Ma T. Perfusion bioreactor system for human mesenchymal stem cell tissue engineering: dynamic cell seeding and construct development. Biotechnol Bioeng. 2005;91(4):482–93.
115. Nold P, Brendel C, Neubauer A, Bein G and Hackstein H. Good manufacturing practice-compliant animal-free

expansion of human bone marrow derived mesenchymal stroma cells in a closed hollow-fiber-based bioreactor. Biochem Biophys Res Commun. 2013;430(1):325–30.

116. Diederichs S, Röker S, Marten D, Peterbauer A, Scheper T, van Griensven M and Kasper C. Dynamic cultivation of human mesenchymal stem cells in a rotating bed bioreactor system based on the Z RP platform. Biotechnol Prog. 2009;25(6):1762–71.
117. Clark P, Trickett A, Stark D and Vowels M. Factors affecting microbial contamination rate of cord blood collected for transplantation. Transfusion. 2012;52(8):1770–7,
118. Cobo F, Stacey GN, Hunt C, Cabrera C, Nieto A, Montes R, Cortés JL, Catalina P, Barnie A and Concha A. Microbiological control in stem cell banks: approaches to standardisation. Appl Microbiol Biotechnol. 2005;68(4):456–66.
119. Clark P, Trickett A, Saffo S and Stark D. Effects of cryopreservation on microbial-contaminated cord blood. Transfusion. 2013;1–9.
120. Honohan A, Olthuis H, Bernards AT, van Beckhoven JM and Brand A. Microbial contamination of cord blood stem cells. Vox Sang. 2002;82(1):32–8.
121. a Jochems CE, van der Valk JBF, Stafleu FR and Baumans V. The use of fetal bovine serum: ethical or scientific problem. Altern Lab Anim. 2002;30(2):219–27.
122. Gstraunthaler G. Alternatives to the use of fetal bovine serum: serum-free cell culture. ALTEX. 2003;20(4): 275–81.
123. Simões IN, Boura JS, dos Santos F, Andrade PZ, Cardoso CMP, Gimble JM, da Silva CL and Cabral JMS. Human mesenchymal stem cells from the umbilical cord matrix: successful isolation and ex vivo expansion using serum-/xeno-free culture media. Biotechnol J. 2013;8(4):448–58.
124. Doucet C, Ernou I, Zhang Y, Llense JR, Begot L, Holy X and Lataillade JJ. Platelet lysates promote mesenchymal stem cell expansion: a safety substitute for animal serum in cell-based therapy applications. J Cell Physiol. 2005;205(2):228–36.
125. Johansson L, Klinth J, Holmqvist O and Ohlson S. Platelet lysate: a replacement for fetal bovine serum in animal cell culture. Cytotechnology. 2003;42(2):67–74,
126. Berz D and Colvin G. "Cryopreservation of Hematopoietic and Non-Hematopoietic Stem Cells–A Review for the Clinician. cdn.intechopen com. 2010.
127. Windrum P, Morris TCM, Drake MB, Niederwieser D and Ruutu T. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. Bone Marrow Transplant. 2005;36(7):601–3.
128. Naaldijk Y, Staude M, Fedorova V and Stolzing A. Effect of different freezing rates during cryopreservation of rat mesenchymal stem cells using combinations of hydroxyethyl starch and dimethylsulfoxide. BMC Biotechnol. 2012;12(49).
129. Rodrigues JP, Paraguassú-Braga FH, Carvalho L, Abdelhay E, Bouzas LF, and Porto LC. Evaluation of trehalose and sucrose as cryoprotectants for hematopoietic stem cells of umbilical cord blood. Cryobiology. 2008;56(2):144–51.
130. Sohni A and Verfaillie CM. Mesenchymal Stem Cells Migration Homing and Tracking. Stem Cells Int. 2013; 130763.
131. Rangappa S, Entwistle JW,. Wechsler S, and Kresh JY. Cardiomyocyte-mediated contact programs human mesenchymal stem cells to express cardiogenic phenotype. J Thorac Cardiovasc Surg. 2003;126(1): 124–132.
132. Meier RPH, Müller YD, Morel P, Gonelle-Gispert C and Bühler LH. "Transplantation of mesenchymal stem cells for the treatment of liver diseases, is there enough evidence. Stem Cell Res. 2013;11(3):1348–64,
133. Maxson S, Lopez EA, Yoo D, Danilkovitch-Miagkova A and Leroux MA. Concise review: role of mesenchymal stem cells in wound repair. Stem Cells Transl Med. 2012; 1(2):142–9.
134. Lau K, Paus R, Tiede S, Day P and Bayat A. Exploring the role of stem cells in cutaneous wound healing. Exp Dermatol. 2009;18(11):21–33.

135. Sato K, Ozaki K, Mori M, Muroi K and Ozawa K. Mesenchymal stromal cells for graft-versus-host disease : basic aspects and clinical outcomes. J Clin Exp Hematop. 2010;50(2):79–89.
136. Nauta AJ, Kruisselbrink AB, Lurvink E, Willemze R and Fibbe WE. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol. 2006;177(4):2080–7.
137. Di Nicola M, Grisanti PS and Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood. 2002;3838–3843.
138. Corcione A, Benvenuto F, Ferretti E, Giunti D, Cappiello V, Cazzanti F, Risso M, Gualandi F, Mancardi GL, Pistoia V and Uccelli A. Human mesenchymal stem cells modulate B-cell functions. Blood. 2006;107(1): 367–72.
139. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC and Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood. 2006;107(4): 1484–90.
140. da Silva CL, Gonçalves R, dos Santos F, Andrade PZ, Almeida-Porada G, and Cabral JMS. Dynamic cell-cell interactions between cord blood haematopoietic progenitors and the cellular niche are essential for the expansion of CD34+, CD34+CD38- and early lymphoid CD7+ cells. J Tissue Eng Regen Med. 2010;4(2): 149–58.
141. Yoo J, Kim HS and Hwang DY. Stem cells as promising therapeutic options for neurological disorders. J Cell Biochem. 2013;114(4):743–53.
142. Wohn DY. Korea okays stem cell therapies despite limited peer-reviewed data. Nat Med. 2012;18(3): 329.
143. Sharma A. Stem cell research and policy in India: current scenario and future perspective. J Stem Cells. 2009; 4(2):133–40.
144. Hyun I. Allowing innovative stem cell-based therapies outside of clinical trials: ethical and policy challenges. J Law Med Ethics. 2010;38(2):277–85.
145. Gunter KC, Caplan AL, Mason C, Salzman R, Janssen WE, Nichols K, Bouzas LF, Lanza F, Levine BL, Rasko JEJ, Shimosaka A and Horwitz E. Cell therapy medical tourism: time for action. Cytotherapy. 2010;12(8):965–8.
146. Sipp D. The unregulated commercialization of stem cell treatments: a global perspective. Front Med. 2011;5(4):348–55.
147. Jotwani G. Guidelines for Stem Cell Research Indian Council of Medical Research. Indian Counc Med Res. 2012;1–45.