

New Frontiers in Regenerative Medicine in Cardiology: The Potential of Wharton's Jelly Mesenchymal Stem Cells

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Abstract: Cardiomyopathies are still the first cause of death in the world. The identification of resident stem cells, comprising those derived from sub-endocardial stroma, suggests the possible self regeneration of the heart under autocrine/paracrine modulation in the cardiac microenvironment. Nevertheless, because of the limited *in vivo* regeneration potential of damaged cardiac tissue, the use of drugs and ultimately cardiac transplantation remain the common treatments of heart diseases and defects. The differentiative potential of embryonic and mesenchymal stem cells (MSCs) derived from different tissues (such as bone marrow and adipose tissue) was extensively explored in cell therapy for regenerative medicine. Many groups have been focused, in recent years, on isolation, characterization, and differentiation potential of MSCs derived from perinatal (or extraembryonic) tissues, mainly the placenta and the human umbilical cord. In this review, we summarized recent works about the stemness of Wharton's jelly stromal cells and their potential in cardiac regeneration with favourable use in cell therapy and regenerative medicine. The peculiar features of these cells, as the expression of cardiac-specific transcription factors and immunomodulatory molecules suggest that human umbilical cord may be considered as a reliable alternative source of MSC useful for advanced therapy in cardiac regenerative medicine.

Keywords: Heart failure, mesenchymal stem cells, regenerative medicine, Wharton's jelly, immune modulation, tissue repair.

1. HEART RESIDENT STEM CELLS AND THEIR LIMITATION IN CARDIAC REGENERATION

Cardiomyopathies (congenital heart diseases, myocardium infarction, heart failure) are still the first cause of death in the world. As well reviewed by Anversa and colleagues, the concept on the heart as a post-mitotic organ featuring irreplaceable myocytes has changed during the last two decades, creating a strong debate in the scientific world [1]. In fact, the identification of human cardiac stem cells (hCSCs) which feature the essential properties of all stem cells (self-renewal, clonogenicity, multipotency) was the basis for *in vitro* and *in vivo* studies on animal models that highlighted their ability to regenerate cardiomyocytes [2]. The regenerative ability of cardiac tissue is probably based on the activation of numerous genetic and epigenetic events, as the activation of chromatin remodelling factors and transcription factors (such as GATA 4, Nkx2-5, Tbx 5) [3]. Autocrine/paracrine modulation in the cardiac microenvironment is thought to act on these resident stem cells, perhaps through the secretion of growth factors by stressed car-

diomyocytes, such as insuline-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF), and stromal-derived factor 1 (SDF-1), which exert their paracrine effects on progenitor cells. In addition, these progenitor cells seem to secrete cardioprotective factors, such as adrenomedullin, connective tissue growth factor, and interleukin-1 receptor-like 1 [4]. Resident cardiac stem cells were characterized by various groups as different populations, such as Lin⁻ c-kit⁺ (isolated from adult rat heart), Sca-1⁺, isl-1⁺ cells, and side population (SP). Functional characterization experiments showed that these populations possess different efficacy in generating action potentials, expressing cardiac markers, mature sarcomeric structures formation, thus resulting in improved cardiac functionality. By contrast, these cells constitute a low percentage of total resident ones and need to be co-cultured *in vitro* to improve their cardiogenic potential [5-11]. The latter evidence may explain in part the poor regenerative power of the heart by itself. As well discussed by Vandervelde and co-workers, there are many signaling factors released by myocardium after injury that induce mobilization and homing of bone marrow-derived stem cells from peripheral blood to the site of cardiac damage [12]. Among these factors, hematopoietic factors (granulocyte-colony stimulating factor, G-CSF and stem cell factor, SCF), Interleukin-8 (IL-8), tumor necrosis factor-alpha (TNF- α), vascular endothelial growth factor (VEGF), SDF-1 (together with its receptor CXCR4), fibroblast growth factor (FGF), IGF, HGF,

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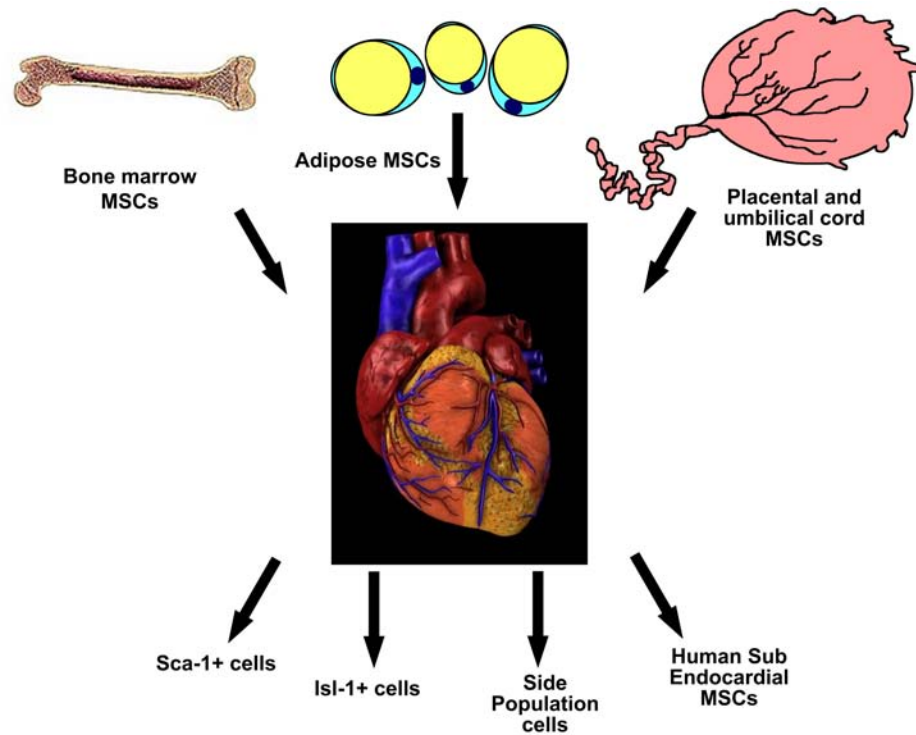


Fig. (1). Schematic representation of the main cellular sources for heart therapy compared to the main cellular populations of heart resident stem cells. Bone marrow, adipose tissue and perinatal tissues represent the main alternatives to be explored for cellular therapy of human heart. Conversely, Human hearts have been proved to contain stem/progenitor cells populations which biology has only been partly explored. No data exist on the possible interactions between endogenous and exogenous stem cells in heart repair mechanisms.

platelet-derived growth factor (PDGF), and others, seem to play pivotal roles in mobilization, homing, proliferation, differentiation, and cardiac protection [12-14]. Pathological conditions of cardiac tissue are also related to the onset of oxidative stress cascade, leading to the activation of pro-survival responses such as the expression of erythropoietin (EPO) [12], cyclooxygenase-2 and heme oxygenase-1 [15], myeloperoxidase (MPO) [16-18] and heat shock proteins (HSPs) [19-27], as well as biomarkers of nitrosative stress [18, 28]. Because of the limited *in vivo* regeneration potential of damaged cardiac tissue, the use of drugs and, at last, cardiac transplantation are, at instance, the common treatments of heart diseases and defects. Nevertheless, there are many limitations to these approaches, especially related to the number of organ donors. For this reason, new frontiers in therapeutic approaches were recently focused on the application of cell therapy, strictly related with cardiac tissue engineering by design appropriate *in vitro* approaches and/or *in vivo* transplantation [29, 30]. Very recently, our group has described a new and reproducible non-enzymatic isolation method in order to obtain mesenchymal stem cells derived from sub-endocardial zone (HSE-MSCs) of human left ventricle from patients undergoing heart transplant for post-infarct chronic heart failure. We showed that these cells expressed markers and featured a differentiation potential similar to other MSC populations, as well as we reported for the first time the expression of immunomodulatory molecules, namely non-classical major histocompatibility complex (MHC) class I HLA-E and HLA-F (class Ib HLA) and

costimulatory molecules B7-1 (but not B7-2) and the immunosuppressive marker B7-H3, suggesting an inhibitory effect on immune system and a favourable outcome from an immune modulation after transplantation. Moreover, these cells showed the expression of cardiac markers (connexins-26, -43, and -45, myosin heavy chain) and cardiac-specific transcription factors (Isl-1, Nkx 2.5, MEF2C, myocardin) [31]. The latter findings suggested and highlighted the existence of a regeneration potential in failed human hearts and deserve further investigations about new sources of MSCs with similar features to HSE-MSCs. Apart heart-derived cells, different sources of stem cells were explored to be applied in cardiac regenerative medicine, as outlined in the following sections of the review.

2. EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

Embryonic stem cells (ESCs), derived from inner cell mass at blastocyst stage of the embryo, are able to generate all tissue cell types, leading to the formation of the three germ layers (ectoderm, mesoderm, and endoderm), and can also differentiate into cardiomyocytes [32 and refs. therein], but they may conversely generate tumor formation and immune rejection response by the host [33, 34]. Moreover, the ethical problems related to their procurement and use, together with tumorigenicity and rejection response [35], made necessary to find other sources of cells with similar behaviour. Induced pluripotent stem cells (iPSC), firstly described by Takahashi and Yamanaka in the 2006 [36] were derived

from adult somatic cells and *in vitro* genetically manipulated by both viral and nonviral methods to express four factors, Oct 3/4, Sox2, Krüppel-like factor 4 (klf-4), and cellular myelocytomatosis oncogene (c-myc) (in mouse fibroblast), or together with Oct 3/4, Sox2, Nanog, and Lin28 transcription factors (in human fibroblasts) [37 and refs. therein]. Progenitor cells from other adult tissues were hypothesized as other possible source for potentially cardiogenic transdifferentiation, by genetic incorporation of several transcription factors. Fibroblasts were reprogrammed towards cardiomyocytes or endothelial progenitor cells [38]. Human embryonic stem cell-derived mesenchymal stem cells, were reprogrammed to express fusogens that can create multinucleate cells [39], and selected stem cell antigen 1-negative (Sca-1⁻) skeletal muscle [40]. A comparison between different adult somatic cells from bone marrow, hair keratinocytes, and skin fibroblasts, induced towards cardiomyocytes, showed a higher differentiation efficiency for bone marrow cells than other cell types [41]. Several differentiation methods have been reported using embryoid body formation, coculture with stromal cells (visceral endoderm-like cells), or 2D system (Matrigel) culture, and supplementation of cytokines and bone morphogenic protein (BMP) [37]. Nevertheless, some problems still remain concerning the incomplete integration of vectors and the associated risk of tumorigenesis [42]. Moreover, transplantation methods and post-transplantation efficiency of these cells need to be better improved, due to their poor survival, the reduced engraftment potential, and the safety issues. As reviewed in literature, the drug or hypoxic preconditioning of stem cells populations, in addition with growth factor cocktails, resulted in an upregulation of all those factors involved in survival, migration, myocardial tolerance, and possible differentiation towards a cardiac fate [43, 44].

3. BONE MARROW AND ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

Adult tissues (blood, bone marrow, muscle, bone, dermis, adipose tissue, and others) host adult mesenchymal stem cells (MSCs) that can potentially differentiate into mesodermal derivatives, such as adipocytes, fibroblasts, chondrocytes, osteoblasts/osteocytes, stromal cells (reviewed in [45]) and towards neural cells (as for bone marrow-derived stem cells, BMSCs) [46], or eventually, neuron protecting cells (as for adipose-derived stem cells, ASCs) [47]. As reviewed by Elnakish and colleagues, the use of MSCs in preclinical trials (especially in experimental animal models) showed an improvement of left ventricular function, a decreased size of infarcted area, and a decreased mortality [48]. Nevertheless, bone marrow (BM) and adipose tissue (AT) were extensively studied and still remain the major sources of adult stem cells used in most clinical trials, since their transplantation seems to be related to an improvement of heart physiology [49-53]. Coculture protocols with neonatal rat ventricular myocytes highlighted the cardiogenic potential of BM-MSCs, suggesting the influence of soluble factors [54]. Studies in rat models showed the efficiency of both intravenously-injected BM-MSCs and endogenous BM-MSCs in homing and regenerating myocardium under the oral administration of cardiogenin, a natural active compound extracted from *Geum*

japonicum, even if the use of exogenous BM-MSCs may be associated with the risks related to dangerous intramyocardial injection, as well as possible immune rejection response, and the oral administration of cardiogenin is not yet studied at pharmacodynamic and pharmacokinetic level for clinical therapy [55]. In a similar way, rat adipose-derived MSCs (ACSS) treated with phorbolmyristate acetate, a protein kinase C (PKC) activator, showed the expression of cardiac-specific markers (such as cardiac troponin T, myosin light chain, myosin heavy chain) and a reduction of infarct size, interstitial fibrosis, and apoptotic index, suggesting a possible role in restoration of electromechanical function in infarcted rat hearts [56]. Zuk and co-workers analyzed the differentiation potential of adipose MSCs from human lipoaspirate towards adipogenic, osteogenic, chondrogenic, neurogenic and myogenic lineages [57]. Cardioprotection induced by BM-MSCs and ASCs seems to be related also to hypoxia condition and reduced both senescence [58] and apoptosis due to the release of VEGF and IGF-1 [59]. In parallel, the oxidative stress resulting after cardiac injury may play an important role on the prevention of apoptosis, perhaps by expression of specific receptors on BM-MSCs, such as Toll-like receptor 4 (TLR-4) (reviewed in [60]). BM-MSCs are able to differentiate *in vitro* towards adipocytes, chondrocytes, and osteocytes, after treatment with tissue-specific growth factor-supplied culture media [61]. As summarized by Pourrajab and co-workers, it was also supposed that the mechanisms by which BM-MSCs can reach the site of injury and exert their regulation in cardioprotection is related to the modification of extracellular matrix (ECM) under the stressed cardiac cells releasing of proteinases, such as metalloproteinases (MMPs), on the different collagen molecules [60]. MMPs constitute a broad family of extracellular proteinases which are involved in the development, function and pathogenesis of several tissues and organs [62-64] as well as in immunomodulation by mesenchymal stem cells (reviewed in [65]). ECM fragments seem to be involved in BM-MSCs chemotaxis and were also associated to the subsequent downregulation of collagen synthesis by resident cardiac fibroblasts [60]. Remodelling of ECM and its alterations exerted by MMPs under stress conditions may critically affect microenvironmental influence on adult cardiac progenitor cells and cardiac regeneration potential [66]. These findings highlighted that paracrine molecules may have crucial therapeutic effects in cardioprotection and regeneration. Despite their extensive use in research, adipose tissue and bone marrow are still affected by heterogeneity of the cell phenotype [51, 67], highlighting the complexity to isolate specific subpopulation, such as mesenchymal stem cells (MSCs). Moreover, the amount of MSCs that can be isolated from bone marrow is too low (estimated at about 0.001 to 0,01% for 1.073 g/ml of bone marrow aspirate) [61], because also the problem related to the availability of bone marrow tissue. In contrast to other adult stem cells, BM-MSCs were phenotypically described as non-hematopoietic (CD14⁻, CD34⁻, and CD45⁻) fibroblast-like cells, that are negative also for endothelial marker CD31, and possess a specific immunomodulatory activity, due to the absence of MHC class II and co-stimulatory molecules (CD80⁻, CD86⁻) that may render T lymphocytes anergic (reviewed in [68]), suggesting the possibility of allogeneic transplantation.

4. HUMAN UMBILICAL CORD: THE HIDDEN POTENTIAL OF A DISCARDED TISSUE

Despite the extensive use of BM-MSCs in pre-clinical studies, many groups were involved, in recent years, on isolation, characterization, and differentiation potential of MSCs derived foetus-associated organs, known as perinatal (or extraembryonic) tissues, mainly the placenta and the human umbilical cord. Currently, they are both discarded as a waste of the labour, but they proved to contain large numbers of multipotent mesenchymal cells, which were supposed to feature intermediate characteristics between embryonic and adult stem cells [69]. The amnios has shown to bear adherent MSCs with classical pattern of molecular markers and multipotency (as shown by *in vitro* differentiation towards endodermal, mesodermal, and ectodermal lineages) [70]. These cells express cardiac-specific transcription factors (such as those belonging to GATA family), and MHC class I molecules HLA-A, -B, and -C. It has been demonstrated that these cells exert *in vivo* a differentiative potential towards cardiomyocytes and result in improved cardiac function [71, 72]. Moreover, as well demonstrated by Park *et al.* (2011), perivascular cells from placental villi showed adhering and migratory behaviour and myogenic differentiation in both *in vitro* and *in vivo* experiments [73]. Parolini's group well reviewed and analyzed regenerative potential of placenta-derived cells [74]. Moreover, they demonstrated the effect of human amniotic membrane fragments on restoration of cardiac ischemia (in a rat model), showing an amelioration of cardiac dysfunction [75, 76]. These effects may be due to release of mediators rather than direct differentiation of amniotic cells. Placenta-derived amniotic MSCs were also analyzed and proposed for use in therapy, due to their immunomodulatory activity (exerted by the inhibition of T lymphocyte proliferation) for which the release of prostaglandins has been suggested at the main mechanism [77]. Recent data highlighted the human umbilical cord (hUC) as a source of mesenchymal stromal cells. hUC is an extraembryonic formation that originates at day 13 of the embryonic development [78] that connects foetus and mother during pregnancy through the placenta. At term, HUC consists of a simple squamous epithelium (except the cubical amniotic epithelium at the junction of the cord with the placenta), which embrace three major vessels (two arteries and one vein) immersed in a surrounding connective tissue (mature mucous connective tissue), which does not present neither lower calibre vascular structures nor neural elements [79]. The stromal connective tissue consists of an abundant ECM distinguishable in three zones, the subamniotic stroma, the Wharton's jelly (WJ), and the vessels' adventitia, and rich in collagen (types I, III and VI) and in basement membrane molecules such as collagen type IV, laminin and heparan sulphate proteoglycan [80, 81]. Type VII collagen is expressed in the epithelium and in the endothelial cells, but it was found as predominately expressed by fibroblast-like stromal cells [82]. ECM components are a storage of growth factors (including IGFs, FGFs and TGF- β) that sustain these stromal cells [83] (Sobolewski *et al.*, 2005). The abundant ECM of umbilical cord stroma contain dispersed stromal mesenchymal stem cells (MSCs). Studies by Takechi and colleagues [84] referred to these cells as 'myofibroblasts', a term firstly used by Majno and colleagues (1971) [85, 86].

Wharton's jelly cells (WJCs) are fibroblast-like cells sharing common markers (such as CD13, CD29, CD44, CD73, CD90, CD105, α -smooth muscle actin, and vimentin) with other MSCs [87-91]. Since many protease-based isolation protocols described in literature could lead to damages to surface molecules, we set up and standardized an *in vitro* non-enzymatic isolation protocol of stromal cells from WJ, named Wharton's jelly cells (WJCs) or 'human extraembryonic mesoderm stem cells' (HEMSCs), on the basis of the migratory capability of mesenchymal cells to the plastic surface of culture flasks, thus preventing cell membrane proteins integrity [92]. These cells showed a fibroblastoid morphology, genetic stability, telomere activity, and good cloning efficiency (10-12%) after several passages in culture, a pattern of markers similar to other mesenchymal stem cells (including CD10 and CD13), at both protein and RNA level. They featured multipotency and were differentiated into osteoblasts and adipocytes. We also suggested a possible immune suppression mechanism due to the expression of molecules involved in T cell response inhibition, such as a particular MHC class I, HLA-G, normally expressed by trophoblastic lineage in order to inhibit NK cells response versus allogeneic foetus [92]. As reviewed by Prasanna and Jahanavi (2011), WJCs showed a lymphoproliferative suppression stronger than those exerted by BM-MSCs [93]. Moreover, we demonstrated that WJCs maintained immunomodulatory molecules (HLA-G, -E, and -F) also after differentiation *in vitro*, supporting our concept about the large plasticity of these cells and their use in regenerative medicine for allogeneic therapy [92, 94-98]. As for placenta-derived MSCs described above, also in WJCs lymphoproliferative inhibition was linked to the expression and release of soluble factors as prostaglandins [99] and IL-10 [100]. Immune tolerance instauration during pregnancy (thus preventing rejection of allogeneic foetus) is also ensured by the release of soluble 10 kDa Heat Shock Protein (HSP10), also known in literature as Early Pregnancy Factor (EPF) by placental structures [101]. In addition, we demonstrated that these cells do express CD68, a typical monocyte/macrophage cells marker at both protein and mRNA level [102]. This result highlighted the need to understand and to explain the biological function of 'non-classical' patterns, taking into account the novel panel of markers which we previously described, such as the expression of costimulatory CD80 (B7-1), but not of CD86 (B7-2), and the expression of HLA-A and especially HLA-G, but not of HLA-DR (MHC class II) [92]. Moreover, Zhou and colleagues described a reduced secretion of TGF- β and IFN- γ by lymphocytes induced in co-culture with WJCs [103]. Thus their immunomodulatory capability support our hypothesis about their possible use in regenerative medicine. Moreover, the expression of vimentin, α -smooth muscle actin, and cardiac-specific molecules, mainly connexin 43, c-kit/CD117, GATA 4 [92, 104], reinforces the concept that hUC is a novel and promising source of readily available autologous cells for cardiac tissue engineering. This concept would be applied not only to improve damages related to heart failure or ischemia, but also to provide innovative therapies for paediatric patients with congenital heart pathologies and defects [105-107]. Costa Pereira and colleagues isolated WJCs with an enzymatic protocol. The cells underwent differentiation towards car-

diomyocytes, using 5-azacytidine. After 21 days the adherent cells showed cardiomyogenic morphology and a slight spontaneous beating [108]. Hollweck *et al.* (2011) described the effect of six different cardiac differentiation protocols on WJCs, assessing the efficiency of oxytocin with respect to 5-azacytidine [109]. Nevertheless, the use of enzyme isolation protocols may drastically induce damages on surface markers that could result in modification in phenotype characterization and regarding their response when transplanted *in vivo*. Future investigation is needed in order to unify isolation protocols, create co-banking of WJCs, and establishing hUC as a reliable source of stem cells without ethical and safety issues [110]. Moreover, little is known about differentiation of WJCs towards cardiomyocytes with both *in vitro* and *in vivo* experiments for further application in pre-clinical and clinical trials.

CONFLICT OF INTEREST

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