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From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera?

GAETANA A. TONTI and FERDINANDO MANNELLO*

Department of Biomolecular Sciences, Section of Clinical Biochemistry, University "Carlo Bo", I-61029 Urbino (PU), Italy

ABSTRACT Bone marrow-derived mesenchymal stem cells are a multipotent adult cellular population endowed with broad differentiation potential. Their regeneration capability, ease to undergo gene modifications, and immuno-suppressive capacity makes them optimal tools for tissue engineering, gene- and immuno-therapy. Due to the ever-increasing number of studies on the clinical applications of mesenchymal stem cells in regenerative medicine, these cells have become attractive targets in clinical transplantation. However, the identification and definition of mesenchymal stem cell culture media for their clinical application in cell therapy is currently a matter of strong discussion. Up to now, clinical studies have been conducted with mesenchymal stem cells cultured in foetal calf serum, and the chance of contamination or immunological reaction towards xenogeneic compounds must be taken into consideration. On the other hand, a serum-free medium without the addition of growth factors is not able to expand these cells in vitro; so the evaluation of which is best, among foetal calf serum, human serum (whether autologous or allogeneic) and platelet-rich plasma, is a "hot topic" urgently needing further research efforts. The need for the establishment of standardized protocols for mesenchymal stem cell preparations, in order not to interfere with their self-renewal and differentiation processes, assuring durable engraftment and long-term therapeutic effects, is evidently crucial. Therefore, the search for optimal culture conditions for the effective clinical-scale production of vast numbers of mesenchymal stem cells for cellular therapy is of paramount importance and the need for a robust passage from basic to translational research is fundamental.

KEY WORDS: *AB serum, autologous serum, mesenchymal stem cell, platelet-rich medium*

The starting point

The first isolation of fibroblast-like colonies from bone marrow falls back to 1970, when Friedenstein reported the isolation of marrow-derived fibroblast-like cells, able to adhere to the plastic substrate of the cell culture plate (Friedenstein *et al.*, 1970). On this basis, many approaches have been investigated and several protocols have been established to prepare primary cultures of bone marrow-derived mesenchymal stem cells (MSC) with more homogeneous cell population (Pittenger *et al.,* 1999; Jiang *et al.,* 2002). These MSCs are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into lineage-specific cells that form bone, cartilage, fat, tendon and muscle tissue; in addition to the capacity to differentiate into their natural derivatives, MSCs have the capability to differentiate into other types of cells, giving rise to hepatic, renal, cardiac and neural cells (Song and Tuan, 2004; Pansky *et al.,* 2007). Moreover, MSCs have been isolated not only from bone marrow, but also from other tissues such as adipose tissue, skeletal muscle, trabecular bone, liver, brain, placenta, deciduous teeth, pancreas and tissue engineering (Zuk *et al.,* 2002; Tuli *et al.,* 2003; Porada *et al.,* 2006; Satija *et al.,* 2007; Spitkovsky and Hescheler, 2008).

*****Address correspondence to:** Prof. Ferdinando Mannello. Dipartimento di Scienze Biomolecolari, Sezione di Biochimica Clinica, Via O. Ubaldini 7, Università Studi "Carlo Bo", 61029 Urbino (PU), Italy. Fax: 39-0722-322370. e-mail: ferdinando.mannello@uniurb.it

These cells have generated a great deal of interest; in fact, based on their facility to be isolated, extensive expansion rate, systemic engraftment capabilities and differentiation potential, MSCs find great utility and applicability in several therapeutic fields. MSCs are hypo-immunogenic, rendering them suitable for allogeneic transplantation, and exert the capacity to produce immuno-suppression after transplantation (Deans and Moseley, 2000; Porada *et al.,* 2006, Spitkovsky and Hescheler, 2008; Le Blanc *et al.,* 2008). The high plasticity and the capacity to differentiate, in addition to the capacity to migrate to the sites of injury, inflammation, or to tumour areas, makes MSCs promising candidates for use in regenerative medicine and tissue engineering (Satija *et al.,* 2007), as well as serving as efficient delivery vehicles in site-specific therapy (Barry and Murphy, 2004, Chen *et al.,* 2008).

So, much interest and hope have raised towards the potential clinical applications of MSCs; but multiple designations have been given to different MSC preparation protocols. In fact, MSCs can be derived from different tissues as starting material, with diverse isolation protocols, cultured and expanded in different media and conditions, rendering the preparation of these cells very heterogeneous (Mannello and Tonti, 2007). Taken together these variables may lead to such consequences involving: a) the selection of cell types and the composition of heterogeneous sub-populations, b) the expansion of different cell population with different potential, and c) the alteration of long-term fate of adult stem cells upon *in vitro* culture (Wagner and Ho, 2007; Spitkovsky and Hescheler, 2008). The recent controversies on the multi-lineage differentiation potential of some MSC preparations could depend on the lack of common standard protocols; in order to obtain the best cell culture condition, a number of methodological decisions need to be made; in particular special considerations are fundamental when MSCs are going to be used for patients' treatment (Sotiropoulou *et al.,* 2007; Brinchmann, 2008), as culture conditions exert a prominent impact on transcriptome, proteome and cellular organization of bone marrow-derived MSCs (Wagner, *et al.,* 2006; Phinney, 2007).

In this Issue of *The International Journal of Developmental Biology*, Dahl and colleagues present an interesting and insightful study, evidencing the importance of highly controlled culture conditions which may favour cell proliferation and differentiation, and that, in light of the therapeutic use of MSCs, must support their genomic and epigenetic stability (Dahl *et al.,* 2008). Currently, there is extensive inconsistency among laboratories concerning the media, the culture surfaces, and the addition of supplementary factors for the successful isolation and expansion of MSCs (Fig. 1), that may thus end up with heterogeneous cell populations both *in vivo* experiments and in clinical trials. On these bases, and in view of the therapeutic utility/applicability of MSCs, the need to define a more precise and standardized protocol for MSC expansion is urgently needed.

From foetal bovine to autologous and/or allogeneic sera

Bone marrow-derived MSCs (BMMSCs) were first identified by Fridenshtein, who demonstrated that when bone marrow is plated in foetal calf serum-containing medium, colonies of adherent fibroblast-like cells develop, which differentiate into bone and adipocytes (Friedenstein *et al.,* 1970). Accordingly, most current protocols for *in vitro* culture of MSCs derived from bone marrow include foetal bovine or calf serum (FBS or FCS, respectively) as nutrient supplement (Satija *et al.,* 2007). The widely accepted use of FBS or FCS is also true for human clinical trials approved by the
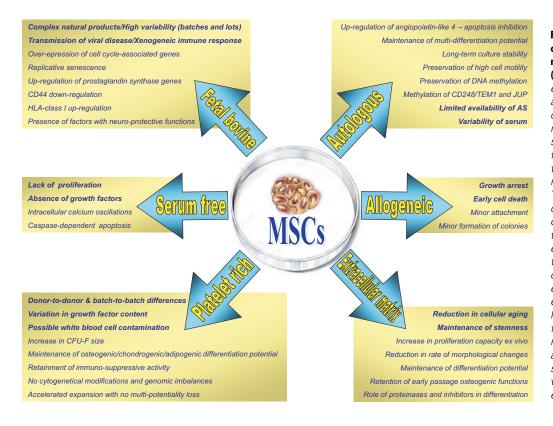

Fig. 1. Schematic representation of the pros and cons of cell culture media for mesenchymal stem cells (MSCs). *Bone marrow-derived mesenchymal stem cells are a multipotent adult cellular population with multi-differentiation potential. Due to their regeneration and immuno-suppressive capabilities, together with their facility to undergo gene modifications, they are useful tools for tissue engineering, gene- and immuno-therapy. The identification of a culture medium for the isolation and expansion of mesenchymal stem cells is a matter of strong literature discussion, as evident from the positive and negative effects which the various types of culture media have on both mesenchymal stem cell expansion/proliferation/differentiation and on bio-molecular pathways. These media range from serum-free to media supplemented with bovine foetal serum, autologous and allogeneic human sera, and platelet-enriched serum, as well as serum supplemented with extracellular matrix components.*

Fetal bovine
Complex natural products/High variability (batches and lots)
Transmission of viral disease/Xenogeneic immune response
Over-epression of cell cycle-associated genes
Replicative senescence
Up-regulation of prostaglandin synthase genes
CD44 down-regulation
HLA-class I up-regulation
Presence of factors with neuro-protective functions

Autologous
Up-regulation of angiopoietin-like 4 → apoptosis inhibition
Maintenance of multi-differentiation potential
Long-term culture stability
Preservation of high cell motility
Preservation of DNA methylation
Methylation of CD248/TEM1 and JUP
Limited availability of AS
Variability of serum

Serum free
Lack of proliferation
Absence of growth factors
Intracellular calcium oscillations
Caspase-dependent apoptosis

Allogeneic
Growth arrest
Early cell death
Minor attachment
Minor formation of colonies

Platelet rich
Donor-to-donor & batch-to-batch differences
Variation in growth factor content
Possible white blood cell contamination
Increase in CFU-F size
Maintenance of osteogenic/chondrogenic/adipogenic differentiation potential
Retainment of immuno-suppressive activity
No cytogenetical modifications and genomic imbalances
Accelerated expansion with no multi-potentiality loss

Extracellular matrix
Reduction in cellular aging
Maintenance of stemness
Increase in proliferation capacity ex vivo
Reduction in rate of morphological changes
Maintenance of differentiation potential
Retention of early passage osteogenic functions
Role of proteinases and inhibitors in differentiation

U.S. Food and Drug Administration (Horwitz *et al.,* 1999); however, as FBS or FCS are complex natural products (which may vary from lot to lot even from a single manufacturer, and the quality and concentration of both bulk and specific proteins can affect cell growth), obtaining and maintaining successful and consistent cell fermentation can be difficult (Zheng *et al.,* 2006; Mannello and Tonti, 2007). On the bases of the lack of a uniform approach for MSC expansion, a comprehensive study evaluated many culture parameters that may influence MSC expansion and differentiation. Several types of culture media, all supplemented with FBS, have been analyzed, reporting that variables such as basal medium, glucose concentration, stable glutamine, bone marrow mononuclear cell plating density, MSC passaging density, and plastic surface quality, all affect the final outcome of MSC isolation and expansion (Sotiropoulou *et al.,* 2006). For example, the use of basic fibroblast growth factor, the most common growth supplement in MSC culture media, greatly increased the proliferation rate, and due to its down-regualtion of CD44 (involved in cell-cell and cell-matrix adhesion, and responsible for extravasation and homing), it may possibly reduce effective engraftment of transplanted MSCs. Basic fibroblast growth factor also up-regulated HLA-class I and induced low HLA-DR expression, increased the *in vivo* immuno-suppressive potential of MSCs and affected MSC multi-lineage differentiation capability, favouring differentiation toward the osteogenic lineage and limiting neurogenic potential (Sotiropoulou *et al.,* 2006).

Even though extensively used in protocols for the expansion and differentiation of MSCs, FBS raises potential hazards that can not be neglected and its use has recently become a hot and very discussed topic due to the many complications and problems it raises (Mannello and Tonti, 2007); FBS may be an undesirable additive to cells that are expanded for therapeutic purposes in humans because bovine serum proteins may be internalized in stem cells and thus its use carries the risk of transmitting viral/prion disease and proteins that may initiate xenogeneic immune responses (Shahdadfar *et al.,* 2005, Dimarakis and Levicar, 2006) and may also cause immunological reactions due to the bovine protein attachment to cells in culture that act as antigenic substrates once transplanted (Heiskanen *et al.,* 2007). Despite the very early recognition and adaptation of this concept in clinical work (Holst *et al.,* 1990), only recently has wider application been allowed in emerging areas of stem cell research (Amit *et al.,* 2004; Li *et al.,* 2005), and MSC research has not been unscatched by this principle (Tao *et al.,* 2005).

At a preclinical level, cell culture protocols using human blood derivatives should avoid the risk of problems associated with FBS, and autologous serum (AS) should also be taken into consideration until an "off-the-shelf" alternative becomes available (Dimarakis and Levicar, 2006). The clinical impact of these observations remains to be more accurately evaluated, but the use of AS instead of FBS prevented life-threatening arrhythmias after cellular cardiomyoplasty (Chachques *et al.,*2004); so, these bases strongly support the motivation to evaluate for MSC culture the use of AS serum or allogeneic human serum (alloHS) other than FBS. It has been demonstrated that expansion of human MSCs in AS in the absence of FBS and without any cytokines or growth factors is as effective as supplementing the culture with FBS with regard to both isolation and expansion (Stute *et al.,* 2004; Shahdadfar *et al.,* 2005). Human MSCs expanded in AS

proliferate faster but differentiate more slowly than those expanded in FBS, and these differences are also evident in gene expression. For example, genes associated with the cell cycle are over-expressed in MSCs cultured in FBS and associated with prolongation of the cell cycle, leading to premature replicative senescence; on the other hand, angiopoietin-like 4, which is up-regulated in MSCs expanded in AS, has been shown to inhibit apoptosis. In addition, other genes over-expressed in MSC in FBS are linked to differentiation into osteoblasts, adipocytes and chondrocytes, suggesting that FBS may be conductive toward differentiation. All together these patterns of gene expression suggest that MSCs grown in FBS have gone through some steps along the differentiation pathways that MSCs in AS have yet to pass through (Shahdadfar *et al.,* 2005). Moreover, some of the genes up-regulated in MSC cultured in FBS, compared with those in AS, may be of interest for clinicians involved in defining protocols where the immunosuppressive properties are being exploited; in particular, the fact that several prostaglandin synthase genes are highly up-regulated in MSCs supplemented with FBS may move the "balance of power" towards FBS as serum supplement for these particular protocols (Shahdadfar *et al.,* 2005). An interesting study reports that medium supplemented with AS, besides providing sufficient *ex vivo* expansion of bone-marrow MSCs maintaining their multi-differentiation potential, is more effective in promoting and preserving high motility in respect to medium with FBS (Kobayashi *et al.,* 2005).

These reports point out that for what concerns the use of AS, if human MSCs are needed for therapeutic purposes, they can be expanded with AS serum supplement without risk of transcriptome instability: compared with cells expanded in FBS, human MSCs in AS are less differentiated and remain transcriptionally stable over time in culture. So, the alternative of using AS seems quite feasible, even though it has been argued that it can be used only for the minority of clinical protocols involving low numbers of MSCs (Sotiropoulou *et al.,* 2006); in fact, in this case, it is possible to obtain the appropriate volume of peripheral blood. Most types of cellular therapy need wide numbers of MSCs, which thus require large amounts of culture media and subsequently forbiddingly large volumes of peripheral blood (Sotiropoulou *et al.,* 2006). However, the limitation of AS due to its availability, may be somehow revisited, because the concept that cells must be amplified before therapeutic use is based on a doubtful basis: the need to amplify cells because they are rare in the bone marrow (Berger *et al.,* 2006). In fact, nothing demonstrates that daughter cells produced by native MSCs compensate by they numbers the properties lost or modified during amplification (Yamamoto *et al.,* 2003; Stute *et al.,* 2004; Schecroon and Delloye, 2004). Moreover, culture conditions could be adapted to the clinical application (e.g. in bone reconstruction, where the number of progenitors required could be limited and human serum seems of interest as it favours osteoblast differentiation) (Berger *et al.,* 2006) and the most primary phenotypes have been described in poor media (Reyes *et al.,* 2001; Pochampally *et al.,* 2004); so it seems possible that low serum concentrations, while limiting cell proliferation, may favour production of cells for therapeutic use, justifying further studies.

The use of pooled human serum could not help to solve the possible problem of AS limited availability as evidenced by the contradictory results regarding allogeneic serum/plasma. Alloge-

neic human serum (alloHS) results in the arrest of MSCs and their death (Shahdadfar *et al.,* 2005) and human MSCs cultures supplemented with alloHS are dramatically different from those expanded in FBS or AS; in fact, fewer cells attach and form colonies and the attached cells survive through a few cell divisions but then die; human MSCs supplemented with alloHS never reach 60% sub-confluence in the first flask (Spees *et al.,* 2004; Shahdadfar *et al.,* 2005). These reports indicate that allogeneic differences in serum composition affect MSC survival and proliferation *in vitro* to a much greater extent than xenogeneic differences; however, the allogeneic proteins responsible for this growth inhibition are not yet known. On the other side, some studies have been successful in isolating and expanding MSC using AB serum (Yamaguchi *et al.,* 2002; Anselme *et al.,* 2002), even though other Authors reported growth arrest of MSCs after the first passage (Spees *et al.,* 2004; Shahdadfar *et al.,* 2005). It has been also demonstrated that adipose tissue-MSCs can be efficiently kept throughout long-term culture with AB serum reaching the same final cumulative population doubling as FCS (Kocaomer *et al.,* 2007).

Comparing the extremes: serum-free and platelet-rich media

In order to reliably and safely produce MSCs, the use of reagents that are defined, qualified and preferably derived from a non-animal source is desirable, and possible infection complications and host immune reactions have fuelled the investigation of alternative culture supplements.

Serum-free media have been investigated, but none seem to support the proliferation of MSCs in the absence of growth factors; in fact, serum-free media have not yet been defined for the isolation and expansion of MSCs, and studies using serum-free media refer only to differentiation studies *in vitro* (Lee *et al.,* 2004; Tao *et al.,* 2005). Serum-free culture experiments on animal models demonstrate the maintenance in culture of MSCs, but only after two passages in FCS-supplemented media (Lemnon *et al.,* 1995), and due to MSC species-specific differences, even maintenance in serum-free conditions may not be applicable in the human system. Serum-free media can not promote MSC growth without the addition of cytokines or growth factors, possibly because serum induces intracellular calcium oscillations which are vital to stem cell proliferation and differentiation (Kuznetsov *et al.,* 1997; Foreman, *et al.,* 2006).

In order to avoid the many problems associated with the use of FBS, and to supply the limitation of a serum-free medium devoid of growth factors and cytokines, a commercial serum-free medium supplemented with a serum substitute (composed of all nutrients necessary for cell growth, such as growth factors, hormones, vitamins and binding proteins) was tested for the culture of MSCs (Meuleman *et al.,* 2006). Its protein content is much lower when compared to FBS and its stable composition ensures a good reproducibility and biological activity from batch to batch. The use of this latter medium allowed to reduce the duration period of MSC amplification when compared to α-MEM medium supplemented with FBS. The number of mesenchymal progenitors after primoculture and after the first passage was higher respect to classical medium, suggesting a better potential for expansion in commercial serum-free than α-MEM, and a higher capacity to conserve MSC multipotentiality. The superiority of commercial serum-free medium (Meuleman *et al.,* 2006) could be due to the presence of inhibitory factors but could also depend on the low levels of insulin, albumin, transferrin, that may play major roles in cell proliferation.

The use of human platelet lysate (PL) in a really serum-free medium seems a first step towards a defined MSC expansion procedure, and as platelets are a natural source of growth factors, PL may represent a powerful substitute for FBS, even though PL preparations may also have great differences from donor to donor, or from batch to batch; these differences make it difficult to standardize culture conditions in the presence of PL. Many investigators sought the *in vitro* expansion of MSCs in response to PL-conditioned medium. A study dealing with MSC, derived not from bone marrow but from umbilical cord, demonstrated that human MSCs can be obtained and propagated to a clinical quantity in a completely bovine serum-free system, highlighting the applicability of clinical-grade MSCs propagated with human PL-conditioned medium, for hematopoiesis support, immune regulation and vascular regeneration (Reinisch *et al.,* 2007). It has also been demonstrated that PL from platelet-rich plasma is able to promote BMMSC expansion, to decrease the time required to reach confluence, and to increase CFU-F size, as compared to FCS serum; moreover, MSCs cultured in PL and in fresh frozen plasma with platelets maintain their osteogenic, chondrogenic, and adipogenic differentiation properties and retain their immuno-suppressive activity (Doucet *et al.,* 2005, Muller *et al.,* 2006). In a study reporting the high efficacy of expansion of MSCs with human PL compared to FBS it is not clear whether the more robust MSC cultures produced by human PL only resulted from the rather constant growth factor activities (*e.g.,* EGF, FGF and PDGF), evidencing that further studies are needed to understand the behaviour of MSCs prepared with human PL compared to that with FBS (Schallmoser *et al.,* 2007). The use of platelet-rich plasma was also tested in an *in vivo* study on bone formation capacity as well as for the growth and differentiation capacity *in vitro* of MSCs, reporting that platelet-rich plasma supplemented medium yielded two-fold higher cell numbers compared to medium with FCS, retained a similar capacity to differentiate towards osteogenic, chondrogenic and adipogenic lineages and equalled *in vivo* bone formation (Vogel *et al.,* 2006, Carrancio *et al.,* 2008). The high reliability of the use of PL has been strongly confirmed by an interesting study evidencing that, compared to FCS-supplemented culture conditions, PL-supplemented culture significant increased both CFU-F as well as cumulative stem cell numbers after expansion (Lange *et al.,* 2007). These cells met all criteria for MSCs, such as plastic adherence, spindle-shaped morphology, surface marker expression, lack of hematopoietic markers and differentiation potential into three mesenchymal lineages; moreover, MSCs were cytogenetically normal and retained their immune-privileged potential by suppressing allogeneic reaction of T-cells (probably due to the down-regulation of both MHC class I and class II genes) (Lange *et al.,* 2007). Finally, gene expression profiles showed altered mRNA levels of some genes (e.g., involved in cell cycle and DNA replication), supporting the observation of accelerated MSC expansion in platelet-supplemented medium without loss of multi-potentiality.

It has been also demonstrated that PL preparations exert a dose-dependent effect on MSC expansion, probably due to the high concentration of natural growth factors contained in it; there is

also a subtle difference in the morphology of MSCs cultured in the presence of different concentrations of PL, compared to cells grown in standard FCS conditions (Bernardo *et al.,* 2007). The biosafety of MSCs was also confirmed by karyotype analysis, reporting absence of any genomic imbalances (Bernardo *et al.,* 2007). It has been also supported the hypothesis that, on the basis of a remarkable immunological functional plasticity of MSCs, the use of MSCs from PL culture/expansion, which seem to be endowed with a relatively low immune suppressive activity, could be more appropriate in reparative/regenerative cell-therapy approaches or in strategies aimed at improving hematopoietic/immune recovery after hematopietic stem cell transplantation. On the contrary, MSCs from FCS culture, seem to display a more pronounced immune suppressive function, and might be more suitable for preventing or treating alloreactive-related immune complications, such as severe GvHD and graft rejection in hematopietic stem cell transplantation and solid organ transplantation (Bernardo *et al.,* 2007).

The use of thrombin activated platelet-rich plasma has also been investigated, due to the fact that platelet activation with thrombin closely imitates the physiologic activation of platelets, ensuring the bioactivity of secreted growth factors, and, via the release of small-membrane vesicles, has additional mitogenic effects; it has been demonstrated to enhance the proliferation of MSC (even though isolated from adipose tissue) without compromising differentiation capacity and immune-phenotype (Kocaoemer *et al.,* 2007).

Even though the content of growth factors varies according to platelet concentration, preparation mode, white blood cell contamination and mechanism of platelet growth factor release, these results demonstrate that PL may be a powerful and safe substitute for FCS in the development of tissue- and cellular-engineered products in clinical settings using culture-expanded MSCs for transplantation applications. This approach provides a safe and optimal tool to elaborate cell therapy protocols by improving technical culture performances in terms of quality and quantity of MSCs produced, while reducing the patients' exposure to immunological reactions and to the risk of xenogeneic compounds. Based on cumulating evidence demonstrating the efficiency and safety of human PL or equivalent platelet rich plasma-derived preparations, the discussion on what is the best way to cultivate MSCs has surely been re-opened, requiring however further studies.

And what about extracellular matrix components?

An interesting feature of MSCs culture conditions, may be represented by the presence of different extracellular matrix biocompounds during their cultivation. It has been demonstrated that, when compared to tissue culture polystyrene, the growth of BMMSCs on a denatured collagen matrix significantly reduced the main manifestations of cellular aging (*i.e.,* the attenuation of the ability to express major protective stress response component, HSP70), increased the proliferation capacity *ex vivo* of the cells, reduced the rate of morphological changes and maintained the differentiation potential (Mauney *et al.,* 2004). For example, the maintenance of MSC osteogenic differentiation potential is primarily due to the ability of denatured collagen matrix to influence the retention of early passage osteogenic functions in late passage cells during *ex vivo* expansion, in contrast to only enhancing attenuated late

passage cellular functions during osteogenic differentiation, showing also that serum-associated factors play a role in influencing the retention of MSc osteogenic differentiation potential (Mauney *et al.,* 2006). These results were further reinforced by studies evaluating the ability of a denatured collagen type I matrix to preserve MSC adipogenic potential during *ex vivo* expansion, indicating significant preservation of the ability of late passage MSCs *ex vivo* expanded on a denatured collagen matrix to express adipogenic markers in contrast to late passage MSCs expanded on tissue culture plastic (Mauney *et al.,* 2005). An animal study was conducted on bovine MSCs analyzing the chondrogenic response during culture in different types of extracellular matrix in both serum free medium and medium supplemented with transforming growth factor beta1, showing that collagen II has the potential to induce and maintain MSC chondrogenesis, and prior to interaction with transforming growth factor beta1, to enhance differentiation (Bosnakovski *et al.,* 2006); moreover, human MSCs cultured in a collagen type I hydrogel are able to undergo a distinct chondrogenic differentiation pathway, underling their utility in terms of pre-differentiation or *in situ* differentiation of MSCs in collagen hydrogels for articular cartilage repair (Noth *et al.,* 2007).

MSCs were also cultured on an extracellular matrix (ECM) made by bone marrow cells to try to reconstitute the MSC niche. This ECM promoted replication of mesenchymal progenitors and retention of their multi-potentiality, indicating that the marrow ECM facilitates expansion of mesenchymal progenitors hypothesizing that it plays important roles in the maintenance of MSC stemness (Chen *et al.,* 2007). Interestingly, transplantation of these cells, expanded on the marrow-derived ECM, into immuno-compromised mice generated five times more bone and eight times more hematopoietic marrow compared with mesenchymal colony-forming units expanded on plastic (Chen *et al.,* 2007).

So, MSCs when cultured on tissue plastic lose their unique properties, indicating that a critical feature of the marrow microenvironment that facilitates retention of stem cell properties is missing in such culture systems. The ability of matrix components to preserve the peculiar characteristics of MSCs following extensive *ex vivo* expansion represents a novel culture technique to expand functional progenitors for tissue engineering applications.

The important role of the proteinases degrading ECM components in maintaining and influencing the behaviour of MSC has been also recently overviewed (Mannello, 2006), highlightening that matrix metalloproteinases and their inhibitors are implicated in several differentiation events; the proliferative and pro-differentiating effects of the proteolytic cascade due to Matrixins may have regulatory effects on the differentiation of MSC (including those of myoblastic, osteoblastic, chondroblastic, neural and adipoblastic lineages)(Mannello *et al.,* 2006; Tonti *et al.,* 2008).

The "dark side" of *in vitro* MSC expansion

Of great importance in cell culture is not only to remove possible contamination from the culture medium due to animal serum antigens, but also to obtain large numbers of cells for use in biomedical procedures, clinics and therapy. MSCs lack the activity of the immortalized enzyme telomerase which can counteract senescence by maintaining telomerase sequence (Zimmerman *et al.,* 2003; Serakinci, 2004) and thus have defined *ex vivo* proliferation capability, reaching senescence and losing their

multi-lineage differentiation potential after 30-40 population doublings in culture; so, it may be crucial to prolong the replicative capacity of MSCs without impairing their multi-potentiality (Simonsen *et al.,* 2002). To solve these problems, investigators can extend the life span on MSCs via retrovital transfection of human telomerase reverse transcriptase (hTERT) and human papillomavirus type 16 E6 and/or E7 genes (Takeda *et al.,* 2004). Forced ectopic expression of hTERT in MSCs can greatly extend their lifespan maintaining their differentiation potential. However, it has been demonstrated that a hTERT-transduced cell line, showed loss of contact inhibition, anchorage independence and tumor formation (Reiser *et al.,* 2005). These data suggest that MSCs can be prone to the neoplastic transformation and thus these cells may be used in both the development of novel anticancer therapeutics and in tissue engineering and transplantation protocols (e.g., use of hTERT-immortalized MSCs). So, MSCs represent an excellent model for studying telomerase gene expression with direct implications for cell therapies and tissue engineering (Satija *et al.,* 2007).

When hTERT is ectopically expressed in MSCs, neoplastic changes are accumulated suggesting that MSCs can be targets for neoplastic transformation (Serakinci, 2004), and long-term cultures of telomerase-transduced adult human MSCs may evolve spontaneously genetic changes leading to tumorigenicity in immunodeficient mice, indicating that these transduced cells represent cancer cells with hierarchical tumorigenicity, providing new models to explore the stem cell hypothesis of cancer (Burns *et al.,* 2005).

In regard to gene therapy application of MSCs, we can not rule out the possibility that gene-transduced bone marrow cells will become tumourigenic in patients several decades after commencement of cell therapy (Toyada *et al.,* 2007). What must be taken into account is that even when non oncogenic genes are introduced for cell-based therapy to increase cell growth and prolong life span, cases of leukemia have occurred in patients treated with gene-modified lymphocytes (Hacein-Bey-Abina *et al.,* 2003). There are strong links between normal stem cells and cancer stem cells, suggesting that stem cells are targets for neoplastic transformation, thus raising the question regarding the possibilities that MSCs undergo malignant transformation; however, despite having stem cells properties, when passaged in culture, MSCs reduce their proliferative rate and enter replicative senescence (Dick, 2003). A recent study has investigated the susceptibility to transformation of human bone marrow derived-MSCs at different *in vitro* culture points, reporting that telomerase activity and hTERT were not expressed, telomeres shortened during the culture period and alternative lengthening of telomere was not evidenced in MSCs (Bernardo *et al.,* 2007). All these reports highlight contrasting results, suggesting that the research in this field is in its infancy and more studies are needed to clarify if MSCs can be safely expanded *in vitro* and susceptible to malignant transformation, in order to render these cells suitable for cell therapy approaches.

Self-renewal and differentiation potential is the feature of stem cells and the roles for cancer stem cells has been demonstrated for some cancers, however the origin of cancer cells remains elusive (Wu, 2008). Cancer stem cells may derive from mutations occurring in multi-potential stem cells, being not the cause, but the consequence of carcinogenesis, where cancer cells may be caused by disturbance of self-renewal and differentiation (Wu, 2008). In this respect, recent reports have proposed the hypothesis that some carcinomas are derived from mesenchymal precursors (Rubio *et al.,* 2008), and that human MSCs exposed to tumor-conditioned media over a prolonged time assume a carcinoma-associated fibroblast-like myofibroblastic phenotype, exhibiting the ability to promote tumor cell growth both *in vivo* and *in vitro*; this is the first evidence that that human MSCs become activated and resemble carcinoma-associated myofibroblasts (Mishra *et al.,* 2008). It is well known that cancer epigenetic may be also affected by DNA methylation; it is associated with long-term gene silencing and the methylation of tumor suppressor genes may lead to cellular transformation (Laird, 2005). So, a question is reasonably raising: can methylation affect genomic and epigenetic stability of MSCs upon extended culture or may hypermethylation affect the fate of cultured MSCs?

Methylation changes during bone marrow MSC culture: what lies ahead?

The use of MSCs for therapeutic applications is based on their subsequent large-scale *in vitro* expansion, which may lead to genetic and epigenetic instabilities; however, up to now, data on MSC transformation are very few (Rubio *et al.,*2005), showing normal karyotypes and DNA copy numbers in long-term culture (Rubio *et al.,*2005; Zhang *et al.,* 2007; Bernardo *et al.,* 2007). Telomeric deletions have been reported (Meza-Zepeda *et al.,*2008), indicating that chromosomal aberrations may occur, but with a very low incidence (Bernardo *et al.,*2007). To date there is no report highlighting whether both human and animal serum sources affect genomic and epigenetic stability of MSCs in extended culture. In this respect, an important feature of epigenetics is DNA methylation, where methylation of cytosines in cytosine-phosphate-guanine may favour genomic integrity ensuring correct gene expression. In this Issue of *The International Journal of Developmental Biology* a timely study has been reported, demonstrating that MSCs cultured in AS maintain long-term genomic stability and preserve DNA methylation better that in FBS, indicating that up- or down-regulation of genes is unlikely due to genomic alterations (Dahl *et al.,*2008). Although contradictory results have been described about the alterations in DNA copy number on a number of chromosomes during MSCs culture in AS and FBS (Bernardo *et al.,* 2007), Dahl *et al.* report that all AS-supplemented cultures yielded normal gene copy numbers for all donors studied, strengthening the concept that genomic instability in BM-derived MSC culture depends on donor variability and/or variability between bovine serum lots. From these results, it seems that the regulation of genes in AS or FBS is not due to genomic modifications; in fact, there are studies suggesting lack of correlation between DNA methylation and transcription, as no gene with altered methylation pattern significantly modified its expression pattern (Shahdadfar, *et al.,* 2005). Moreover, Dahl *et al.* showed that among those genes undergoing methylation changes in MSCs, most become methylated rather than demethylated; in particular, *CD248/TEM1* (endosialin) and *JUP* (junction plakoglobin or γ-catenin) genes are involved in cell adhesion and migration, genomic stability and oncogenesis. Interestingly, among the differently methylated genes, there were more genes stably unmethylated in AS respect to FBS, suggesting that AS has a

greater capacity that FBS in maintaining the unmethylated state upon long-term culture. According to the hypothesis that BMMSCs cultured in FBS seem to differentiate more readily than in AS (suggesting an early differentiation program in FBS) (Shahdadfar *et al.,* 2005), it is conceivable that AS contributes to perpetuating a less differentiated state than FBS; however, whether this will be detectable at the DNA methylation level in a whole-genome scale or if most unmethylated genes in BMMSCs are also unmethylated *in vivo* is a matter of future perspectives due to the ever growing potential use of MSCs in regenerative medicine.

MSCs are believed as potential therapeutic targets for cell and gene-therapy-based approaches towards several different kinds of diseases, such as myocardial infarcts, graft-versus-host disease, Crohn's disease, cartilage and meniscus repair, neurodegenerative and muscle degenerative disease, stroke and spinal cord injury (Caplan, 2007, Le Blanc *et al.,* 2008; Dezawa, 2008). Due to their self-renewal potential, MSCs are strong candidates for delivering genes; however, although expanded MSCs have great proliferation and differentiation potential *in vitro*, there are limitations with the biology of these cells *in vivo*. Up to now, MSCs didn't maintain long-lasting therapeutic effects from a true self-renewing stem cell population. The loss of extensive *in vivo* self-renewal capacity may be due to the extensive expansion of MSCs in existing *in vitro* expansion systems, suggesting that the original stem cell population may no longer exist. Rather, the *in vivo* biology of expanded cell populations suggests that they may be a heterogeneous population of MSCs produced after several generations *in vitro*, and contain different types of mesenchymal cell progeny that have limited proliferation potential and responsiveness for terminal differentiation along mesenchymal and non-mesenchymal lineages (Reiser *et al.,*2005).

The "sore point" of the actual idea of MSCs may lie in the extensive proliferation capacity that we may be taking advantage of, where, extensive passages may produce cells terminally ready to differentiate, but can not grow anymore or anywhere (Rombouts and Ploemacher, 2003). The lack of telomerase activity in MSCs may not be a paradox after all. The stem cell nature of the original MSC may be lost with current expansion systems that appear to give great promise *in vitro*, but without *in vivo* efficacy. Therefore, it may no longer be surprising that their *in vivo* engraftment may no longer exist (Reiser *et al.,* 2005).

For the clinical use of MSCs, standards are needed and the clinical grade production necessitates to adhere to "good manufacturing practices" to insure the delivery of a "cell drug" that is safe, reproducible and efficient (Sensebè, 2008). Currently, there is extensive inconsistency among laboratories regarding the media and the addition of supplementary factors for the successful isolation and expansion of MSCs, that ends up with heterogeneous cell populations both in *in vitro* experiments and in clinical trials. The source and the freshness of the starting material, culture media used, presence of animal sera, cytokines, growth factors, all have a significant impact on: the cell type components and heterogeneity of the initial population, differential expansion of specific subsets, with different potentials of the end products, and long-term functional fate of MSCs as well as of other types of progenitors that are co-cultivated with them (Caterson *et al.,* 2002; Ho *et al.,* 2008).

The elaboration of a culture medium, adapt to the production of MSCs for the clinical application of cell therapy, remains a crucial matter. Since, at the moment, a serum-free medium with no growth factors is not able to amplify these cells *in vitro*, the type of serum used (FBS or human serum, allogeneic or autologous, serum or plasma) is a matter of strong discussion and effort (Berger *et al.,* 2006; Mannello *et al.,* 2007). Even though clinical studies with MSCs cultured with FBS have not shown any marked secondary effect, the possible contamination or immunologic reactions have not been investigated; and moreover, follow-up is too short to evaluate the long-term effects (Horwitz *et al.,* 2002; Le Blanc *et al.,* 2004). In theory, using human serum should avoid the risk of these secondary effects, suggesting not to discard the idea of using human serum, in particular autologous serum (Mannello, 2006). The choice of the best media for MSC may rely on its ability to obtain MSC with characteristics more similar as possible to those of native MSCs, which requires to identify progenitors in fresh bone marrow (involving quality control of the mesenchymal compartment of non-manipulated grafts) and to evaluate the self-renewal capacity of MSCs with suitable tools (Berger *et al.,* 2006; Mannello *et al.,* 2007)

In conclusion, different pre-operative protocols lead to MSC-like cell cultures and the quality of preparation from different laboratories is very diverse with heterogeneous products (Fig. 1). All these reports, point out to the urgent need for the establishment of standardized approaches and protocols for MSC preparations, in order not to interfere with MSC self-renewal and differentiation processes as well as gene methylation, assuring durable engraftment and long-term therapeutic effects of MSCs; in particular special considerations are necessary if the cells are to be used for the treatment of patients. Therefore, the search for the optimal culture conditions for the effective clinical-scale production of vast numbers of MSCs for cellular therapy in transplantation, immuno-therapy and regenerative medicine is of paramount importance and the need for a robust passage from basic to translational research is thus fundamental.

Although we have not overviewed all aspects of the best conditions for MSC culture and even though at the end many questions remain unsolved, it is essential to know and consolidate the foundations in order to reach the future perspectives; the resolution of this demanding task is not immediately at hand, but such a challenging issue as the search of MSC optimal culture conditions, surely makes up with all the efforts.

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