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Review

Characterization of mesenchymal stem cells and their application in experimental embryology

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Abstract

The efficiency of somatic cell cloning (somatic cell nuclear transfer; SCNT) as well as *in vitro* fertilization/*in vitro* embryo production (IVF/IVP) in mammals stay at relatively same level for over a decade. Despite plenty of different approaches none satisfactory break-through took place. In this article, we briefly summarize the implementation of mesenchymal stem cells (MSCs) for experimental embryology. The advantages of using MSCs as nuclear donors in somatic cell cloning and *in vitro* embryo culture are described. The description of results obtained with these cells in mammalian embryo genomic engineering is presented.

Key words: mesenchymal stem cell, *in vitro* culture, somatic cell cloning, embryo

Characteristics of mesenchymal stem cells and their potential for differentiation

Mesenchymal stem cells (MSCs), also termed multipotent marrow stromal cells or mesenchymal stromal cells are a heterogeneous population of plastic-adherent, fibroblast-like cells, which in culture are able to self-renew and differentiate into bone, adipose and cartilage tissue (Horwitz et al. 2005). The MSCs have traditionally been isolated from the bone marrow (BM) but isolation of cells with MSC characteristics from a variety of tissues including cord blood, peripheral blood, fetal liver and lung, adipose tissue, skeletal muscle, amniotic fluid and the circulatory system is applied. The MSCs are rare in the BM (occurring at an estimated frequency of 1 in 1×10^5 cells in the human bone marrow) but can be isolated by selecting the adherent, spindle-shaped cells that expand from mononuclear cells in humans, rodents and pigs (Caplan 2007). Different cell types of predominantly mesodermal origin could be identified in the adherent fraction of bone marrow mononuclear cells including embryonic-like stem cells, lineage committed progenitors as well as mature cells such as osteoblasts and fibroblasts (Jiang et al. 2002, Ratajczak et al. 2004, 2008). MSCs may also generate mature cells typically arisen from endoderm or ectoderm (Oh et al. 2007, 2008, Tondreau et al. 2008) suggesting that *in vitro* cultures of bone marrow-derived MSCs may represent a mixture of phenotypically, functionally and bio-

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chemically different cells (Phinney 2007, Jones and McGonagle 2008).The amount of primitive stem cells in these cultures is rare and varies depending on the method of cell isolation or culture conditions and the donor's age (Wagner and Ho 2007, Ho et al. 2008). There are morphological differences between MSC colonies and within initial colonies (Vacanti et al. 2005, Horn et al. 2008, 2010). Colonies of larger or smaller cells with prevailing round or elongated cellular morphology and with tight or disperse growth pattern can be recognized. Faster proliferating cells will overgrow slowly dividing cells. Nevertheless, variable kinetics of mitotic cell divisions between the established MSC subpopulations (i.e., their diverse migration rapidity and proliferative activity) is intensified by different methods of cell isolation/culture, different types of cell expansion media and their supplementations (i.e., various qualitative and quantitative composition/differential formulation of the media). Generally, certain media will favour different subpopulations of MSCs (Schallmoser et al. 2008, Baer et al. 2009, Baer and Geiger 2010).

MSCs cell surface markers

Despite the variation in phenotypes of mesenchymal subpopulations, it is widely accepted that the cultured cells, regardless of the methods employed in their isolation and culture lack expression of hematopoietic antigens including clusters of differentiation (CDs): CD45 (receptor-type tyrosine-protein phosphatase C), CD34 (single-pass transmembrane sialomucin glycoprotein), CD11b (antigen-like family member B), CD14 (leukocyte-specific pathogen recognition/primitive pattern recognition co-receptor) and CD133 (prominin) expression. Simultaneously, MSCs have been reported to express: CD105 (endoglin), CD73 (5'-ribonucleotide phosphohydrolase/ /ecto-5'-nucleotidase; NT5E), CD90 (also known as thymus cell antigen-1/thymocyte differentiation antigen-1; Thy-1), CD29 (integrin f-1), CD44 (hyaluronate receptor), CD71 (also known as transferrin receptor), CD106 (vascular cell adhesion protein 1/vascular cell adhesion molecule 1; VCAM-1), CD166 (also called activated leukocyte cell adhesion molecule; ALCAM), STRO-1 (stromal precursor cell marker), GD2 (disialoganglioside) and CD146 (melanoma cell adhesion molecule; MCAM or cell surface glycoprotein; MUC18) (Pittenger et al. 1999, Martinez et al. 2007, Sacchetti et al. 2007). Methodology employed in the isolation of mesenchymal stem cells is heavily reliant on the ability of these cells to adhere to and subsequently proliferate on tissue culture plastic. No single, unique marker allowing for MSCs isolation has been reported, but rather a range of composite cell surface phenotypes are utilised.

Promotion of in vitro cell viability by MSC-mediated paracrine activities

An extensive study by Crisan et al. (2008) has established the links between MSCs and pericytes. Pericytes are thought to stabilize blood vessels, contribute to tissue homeostasis under physiological conditions and play an active role in response to focal tissue injury through release of bioactive molecules which have trophic and immunomodulatory properties. Based on this assumption it can be hypothesized that MSCs can exert the positive effect on the developmental capabilities and quality of *in vitro* cultured embryos. Also, Potapova et al. (2007) supported hypothesis that human MSCs (hMSCs) serve as trophic mediators for endothelial cells. They cultured hMSCs as a monolayer or as three-dimensional aggregates in hanging drops (hMSC spheroids). They showed that medium conditioned by hMSC spheroids was more effective in stimulation of umbilical vein endothelial cell proliferation, migration, and basement membrane invasion than medium conditioned by a monolayer of hMSCs. This medium also promoted *in vitro* survival rate of endothelial cells. However, it was already proved that maintaining MSCs as spheroids reduces the volume of medium required for cell culture and affects concentrations of cytokines in conditioned medium (Potapova et al. 2007).

Certainly, to be sure that the cells used for experimental embryology involving *in vitro* embryo culture (IVC) or somatic cell cloning procedures are really homogenous populations of mesenchymal stem cells, their characteristics must be performed. As mentioned previously, the broad variety of tissue sources from which MSCs are isolated, in conjunction with disparate culture conditions, has led to a lack of consensus regarding the phenotype of MSCs. In addition, differences in the formulations of media used to culture the cells, the plating density and the oxygen tension may affect the phenotype of the cell population of mesenchymal origin/lineage (Pittenger et al. 1999, Reyes et al. 2001, D'Ippolito et al. 2004).

MSCs in tissue engineering and regenerative medicine

In recent years there has been increased interest in potential utility of MSCs in both tissue engineering and regenerative medicine that offer the novel clinical

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approaches for replacement or repair of damaged, deteriorated, defective or senescent/aging cells, tissues and organs; ii) to restore, maintain or improve the tissue or organ function iii) to avoid/prevent or reverse the tissue and organ failure or insufficiency. Animal studies have provided a useful tool for defining a number of diverse potential applications for MSCs, e.g., to contribute to the functional repair of a skeletal defect (Bruder et al. 1998).

Mesenchymal cells also exhibit immunomodulatory and anti-proliferative effects on T cells, an attractive feature for cell therapy. Cultured MSCs do not express antigens of the class II major histocompatibility complex (MHC-II) on their cell surface and they suppress a primary mixed lymphocyte reaction, which has prompted the question as to whether these cells can suppress ongoing immune cell-induced reactions in humans and hence be used for the treatment of graft-versus-host disease (GVHD) (Tse et al. 2003). Therefore these cells can also be used in the preclinical and clinical research programs aimed at xenotransplantation of tissues and organs (xenograft transplantation surgery).

Several studies have provided evidence of a strong correlation between age and the proliferative potential exhibited by MSCs *in vitro*, with decreasing progenitor proliferation associated with increasing age (Clarke and McCann 1989). This diminishment could be caused by senescence of the culture-expanded cells or by terminal differentiation through the course of cell culture and passaging (Banfi et al. 2000, Vacanti et al. 2005), which may be related to epigenetic changes of mesenchymal stem cells during prolonged culture (Shibata et al. 2007). A decline in MSC function may therefore be important in several disease states (Minguell and Marttnez 1983, Scopes et al. 2001) as well as in works using MSCs for *in vitro* embryo production (IVP) and somatic cell cloning.

Cultured mesenchymal cells have been shown to exhibit some unique properties that challenge the dogma that stem cells derived from the adult tissue produce only the cell lineages characteristic of tissues in which they reside. The apparent ability of MSCs to give rise to cells of multiple germ layers, however, must be examined cautiously, as undifferentiated mesenchymal cells have been shown to spontaneously express neural markers (Deng et al. 2006) as well as markers of smooth muscle cells. The mechanism by which MSCs repair damaged tissues *in vivo* is still poorly understood. However, recent evidence suggests that repair is likely achieved through paracrine factors released by mesenchymal cells rather than transdifferentiation of mesenchymal cells into specific tissue cell types (Hofstetter et al. 2002). Studies examining the efficacy of transplanted mesenchymal cells in animal models of myocardial infarction, lung injury, kidney damage and neurological diseases are ongoing, and may provide further insights into mechanisms underlying MSC-mediated tissue repair (Moscoso et al. 2005). Moreover, for these therapeutic applications, MSCs are used in an allogeneic setting that might facilitate efficient elimination of oncogenically-transformed cells by the immune system (Wagner et al. 2010a,b). Autologous application might be more prone to tumour formation. Therefore, the use of early cell passages and additional quality control, should be implemented. The danger of malignant transformation needs to be taken into account although tumour formation has not yet been described in clinical trials.

The use of MSCs in mammals SCNT – epigenetic aspect

In vitro cultured fibroblast cells, which had been derived from the tissue of foetuses and adult specimens, or derived from the follicle (granulose cells/cumulus cells) are the commonly used source of nuclear donor cells in the somatic cell cloning of mammals (Skrzyszowska et al., 2008, Jeon et al. 2008, Van Thuan et al. 2009, Zhao et al. 2010, Dai et al. 2010, Samiec and Skrzyszowska 2010a, 2012a). The degree of molecular and epigenetic differentiation of these cells, which is related both to the advanced methylation profile of DNA cytosine residues and to the lysine deacetylation profile of histones forming nucleosomal core of nuclear chromatin, often seems to make impossible converting the covalent modifications back to a totipotent state of embryonic (zygotic) cells. This leads mainly to a decrease in the abilities of differentiated fibroblast or cumulus cells for supporting the *in vitro* development of cloned embryos to the blastocyst stage (Iager et al. 2008, Costa-Borges et al. 2010, Das et al. 2010, Lee et al. 2010, Samiec and Skrzyszowska 2010b, Kim et al. 2011, Samiec et al. 2012). The MSCs exhibit the low activity of histone deacetylases (HDACs) and DNA (cytosine-5)-methyltransferases (DNMTs), which gives rise to the hyperacetylation of histone lysine moieties and to wide-range demethylation of DNA cytosine residues (Vacanti et al. 2005, Inoue et al. 2007, Martinez-Diaz et al. 2010). The last two processes impact the inhibition of transcriptional suppression of many regions in the nuclear genome of multipotent MSCs. All these properties are responsible for the high susceptibility of the MSC nuclei to correct and complete epigenetic reprogramming in the cytoplasm of NT embryo blastomeres. That is why the genome of undifferentiated stem cells may be more easily reprogrammed to

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resemble the genome of the zygote, which may make stem cells more efficient as nuclear donors in the somatic cell cloning (Yamanaka et al. 2009, Dai et al. 2010).

Such possibility has been indirectly confirmed by the expression profiles of different genes at different stages of MSC/NT embryos preimplantation development which more closely mimic *in vivo*-produced pig embryos when compared with NT embryos reconstituted with fibroblast cells (Kumar et al. 2007). The panel of genes analysed involved: genes engaged in transcription and pluripotency (POU domain transcription factor *Oct4*, signal transducer and activator of transcription *Stat3*, homeobox containing transcription factor *Nanog*), genes involved in DNA methylation and histone deacetylation (DNA methyltransferase 1 *Dnmt1*, DNA methyltransferase 3a *Dnmt3a*, histone deacetylase 2 *Hdac2*) genes involved in growth factor signalling and genomic imprinting (insulin-like growth factor 2 *Igf2*, insulin-like growth factor 2 receptor *Igf2r*) as well as pro- and anti-apoptotic genes (B-cell leukemia/lymphoma 2 protein *Bcl-2 and* Bcl-2-associated X protein *Bax*).

SCNT efficiency with the use of somatic cells vs. undifferentiated and/or differentiated MSCs

Generation of cloned embryos that had originated from bovine and porcine enucleated oocytes receiving the cell nuclei of undifferentiated adult bone marrow-derived MSCs gave rise to consistently higher preimplantation development compared to creation of NT embryos using adult fibroblast cells (Kato et al. 2004, Colleoni et al. 2005).

Furthermore, porcine bone marrow MSCs were able to undergo transient and stable genetic modifications with non-viral and viral DNA vectors and were found to be an attractive nuclear donor cell type for production of transgenic cloned embryos/offspring (Colleoni et al. 2005, Bosch et al. 2006). Generally, NT blastocysts yields originated from porcine MSCs were significantly higher than NT blastocysts yields originated from adult and foetal fibroblast cells (Faast et al. 2006, Jin et al. 2007, Kumar et al. 2007, Lee et al. 2010). Interestingly and contrary to the abovementioned conclusion, Bosch et al. (2006) observed no difference in the rate of development of cloned pig embryos to the blastocyst stage when as donor nuclei served transfected fibroblast cell and genetically-modified MSC. In another study convincing of superior MSCs utility over somatic cells for cloning purposes, NT blastocysts yields originated from porcine MSCs were significantly higher than NT blastocysts yields originated from MSCs differentiated *in vitro* into osteocytes, adipocytes and chondrocytes. Still, the developmental outcome of the latter was 2 times higher in comparison to NT embryos derived from fetal fibroblasts (Lee et al. 2010). Therefore, regarding cloning it can be stated that MSCs and MSCs subjected to differentiation are more efficient and more useful than somatic cells.

As MSCs are relatively difficult to isolate from the bone marrow of live animals, attempts were undertaken to determine whether MSCs could be isolated from the peripheral blood circulation system and subsequently used for efficient production of NT embryos. Although these cells exhibited the same antigen profile as bone marrow MSCs the enhancement in preimplantation developmental capacity of NT embryos reconstructed with blood-derived MSCs did not occur (Faast et al. 2006).

On the basis of the above-mentioned findings, it is hypothesized that the use of undifferentiated MSCs isolated from the adult bone marrow, allows to improve the preimplantation developmental potential of mammalian cloned embryos.

Conclusions

The origin and type of nuclear donor somatic cells could be considered the prime causes affecting the developmental capability of cloned embryos, and the progressive diminishment in SCNT efficiency has been related to the molecular and epigenetic differentiation status of the nuclear donor cells (Inoue et al. 2007, Kumar et al. 2007). For this reason, the MSCs bearing a relatively undifferentiated genome might serve as suitable source of nuclear donors the epigenetic intracellular memory of which could be more faithfully reprogrammed to establish *de novo* and recapitulate the early embryonic gene expression patterns (i.e., both DNA transcriptional and mRNA translational activities) following somatic cell cloning.

It will be important to further characterize the biology of different subpopulations of MSCs and to better determine their potential in various applications including assisted reproductive technologies, experimental embryology and embryo genomic engineering. We are still not able to fully predict or control the MSC behaviour, homing potential, molecular mechanisms underlying the MSC genomic/epigenomic plasticity, multipotency level determined by adult or foetal stages of ontogenesis, proliferative activity, robust clonal self-renewal and lineage commitment, and their ability to spontaneously transform. By standardized culture conditions and the use of high-quality MSCs, their spontaneous transformation (i.e., multi-

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lineage transdifferentiation, oncogenic transformation and tumour-supporting potential) may be limited or avoided *in vitro*. Due to extensive characterization of multipotent MSCs, the specific protein factors that are secreted by the MSC clonal lines may be identified, which can play a pivotal role for both developmental competencies and epigenetic DNA reprogrammability of *in vitro* fertilization-derived and somatic cell cloned embryos.

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