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REVIEW

Role of nuclear receptors in breast cancer stem cells

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Abstract

The recapitulation of primary tumour heterogenity and the existence of a minor sub-population of cancer cells, capable of initiating tumour growth in xenografts on serial passages, led to the hypothesis that cancer stem cells (CSCs) exist. CSCs are present in many tumours, among which is breast cancer. Breast CSCs (BCSCs) are likely to sustain the growth of the primary tumour mass, as well as to be responsible for disease relapse and metastatic spreading. Consequently, BCSCs represent the most significant target for new drugs in breast cancer therapy. Both the hypoxic condition in BCSCs biology and pro-inflammatory cytokine network has gained increasing importance in the recent past. Breast stromal cells are crucial components of the tumours milieu and are a major source of inflammatory mediators. Recently, the anti-inflammatory role of some nuclear receptors ligands has emerged in several diseases, including breast cancer. Therefore, the use of nuclear receptors ligands may be a valid strategy to inhibit BCSCs viability and consequently breast cancer growth and disease relapse.

Key words: Cancer stem cells; Hypoxia; Inflammation; Nuclear receptors; Retinoids; Peroxisome proliferator-activator receptors

Core tip: This review examines the roles of breast cancer stem cells (BCSC) in the eliminate breast cancer disease. BCSCs represent the most significant target for new drugs in breast cancer therapy. The use of nuclear receptors ligands may be a valid strategy to inhibit BCSCs viability and consequently breast cancer growth and disease relapse.

Papi A, Orlandi M. Role of nuclear receptors in breast cancer stem cells. *World J Stem Cells* 2016; 8(3): 62-72 Available from: URL: http://www.wjgnet.com/1948-0210/full/v8/i3/62.htm DOI: http://dx.doi.org/10.4252/wjsc.v8.i3.62

INTRODUCTION

The new hypothesis: Cancer stem cells

Several studies in the past years have shown that particular stem cells can have a significant role in cancer formation. These cells were identified in the hematopoietic

system, central nervous system and mammary glands. These cells are a rare cell population of "tumour initiators", with particular biological characteristics[1-4]. These stem cells have the ability to self-renew and to develop into all the cells that form the tumour mass and are called cancer stem cells (CSCs)[5]. In the CSCs hypothesis, cancer derives from normal stem cells that are transformed into tumour cells[6]. Adult stem cells are characterized as long-living with a low proliferative rate and are exposed for prolonged periods to agents that can induce damage and can accumulate mutations that result in neoplastic transformation[7]. Therefore, this condition implies the adoption of a new model to explain the carcinogenesis. Contrary to the "stochastic" model of tumorigenesis, for which the neoplastic transformation would result from random mutations incurred by a healthy cell that, consequently, undergoes clonal expansion. The CSCs hypothesis argues that the tumour begins from a stem cell, probably due to a dysregulation of the pathways involved in self-renewal[6]. However, these mechanisms are not exclusive and we can consider that other mechanisms can participate in the genesis and tumour progression, contributing to the heterogeneity of the tumour.

Breast cancer stem cells

The existence of CSCs in tumours of the mammary gland has been widely demonstrated by several studies, based mainly on transplants. The hypothesis of the origin of the breast cancer stem cells (BCSCs) was confirmed by the finding that only a minority of human breast cancer cells have the ability to induce new tumours when transplanted into immunocompromised mice (NOD/SCID)[8,9]. The presence of BCSCs indicates the onset of a breast tumour and they are distinguishable from other cancer cells by expression of specific membrane markers such as CD44 and an Epithelial Specific Antigen and by the absence or low expression of CD24 protein (CD44 and CD24 are adhesion molecules). Therefore, BCSCs are isolated from the tumour mass as $CD44^+/CD24^-$ by FACS analysis. Approximately 200 cells characterized by this phenotype induced tumour growth in NOD/SCID mice while 20000 cells with a different phenotype did not have this capability. $CD44^+/CD24^-$ breast cancer cells can generate cells of the same phenotype and cells phenotypically different, so that the tumour from which they develop in mice repeats the entire heterogeneity of its initial cancer[4]. The mammary gland epithelial components are thought to arise from stem cells that undergo both self-renewal and differentiation. Self-renewal has been shown to be regulated by the Hedgehog, Notch, and Wnt pathways. Deregulation of the self-renewal in stem cells/progenitors might be a key event in mammary carcinogenesis[10]. Different combinations of cell surface markers such as CD44, CD49f, CD24, and CD29 as well as the activity of certain enzymes such as aldehyde dehydrogenase isoform 1 (ALDH1) have been used to identify BCSCs[11].

The new BCSCs hypothesis has important therapeutic implications. BCSCs have many similar characteristics to normal stem cells, such as apoptosis resistance, the capacity to repair DNA damage and multidrug-resistance (MDR). MDR is important to explain the capacity of breast cancer to overcome chemotherapy. BCSCs are characterized by expression of genes encoding ATP-binding cassette (ABC) proteins, which are transmembrane transporters involved in the extrusion of drugs from cancer cells, as ABCC1, ABCG2 and ABCB1. The principal MDR proteins that are expressed in BCSCs are the P-glycoprotein, the multidrug-resistance protein 1 (MDR1) and the cluster differentiation 243 (CD243). Chemotherapy drugs with anti-proliferative effects are less effective on CSCs population, because these cells divide less frequently than cancer cells[6,7]. For these reasons chemotherapy destroy many neoplastic cells but does not affect the minority component of tumour such as BCSCs. Accordingly, BCSCs induce tumour relapse and metastases[8,9]. So a change in chemotherapy strategy is necessary to kill also BCSCs.

The stem cell niche and CSC

Stem cells are localized in a niche that is a local tissue microenvironment. The niche has a limited area where the cells can maintain their peculiarity. Significant progress has been made by the studies on the interactions between the stem cells and the microenvironment in Caenorhabditis elegans and mammals[12,13]. Comparing the stem cell niches in these systems, various common features and functions have emerged. The niche is formed by a group of cells (fibroblasts of the stroma) which have a support function for stem cells, serving as the anchor point for the stem cells and physical adhesion molecules mediate the interactions between the support cells and stem cells (as well as those between the stem cells and the extracellular matrix). The niche generates factors that control number, proliferation and differentiation of stem cells. Normally, it maintains the stem cells in a quiescent state, providing them with the signals that inhibit the growth and proliferation. Only after implementing a stimulus transient activator, stem cells are able to divide in order to participate in tissue regeneration. This suggests that control stem cell dependent signaling mechanisms, resulting from dynamic niche and maintaining the balance between the proliferative and anti-proliferative signals, are the key to the homeostatic regulation of the stem cells[12,13]. When there is a change in the niche and growth and proliferation signals prevail, the stem cell population is exposed to an uncontrolled expansion which can lead to spread CSCs[13].

Inflammation and breast cancer stroma

The idea that inflammation could play a role in carcinogenesis was born in 1863, when Rudolf Virchow noted the presence of leukocytes in neoplastic tissues. After this observation, more and more data have demonstrated that malignancy may begin at sites of infection or chronic inflammation and approximately 25% of all cancers are

associated with such conditions. In fact, although the inflammation represents a defensive response adaptive to infection or injury and is, under normal conditions, a self-limiting process which culminates in the repair of damaged tissues, an inadequate resolution induces chronic diseases or cancer[14]. Chronic inflammation is involved in all stages of carcinogenesis (initiation, promotion and progression). Inflammation induces an excessive production of reactive oxygen species that could cause genomic instability and mutation and consequently a tumour[14]. Stromal cells as fibroblasts, that are around the tumour, and inflammatory cells, as macrophage, that are infiltrated in the tumour, help to create an environment favoring the increase of inflammation[15]. Fibroblasts are among the most abundant cell types in solid tumours and are especially important in breast, pancreas, colon and prostate cancer[16]. In physiological conditions, fibroblasts have a low proliferative rate and have a constant production of extracellular matrix (ECM). ECM has anchoring function for the epithelial cells maintaining the integrity of structural epithelium. In the carcinoma *in situ*, the stroma is not compromised because it remains separated from the tumour cells through the basement membrane integrity. With the acquisition of infiltrating characteristics, some tumour cells manage to cross the basal lamina whose breaking mimics a traumatic insult to the tissue, causing changes in non-epithelial cell types. Fibroblasts are activated and become tumour associated fibroblasts (TAF) thus contributing to the growth and expansion of tumour in several ways: they produce proteins such as matrix metalloproteinases (MMPs) that have proteolytic activity on the components of the extracellular matrix[17]; release high levels of stromal-derived factor-1 (SDF-1) which attracts endothelial progenitor cells in the tumour mass (thus promoting angiogenesis); directly promote the growth of cancer cells through interaction with their receptor CXCR4[18]; release some growth factors such as epidermal growth factor (EGF) and transforming growth factor β (TGFβ) and release a wide range of inflammatory cytokines[16,19]. Macrophages resident in the stroma and monocytes that act together with tumour chemotactic factors, undergo changes that lead them to favor tumour growth. Thus, tumour associated macrophages (TAM) support tumour angiogenesis through the secretion of pro-angiogenic factors as the vascular endothelial growth factor (VEGF), the interleukin-1β (IL1β) and the angiogenin (Ang). TAM facilitate the migration of cancer cells through the release of tumor necrosis factor-α (TNFα), MMPs (such as the MMP9) and other proteases such as tissue plasminogen activator[20,21]. Moreover, TAM produce factors, such as EGF, that directly promote the growth of cancer cells[22] and have a role in facilitating the invasion of neoplastic cells[23]. Finally, TAF can activate macrophages that produce cytokines to maintain an inflamed microenvironment[24,25]. Inflammatory cytokines, including SDF-1, interleukin-1 (IL-1), IL-6 and IL-8, may affect tumour growth by regulating of CSCs population[26]. In particular, it has been demonstrated that IL-6 can induce the acquisition of malignant characteristics in multicellular spheroids called mammosphere (MS), formed from stem cells and progenitors of the mammary gland; such aggregates were obtained *in vitro* in conditions of non-adherence from MCF-7 breast cancer cell line (MCF-MS) or obtained from breast surgical specimens (normal and tumour, N-MS and T-MS respectively)[27]. High levels of IL-6 mRNA were detected in T-MS, however IL-6 can stimulate the growth and self-renewal in both T-MS and N-MS. In particular, IL-6 induce overexpression of Notch-3 and of its ligand Jagged1, both implicated in the maintenance of stem cells in an undifferentiated state. It has been demonstrated that the pathway IL-6/Notch-3 increases the expression of the protein carbonic anhydrase IX (CAIX), from which depends the survival of MCF7-MS in hypoxic environment, as well as an increase of their invasive potential. IL-6 acting through different signal transduction pathways involving protein kinases such as mitogen activated protein kinase (MAPK) or the phosphatidylinositol-triphosphate kinase (PI3K) and having the ability to directly activate STAT transcription factors (such as STAT3) *via* the kinase JAK2 which is associated its receptor, leads to a number of responses that favor the proliferation, inhibits apoptosis and increases the invasive capacity of tumour cells[28,29]. IL-6 induces the activation of the transcription factor NF-κβ that can code for several cytokines[27]. Other studies show that the TNFα, the major inducer of NF-κβ, involves an increase in the formation of MCF-7-MS cells through up-regulation of the *Slug* gene, a regulator of stem mammary tumour phenotype[30]. TNFα induces, after 10 d of treatment, the acquisition of typical characteristics of BCSCs ($CD44^+$/$CD24^-$) in not transformed mammary epithelial cell line MCF-10A; this effect is accompanied by the reduction of the E-cadherin expression and an increase of mesenchymal markers expression such as vimentin and smooth-muscle actin-α (αSMA)[31,32]. These considerations lead to the hypothesis that the survival of the BCSCs is dependent on the activation of NF-κβ, in turn resulting from the stimulation exerted, for example, by pro-inflammatory cytokines, as confirmed by the effect of inhibition of the proliferation of MCF7-MS due to the use of selective inhibitors of the NF-κβ, as parthenolide[33].

Hypoxia and BCSCs

Hypoxia plays a key role in carcinogenesis. Solid tumours are characterized by poorly vascularized regions and can progress under hypoxic conditions. Hypoxia is a condition that generally is found within the stem cell niche, which requires low concentrations of oxygen in order to minimize the damage that the eventual oxidation of the DNA could generate[34]. Hypoxia is also involved in the maintenance of an undifferentiated cell, thus playing a crucial factor in the stem cells condition and for this reason; could potentially contribute to the generation and or to support the CSCs. In hypoxic condition, there is an induction of the octamer-binding transcription factor 4 (Oct4) and Notch1 expression in CSCs, two proteins that are involved in the self-renewal and differentiation pathways[35,36]. Hypoxia induces *Shc* gene expression in BCSCs, a gene that coding for p66Shc protein, involved in cellular response to oxidative stress, which induce the up-regulation of Notch-3 and its ligand Jagged-1. Interestingly, there is a

correlation of Snail expression with histological grade and lymph node status in breast carcinomas[37]. Snail coding for CAIX protein is a molecule that is overexpressed in hypoxic condition and in BCSCs[38]. Cancer cells in the hypoxic tumour niche overexpressed the hypoxic inducible factor (HIF). HIF is a heterodimeric transcription factor consisting of an α-subunit (HIF-1α or HIF-2α), and a β-subunit (HIF-1β), expressed constitutively. HIF-1 affects a variety of malignant features, such as hypoxic cancer cell survival, *via* the regulation of a large number of genes, including CAIX[27]. The oxygen-dependent HIF activity is mediated by a series of enzymes containing iron (Fe^{2+}), belonging to the superfamily of 2-oxoglutarate-dependent dioxygenase that are oxygen sensitive. Members of this family are the prolyl hydroxylase domain-containing protein (PHD), as PHD1, PHD2, PHD3 and the factor inhibiting HIF. In normoxic conditions, the HIF-1α subunit is characterized by a very short half-life. In hypoxic condition, HIF-1α translocates into the nucleus and leads to gene activation by binding to a specific sequence (59-RCGTG-39) called Hypoxic Responsive Element (HRE), through the recruitment of coactivators CBP/300[39]. HIF-1α causes a metabolic change that allows cancer cells to adapt to poorly oxygenated environments: It results in the use of glycolysis at the expense of oxidative phosphorylation, even in aerobic conditions, with a decrease in mitochondrial respiration and an increased lactate. This phenomenon is called "Warburg effect" and is frequently found in cancer[40]. HIF induces a metabolic "shift" *via* transcriptional activation of genes involved in glucose turnover, including those coding for glucose transporters, glycolytic enzymes and enzymes involved in the production of lactate and in the metabolism of pyruvate[41,42]. HIF induces the VEGF expression and reduce anti-angiogenic factors, such as thrombospondin[43]. A recent study has shown that, in several cancer cell lines (breast, lung, cervical and ovarian), HIF-1α increases cell invasion[44]. Since HIF-1α induces the transmembrane protein CAIX expression, through various mechanisms CAIX can increase the invasive potential of cancer cells[45]. Moreover HIF controls the expression of LOX (lysyl oxidase) and the cytokine receptor CXCR4 expression that are essential for metastasis induction[46]. Finally, HIF reduces E-cadherin expression and induces the epithelial-mesenchymal transition[47-49].

NUCLEAR RECEPTORS

Nuclear hormone receptors (NRs) include receptors for steroid hormones such as estrogen receptors (ERs) and progesterone receptors (PRs), receptors for the thyroid hormone (TRs), receptors for vitamin D (VDRs), retinoic acid receptors (RARs), retinoid X receptors (RXRs) and a number of receptors that respond to intermediary metabolites, among which there are the peroxisome proliferator-activator receptors (PPARs) activated by fatty acids and prostaglandins[50-52]. The members of this superfamily act as transcription factors activated by ligands and have a conserved structure[53]. NRs are characterized by the presence of two conserved domains: (1) A central DNA-binding domain (DBD) which interacts with the core motif, that have specific DNA sequences called "response elements"(monomeric NR recognize a single core motif, while dimeric NR complexes interact with repeated occurrences of this core motif); and (2) A C-terminal ligand-binding domain, which determines specific NRs properties and is highly variable between the different receptors. NRs are characterized by a flexible linker region between the two previous domains. NRs have a carboxy-terminal E-domain that is responsible for the ligand binding, dimerization, and contain an inducible transactivation function dependent on ligand (AF-2). Finally, the N-terminal terminal A/B-domain of the NR molecule contains a constitutive activation function independent on ligand (AF-1). NR can be activated by specific ligands that can modulate gene transcription and induce differentiation and anti-proliferative effects in cancer cells in several tumours[54].

Retinoic acid receptors and retinoid x receptors

Nuclear receptors retinoic acid receptors (RARs) and retinoid x receptors (RXRs) mediate the effects of retinoids. Retinoids are a class of compounds that includes natural metabolites of vitamin A (retinol) and its synthetic analogues. The natural retinoids are produced *in vivo* by oxidation of retinol, a two-step process that leads to the formation of all-trans-retinaldehyde due to the action of alcohol dehydrogenase, followed by oxidized retinaldehyde due to the action of the enzyme dehydrogenase. In the reaction all-trans-retinoic acid (ATRA) is produced, which is then metabolized by CYP26 to produce hydroxylated metabolites[51]. There are three receptor subtypes, encoded by different genes, called RARα, β, γ and RXRα, β, γ. RARs subtypes can bind with high affinity not only ATRA as well as 9-cis retinoic acid (9cRA), the product of isomerization of ATRA, that is able to interact with RXRs, a feature that sets it apart from trans retinoic acid isoforms that do not have this possibility[55]. Following the activation induced by the ligand, the RARs form heterodimers with RXRs (RAR-RXR) that lead to gene transcription by binding to specific DNA sequences in the promoter of target genes, those corresponding to the Retinoic Acid Response Element (RARE), while homodimers formed by RXRs (RXR-RXR) bind to sequences denominated RXRE (Retinoic X Response Element)[52]. The RXRs are the only nuclear receptors that are capable to form both homodimers (RXR-RXR) and heterodimers (NR-RXR), constituting factors required for efficient DNA binding of many other members of the NR superfamily, including RARs and PPARs precisely[52]. These considerations underscore the importance of RXRs ligands because they can mediate effects affecting many biological processes. The NRs partner of RXRs receptors can be "permissive", as PPARs. The heterodimer that is formed can be activated independently from agonists of one or other receptors or, synergistically, by both. RXRs may be "non-permissive", when the heterodimer cannot be activated by RXRs agonists alone, necessitating the presence of a ligand for

the receptor partners (in the case of the dimer RAR-RXR is necessary a RARs ligand as ATRA)[51]. Various studies targetting the identification of a natural endogenous ligand for RXRs did not produce the desired results because the molecules proposed for this role (9cRA, phytanic acid, docosahexaenoic acid) have not demonstrated a selectivity only for binding to RXRs; for this reason synthetic compounds that bind only to RXRs (called rexinoids) could be essential to better understand the role of these receptors[52].

Retinoids and breast cancer

Retinoids are widely used to treat dermatological diseases. Retinoids have recently received considerable attention for the prevention and treatment of cancer due to their role in cell differentiation and their anti-proliferative, pro-apoptotic and anti-oxidant effects[56]. Epidemiological studies show that a low intake of vitamin A leads to a higher risk of developing cancer. Altered expression of RARs and RXRs is associated with malignant transformation both in animal tissues and in cultured cells[57]. Furthermore, in animal models retinoids reduce cancer of skin, lung, breast, bladder, ovary and prostate. In humans, retinoids can reverse epithelial precancerous lesions, induce differentiation of myeloid cells, and have an important role in the lung, liver and breast cancer prevention[58]. Moreover, retinoids regulate stem cell differentiation[59]. Retinoic acid is used today in various diseases: ATRA is the principal retinoid investigated in clinical trials for the treatment of lymphoma, leukemia, melanoma, lung cancer, cervix, kidney, neuroblastoma, and glioblastoma. Its clinical use has more effect in the treatment of the acute promyelocytic leukemia (APL). Since 1995, the FDA approved ATRA to APL treatment[60]. 9cRA differs from ATRA for its ability to activate both RAR and RXR. In addition, 9cRA activates different nuclear receptors such as PPARs, FXRs, PXRs and VDRs through RXR heterodimerization. In preclinical studies, 9cRA is effective in the prevention of prostate cancer and breast cancer and was also approved by the FDA for the topical treatment of cutaneous lesions of Kaposi's sarcoma[61]. The natural retinoid 13-cis retinoic acid (13cRA), binds both receptors RARs and RXRs, has anti-inflammatory activity and is in clinical development for different types of cancer, including cancer of the thyroid[62]. Preclinical and clinical studies have shown the anti-tumoural effects of retinoids in breast cancer. It has been observed that 9cRA inhibits proliferation and induces differentiation and apoptosis in the breast cancer cell line MCF-7 cells. Recently, it has been demonstrated that retinoid have a role also in the regulation of BCSCs self-renewal and differentiation; ATRA reduces BCSCs proliferation demonstrated by ALDH assay[11]. However, clinical studies have shown that natural retinoids can have side effects such as the hypervitaminosis A. It has been demonstrated that retinoids selective for RARs have chemopreventive activity with side effects, while selective RXRs retinoids (called rexinoids) suppress mammary tumorigenesis without side effects[63]. Since hypertriglyceridemia can be induced by rexinoids, recent research has investigated new rexinoids that have anti-tumoural effects without side effects. Among these there is (2E,4E,6Z,8Z)-8-(3′,4′-Dihydro-1′(2H)-naphthalen-1′-ylidene)-3,7-dimethyl-2,3,6 octatrienoinic acid (UAB30) that is currently undergoing clinical evaluation as a novel breast cancer prevention agent[64]. Furthermore, some patients may experience relapses cancer because cancer cells become resistant to retinoids therapies. For these reasons, the synergic use of multiple molecules as NRs ligands with other molecules at lower doses might be a good strategy to block breast cancer growth, while inducing less side effects in patients. Immunotherapy with the use of retinoids and T cell has proved effective in the treatment of neuroblastoma and 13cRA+interferon-α2a significantly increases the survival of patients with metastatic renal cell carcinoma[65]. Lee and co-workers have shown that administration of ATRA increased the effectiveness of EGCG at a low concentration. Indeed, ATRA increased the synthesis of a EGCG molecular targets, the 67 kDa laminin receptor (LR67), which plays a key role in cell adhesion and in the breast metastatic process[66]. ATRA is a regulator of epithelial mesenchymal transition (EMT) that is a determinant of the breast cancer cell invasion and metastatic behaviour. It has shown that in HER2-positive SKBR3 and UACC812 cells, there is an amplification of the *ERBB2* and *RARA* genes and ATRA activated a RARα-dependent epithelial differentiation program. Moreover, ATRA blocked Notch-1 up-regulation by EGF and/or heregulin-β1 and switches TGFβ from an EMT-inducing and pro-migratory determinant to an anti-migratory mediator[67]. ATRA can reduce the MS-forming ability of a subset of breast cancer cells, which correlates with induction of apoptosis, reducing SOX2 expression and inducing of its antagonist CDX2. The SOX2/CDX2 ratio has prognostic relevance in BCSCs[68]. K-Ras mutant BCSCs was resistant to ATRA, which was reversed by MAPK inhibitors. Thus, ATRA can be used in combination to reduce BCSC proliferation[68]. Interestingly, also the combination ATRA and doxorubicin can differentiate and kill the BCSCs. Differentiation of CSCs into non-CSCs can reduce their self-renewal capacity and increase their sensitivity to chemotherapy in a synergistic manner[69].

The new rexinoid IIF can kill BCSCs

In our laboratory, we have investigated the antitumoural effects of ATRA when binded to RARs while with the RXRs ligand, we used the synthetic rexinoid 6-OH-11-O-hydroxyphenanthrene (IIF), a new derivative of retinoic acid, capable of binding selectively to RXR and mainly activating the form RXR-γ[70]. Several *in vitro* studies show that IIF can be used as an anticancer agent: This rexinoid showed a greater anti-proliferative effect than ATRA and 9cRA in leukemic cell line HL-60, which induces apoptosis[71]. IIF induces differentiation in different tumour cell lines, such as colon carcinoma and neuroblastoma[72]. In the glioblastoma mouse model IIF reduces tumour

growth and invasion through the inhibition of MMPs, such as MMP-2 and MMP-9, in combination with increased expression of their inhibitors (TIMP-1 and TIMP-2)[73]. IIF has anti-inflammatory effects in colon cancer by suppressing the expression of cyclooxygenase-2 (COX-2), the inducible form of COX, responsible for the prostaglandin production that is overexpressed in many tumours[73]. Recently, we have demonstrated that ATRA and IIF reduce the inflammation-dependent survival in MS generated from human tumour specimens (T-MS) and from the breast cancer cell line MCF-7 (MCF7-MS), but not in MS derived from normal mammary glands (N-MS). The effect depends on the inhibition of the inflammatory pathway NF-κB/IL-6 which is wired in T-MS. ATRA and IIF, blocking NF-κB axis, reduced expression of genes involved in the maintenance of a tumour stem cell phenotype (such as *Slug*, *Notch-3*, *Jagged-1*) and was accompanied by an increased expression of markers of differentiation such as ERα and keratin-18[74]. A promising strategy is the combination of IIF with natural substances, such as Epigallocatechin-3-gallate (EGCG), that have a cytotoxic effect against breast cancer cells. In a recent study, we demonstrated that the combination of IIF and EGCG had a higher activity than the individual administration. IIF and EGCG can have a common signaling pathway that induces apoptosis by reducing epidermal growth factor receptor activation and its downstream kinase AKT-1[75].

PPAR receptors and their agonists

RXRs receptors can form heterodimers with PPARs receptors. The latter mediate the effects of many synthetic compounds called peroxisome proliferators (PPs-peroxisome proliferators). The PPs influence both the number and the size of the peroxisomes, responsible for various functions within the cell (β-oxidation of fatty acids and cholesterol metabolism). Even PPARs there exist three isoforms (α, β, γ), encoded by different genes and characterized by different tissue localization. They operate as sensors for fatty acids and their derivatives, checking therefore, important pathways concerning lipids and energy metabolism[76]. PPARα is expressed at high levels in organs with significant catabolism of fatty acids. PPARβ has the broadest expression pattern, and the levels of expression depend on the extent of cell proliferation and differentiation. Finally, PPARγ is expressed as two isoforms, of which PPARγ2 is found in the adipose tissues, whereas PPARγ1 has a broader expression pattern and is expressed at high levels in cancer tissue[76]. RXRs dimerization and the presence of coactivators are necessary for PPARs activation as transcription factor[77,78]. There is a wide range of endogenous and exogenous ligands that can interact with PPARs, leading to have different responses. Among PPARs endogenous ligands there are arachidonic acid, eicosapentaenoic acid and prostaglandin J2, while, among exogenous ligands there are the synthetic compounds called thiazolidinediones (TZDs): Pioglitazone (PGZ), rosiglitazone and troglitazone[54]. The TZDs are used in the treatment of type 2 diabetes because they decrease insulin resistance; they increase glucose uptake in peripheral tissues and reduce hepatic production. Some studies show, however, that TZDs could be successfully used also against tumours. In breast cancer, for example, tumour cells often express high levels of PPARs and it was demonstrated that TZDs are able to induce differentiation and inhibit tumour proliferation both *in vivo* (nude mice) and *in vitro* (mammary tumour cell line MCF-7); these effects are increased when combined with retinoids[79,80]. It was also noted that treatment with TZD leads to a reduction in the number of breast cancer cells in S phase and an increase of cells in phase G_0-G_1. Furthermore, TZD and retinoids induced apoptosis in 30%-40% of breast cancer cells through the inhibition of Bcl-2 expression[80]. Among the anticancer mechanisms mediated by PPARs ligands, in addition to the induction of pro-apoptotic proteins and stabilization of cell cycle, the inhibition of the expression or activity of various cytokines and transcription factors involved in inflammatory pathways (as TNFα, IL-1, IL-4, NF-β) could help to slow the growth of transformed cells[54]. Interfering with inflammatory pathway is an ability shown by some ligands of PPARα; fenofibrate and GW7647 (synthetic agonists). For example, they can significantly reduce the levels of pro-inflammatory cytokines such as IL-1, the expression of TNFα, COX-2 and an inducible form of the enzyme nitric oxide synthase in murine microglia BV-2 exposed to radiation. This effect is due to the inhibition of translocation of the NF-κB-p65 subunit or the inhibition of phosphorylation c-jun, a subunit of the transcription factor AP-1, both involved in inflammatory mechanisms[81]. MnSOD expression is significantly amplified in the aggressive breast carcinoma basal subtype. Interestingly, PPARγ activation repressed MnSOD expression and increased chemosensitivity, and inhibited tumour growth in MDA-MB-231 and BT549 breast cancer cell lines[82]. PPARs are also reported to be involved in the modulation of the EMT process in CSCs initiation and in the regulation of CSCs functions[83]. Some data show that activation of PPARα could induce cancer and result in the induction of inflammatory responses. If the stimulation of the PPARs, for example with the TZD, involves the inhibition of neoplastic growth and the induction of differentiation, activation of PPARα significantly increases the proliferation of tumour cells, as demonstrated for the breast cancer cell lines MDA-MB-231 and MCF-7[84]. This stark contrast between these isoforms of PPAR is highlighted by studies that show the effects mediated by an agonist of PPARα, WY-14643. Chronic administration of PP in rats and mice leads to development of hepatocellular carcinoma; as a result of repeated exposure to WY-14643. Mice in which the expression of PPARα is increased, do not develop this type of tumour, as opposed to what happens in wild-type mice for PPARα, demonstrating that the receptor mediates the effects of carcinogenic arising by the stimulation exerted by an agonist[85]. More recently it has been seen that WY-14643 promotes the formation of a MS-tumour (derived from cells of the mammary tumour cell line MCF-7) by stimulating the activation of the NF-κB/IL-6 and, consequently, the expression of genes *Slug*, *Notch-3*, *Jagged-1*, whereas the silencing of PPARα

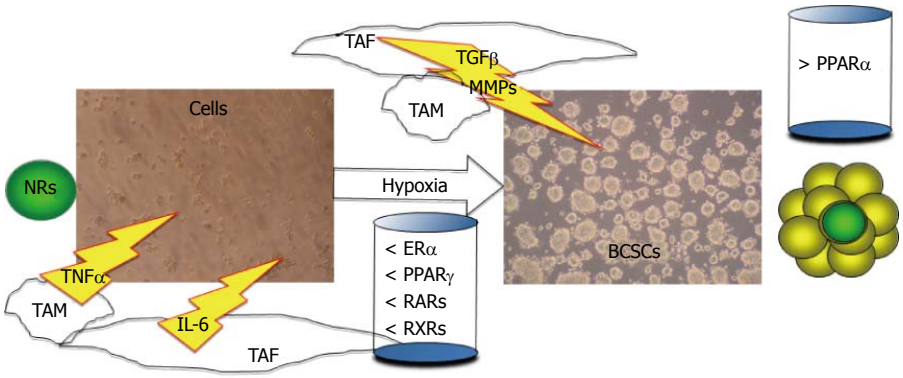

Figure 1 The nuclear receptors phenotype in breast cancer stem cells. In breast cancer, TAF and TAM promote inflammation and invasion through the secretion of cytokines, as IL6, TNFα and TGFβ and the secretion of MMPs, as MMP9. In this hypoxic inflammatory niche, particular stem cells can form the BCSCs as mammospheres. BCSCs are characterized by a particular nuclear receptors phenotype: a lower level of ERα, PPARγ, RARs, RXRs, and a higher level of PPARα is expressed than adherent breast cancer cells. NRs: Nuclear receptors; BCSCs: Breast cancer stem cells; TAF: Tumour associated fibroblast; TAM: Tumour associated macrophage; MMPs: Metalloproteinases; IL6: Interleukin-6; ERα: Estrogen receptor-α; PPARs: Peroxisome proliferator-activator receptors; RARs: Retinoic acid receptors; RXRs: Retinoid x receptors; TNFα: Tumor necrosis factor-α; TGFβ: Trasforming growth factor-β.

with a specific siRNA reduces tumour-MS formation. Furthermore, PPARα expression is positively correlated with the phenotype of BCSCs obtained from specimens of breast cancer patients[86]. Finally, we have recently demonstrated that IIF potentiates the ability of PGZ to hamper the MS-forming capability of human breast tumours and MCF7 cancer cells, reducing the expression of CSCs regulatory genes (*Notch3*, *Jagged1*, *SLUG*, *IL-6*, *Apolipoprotein E*, *HIF-1α* and *CAIX*). Notably, these effects are not observed in normal-MS obtained from human breast tissue[87]. Recently, Wang *et al*[88] demonstrated that PPARγ-binding protein upregulates several genes in the de novo fatty acid synthesis network, which is highly active in ERBB2-positive breast cancer cells. ERBB2 is a prognostic marker occurring in 30% of breast cancers and is associated with aggressive disease and poor outcomes. Inhibition of the PPARγ pathway using PPARγ antagonists (GW9662 and T0070907) reduces the ALDH-positive population and tumour-MS formation in ERBB2-positive breast cancer cells[88].

Vitamin D and BSCSs

Vitamin D-3 exerts most of its cellular effects *via* its nuclear receptor, the vitamin D-3 receptor (VDR), that heterodimerizes with the RXRs. The VDR-RXR complex binds vitamin D responsive elements (VDRE) in gene promoters and regulates transcription of target genes[89]. It has been reported in literature that vitamin D is a potential preventive/therapeutic agent against CSCs. Several proteins, such as Notch, Hedgehog, Wnt and TGF-β, are modulated by vitamin D in CSCs as well as in normal stem cell[90]. Interestingly, MS derived from BRCA1-silenced MCF7 or MDA-MB-231 breast cancer cells were no longer sensitive to the growth inhibitory effects of vitamin D, 1α, 25-dihydroxyvitamin D 3 (1,25D). Since, the active form of vitamin D is a potent inhibitor of BCSCs growth through the down-regulation of BRCA1 expression, which is the most frequently mutated tumour suppressor gene in breast cancer[91]. Treatment with 1α25(OH)2D3 or BXL0124 (two vitamin D compounds) repressed markers associated with the breast stem cell-like phenotype, such as CD44, CD49f, c-Notch1 and NF-κB. Furthermore, 1α25(OH)2D3 and BXL0124 reduced the expression of pluripotency markers, OCT4 and KLF-4 in BCSCs[92]. However, some authors have shown that MS were relatively insensitive to treatment with 125D compared to more differentiated breast cancer cells; instead combined treatment of 125D and DET- NONOate induce a significant decrease in the overall size of MS and reduced breast tumour volume in nude mice[93]. Combination therapy using 125D with drugs specifically targeting key survival pathways in BCSCs could be a best strategy to overcome aggressive breast cancer.

Estrogen receptor and BCSCs

It has been reported in literature that many breast cancers express estrogen receptor-α (ERα) and are dependent on estrogens[94]. Tamoxifen is the most widely used in endocrine therapy for ERα positive (ER^+) breast cancers during the last 30 years. Unfortunately, up to 40% of metastases from ER+ primary breast cancer do not respond to endocrine therapy. Recent study have demonstrated that tamoxifen was effective in reducing proliferation of ERα positive (ER^+) adherent cancer cells, but not their CSCs population[95]. Interestingly, estrogen is essential for the development of the normal breast, but adult mammary stem cells are known to be ERα negative (ER^-)[96]. BCSCs sorted derived from ER^+ breast cancer tissue and established breast cancer cell lines, have low or absent ER expression[74,96]. However, estrogen stimulated BCSCs activity demonstrated by increased MS-formation through the induction of EGF and Notch receptor signaling pathways[96]. Breast cancer cells develop resistance to endocrine therapies by shifting between ER-regulated and growth factor receptor-regulated survival signaling pathways[97]. However, the roles of BCSCs in antiestrogen resistance and the underlying molecular mechanisms have not been well established. Recent, a novel variant of ERα, called ERα36 (molecular weight of 36 kDa) it has been investigated. ERα36 mediates rapid antiestrogen

signaling and is highly expressed in ER^+ breast progenitor cells. Antiestrogens increased the percentages of the BCSCs from ER^+ breast cancer cell through stimulation of luminal epithelial lineage specific and these BCSCs are more resistant to antiestrogens than the bulk cells. Finally, ERα36 mediated antiestrogen signaling such as the PI3K/AKT that plays an important role in antiestrogen resistance of ER^+/BCSCs[98].

CONCLUSION

BCSCs represent the most significant target for new anti-breast cancer drugs. In fact, BCSCs are likely to sustain the growth of the primary tumour mass, as well as to be responsible for disease relapse and metastatic spreading in breast cancer[6-10]. The activity of NF-κB in BCSCs and in the tumour stroma (mainly formed by fibroblasts and inflammatory cells) has been recognized to be of pivotal importance in normal and CSCs survival[99]. It has been proposed that "NF-κB activity addiction" would make CSCs more susceptible to NF-κB inhibitors than their normal counterparts[26,55]. For these reasons the use of molecules, as NRs ligands, capable of inhibiting NF-κB dependent inflammation may be the best strategy to hamper BCSCs growth[74,86]. Recently, we demonstrated that BCSCs have a particular NRs phenotype[86,87] (Figure 1). Therefore, the synergic use of multiple molecules (as ligands of NRs) at lower doses might be a good strategy to kill BCSCs, while inducing fewer side effects in patients. Moreover, the use of NRs ligands in combination with each other (as ligands of PPARs with ligands of RXRs) or with other substances (*e.g.*, EGCG) may be a valid strategy to inhibit BCSCs viability.

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REVIEW

Homing and migration of mesenchymal stromal cells: How to improve the efficacy of cell therapy?

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Abstract

Mesenchymal stromal cells (MSCs) are currently being investigated for use in a wide variety of clinical applications. For most of these applications, systemic delivery of the cells is preferred. However, this requires the homing and migration of MSCs to a target tissue. Although MSC homing has been described, this process does not appear to be highly efficacious because only a few cells reach the target tissue and remain there after systemic administration. This has been ascribed to low expression levels of homing molecules, the loss of expression of such molecules during expansion, and the heterogeneity of MSCs in cultures and MSC culture protocols. To overcome these limitations, different methods to improve the homing capacity of MSCs have been examined. Here, we review the current understanding of MSC homing, with a particular focus on homing to bone marrow. In addition, we summarize the strategies that have been developed to improve this process. A better understanding of MSC biology, MSC migration and homing mechanisms will allow us to prepare MSCs with optimal homing capacities. The efficacy of therapeutic applications is dependent on efficient delivery of the cells and can, therefore, only benefit from better insights into the homing mechanisms.

Key words: Mesenchymal stromal cells; Homing; Bone marrow; Homing receptors; Extravasation

Core tip: Mesenchymal stromal cells (MSCs) are currently under investigation for use in a variety of clinical applications. In most studies, MSCs are administered systemically. This requires efficient homing and migration of the MSCs to a target tissue. However, the homing mechanisms of MSCs are not completely understood. Moreover, the *in vivo* homing and migration of MSCs does not appear to be highly efficient. Therefore, different methods have been investigated to improve homing. Here, we will review the current knowledge of bone marrow homing of MSCs, as well as the different strategies that might improve the homing capacity of these stem cells.

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INTRODUCTION

Mesenchymal stromal cells (MSCs) are non-haematopoietic cells that were first derived from the bone marrow and described approximately 40 years ago by Friedenstein *et al*[1]. In 2006, the International Society for Cell Therapy defined the minimal criteria to define human MSCs. They must adhere to plastic in culture and differentiate into osteocytes, chondrocytes and adipocytes. Additionally they must express CD105, CD90 and CD73 and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules[2].

There is great interest in using these cells in a wide variety of clinical domains, such as Neurology, Orthopaedics, Cardiology and Haematology[3-6]. This interest arises from the following MSC characteristics: They have immunomodulatory capacities, they are multipotent and are thus possible effectors for tissue regeneration, and they tend to migrate to sites of tissue injury/inflammation[7-11]. Additionally MSCs might escape immune recognition, although conflicting observations about this particular phenotype have been published. MSCs do not express MHC class Ⅱ antigens, but the expression of these molecules can be upregulated after exposure to inflammatory cytokines or during MSC differentiation[12]. The data from animal studies suggest that MSCs can elicit allogeneic immune responses and be rejected[13-16]. On the other hand, there is also a report of MSCs that overcame this allogeneic immune response due to their immunomodulatory capacities[17]. von Bahr *et al*[18] addressed this issue and published follow-up data of patients treated with MSCs, showing that there was no correlation between the MSC source (donor-derived or third party) and the patients' response to the MSC treatment. The clinical applications of these cells have been extensively studied in Orthopaedics, where MSCs are used to repair large bone defects, and in Haematology for the treatment of graft-*vs*-host disease and support for the engraftment of hematopoietic stem cells[4,6,19]. In recent years, MSCs have been studied as vehicles to deliver anti-cancer treatments because there is evidence that MSCs home to tumour sites. They can be induced to express anti-cancer proteins [*e.g.*, interleukin (IL) 2], to produce pro-drug activating enzymes, which ensures that the active drug will only be localized in the tumour, or to deliver oncolytic viruses[20-23]. For these applications, the homing and persistence of MSCs in the target tissue are desirable[24].

When MSCs are used in clinical applications, different modes of administration are possible: Systemic administration [intravenous (IV) or intra-arterial (IA) injection] or local administration [intracoronary (IC) injection or direct injection into the tissue of interest]. Of these different options, IV injection is the most widely used because it is minimally invasive, the infusions can be readily repeated and the cells will remain close to the oxygen- and nutrient-rich vasculature after extravasation into the target tissue[25]. However, after IV injection, the cells appear to be trapped in the lungs, and thus efficient homing to the target tissues might be compromised. IA administration requires an invasive procedure that has a higher risk of complications than IV. Although IA injections might improve tissue-specific homing compared to IV, there is a concern that microthrombi might occur as a result of trapping large MSCs in the microvasculature. One example is the concern regarding IC injections of MSCs to treat myocardial infarction[26]. Similar concerns have been raised in studies that used MSCs to treat stroke[27,28]. A true local injection of MSCs might require a surgical intervention, such as that used in the repair of bone defects. In this setting, the MSCs are immediately delivered to the target tissue; however, the cells' survival might be compromised due to a lack of oxygen or nutrients[25]. Currently, haematopoietic stem cell transplantation is performed *via* an IV infusion. Intra-bone marrow transplantation is a more complex procedure, but evidence from an animal model suggests that this might improve the outcome of the treatment[29]. Finally, some animal models of systemic administration, such as intracardiac injection, cannot readily be performed in patients.

The systemic infusion of cells for therapeutic applications implies and requires efficient migration and homing to the target site. Although there is ample evidence of MSC homing, this process appears to be inefficient because only a small percentage of the systemically administered MSCs actually reach the target tissue[30]. The mechanisms by which the MSCs migrate and home are not yet clearly understood.

Currently, in Haematology, MSCs are mainly being tested for their ability to control graft-*vs*-host disease and to support haematopoiesis after haematopoietic stem cell transplantation. Chemo- and radio-therapy can damage the haematopoietic niche. MSCs are part of this niche and secrete a number of haematopoietic growth factors. To facilitate the engraftment of haematopoietic stem cells and stimulate blood formation, the MSCs should successfully home to and persist in the bone marrow[31]. In this review, we discuss current knowledge about MSC homing, specifically focusing on bone marrow homing (based on both *in vitro* and *in vivo* data), and we review the efforts that different groups have undertaken to improve the homing efficiency of these cells.

MSC HOMING AND MIGRATION TO BONE MARROW AND OTHER TISSUES

The exact mechanisms used by MSCs to migrate and home to tissues have not been fully elucidated. It is generally assumed that these stem cells follow the same steps that were described for leukocyte homing. In the first step, the cells come into contact with the endothelium by tethering and rolling, resulting in a deceleration of the cells in the blood flow. In the second step, the cells

are activated by G-protein-coupled receptors, followed by integrin-mediated, activation-dependent arrest in the third step. Finally, in the fourth step, the cells transmigrate through the endothelium and the underlying basement membrane[32].

The first studies addressing MSC homing examined the origin of the bone marrow MSCs after allogeneic bone marrow transplantation. Those groups all concluded that the haematopoietic cells were provided by the donor, but the stromal cells were provided by the recipient[33-35]. However, in these studies, the patients received marrow transplants containing only a limited number of MSCs - approximately 1/250000 nucleated cells at 35 years of age - in contrast to the purified MSC product that is used in the majority of clinical trials[36].

Since then, several studies in animal models and patients have shown that MSCs are capable of migrating and homing to a variety of tissues. Early studies of intra-uterine MSC transplantations in animal models showed that donor-derived non-haematopoietic cells were present in the bone marrow, thymus, spleen and liver[37,38]. Devine *et al*[30] and Chapel *et al*[7] performed MSC transplantations in non-human primates and observed MSCs in a variety of tissues, with highest numbers in the gastro-intestinal tract. The percentage of MSCs in the different tissues was estimated between 0.1% and 2.7%[7,30]. Erices *et al*[39] described the homing and survival of human cord blood-derived MSCs in the bone marrow of immunodeficient (nude) mice after systemic infusion[39]. Several studies in patients have also shown MSC homing[40-43].

A few groups have analysed the dynamics of MSC migration after systemic infusion using different techniques. Immediately after infusion, the MSCs are trapped in the lungs, and, subsequently, the cells are cleared from the lungs and distributed to other tissues[44,45]. The cells could be injected intravenously or intra-arterially for systemic infusion. The former is the least invasive method and the easiest to perform; however, as the MSCs were trapped in the lungs, different administration routes were examined. IA injection, which is already more risky because of the arterial puncture, also appears to entail a risk of development of microvascular occlusions called passive entrapment[27,46]. In addition, there have been reports that MSCs have a procoagulant activity[26,47]. A few years ago, a group from the Karolinska Institute reported that MSCs, particularly those that had been subjected to extended passaging and co-culture with activated lymphocytes, exhibited increased prothrombotic capacities; this effect was dose-dependent[47]. Gleeson *et al*[26] reported that MSCs express functionally active tissue factor. When MSCs were injected in the coronary arteries of a porcine myocardial infarction model, it resulted in a decreased coronary flow reserve. This effect could be reversed by the co-administration of heparin, an antithrombin agent[26].

Kyriakou *et al*[48] have studied the factors influencing short-term bone marrow homing of MSCs. The stem cells were observed in the bone marrow, spleen, liver and lungs 24 h after IV injection. It was observed that homing increased in younger animals and after irradiation but decreased with increasing passage numbers of the cells[48]. Several other groups have also shown that MSC homing improves after irradiation[7,8,30,49-52].

MOLECULES INVOLVED IN MSC (BONE MARROW) HOMING

The expression of molecules involved in MSC migration, homing and functionality has been widely studied.

Different molecules are involved/necessary for the different steps in the homing process. The selectins on the endothelium are primarily involved in the first step. For bone marrow homing in particular, the expression of haematopoietic cell E-/L-selectin ligand (HCELL), a specialized glycoform of CD44 on the migrating cell, is very important[53]. Although MSCs express CD44, they do not express HCELL[54].

The G-protein coupled receptors that are involved in the activation step are typically chemokine receptors. It has been extensively demonstrated that the CXCR4-stromal derived factor-1 (SDF-1) axis is critical for bone marrow homing[55]. Both molecules are very physiologically important, as knock-outs are lethal due to bone marrow failure and abnormal heart and brain development[56,57]. The expression of the chemokine receptor CXCR4 on MSCs is controversial. Some groups did not observe expression of the receptor, while other studies demonstrated that CXCR4 was expressed, albeit at low levels on the membrane, which affected migration in response to SDF-1[58-70].

Integrins are important players in the stable activation-dependent arrest in the third step of homing. It has been shown that the inhibition of integrin β1 can abrogate MSC homing[71]. Integrins form dimers that bind to adhesion molecules on the endothelial cells. Integrin α4 and β1 combine to form very late antigen 4 (VLA-4), which interacts with vascular cell adhesion molecule 1 (VCAM-1). It has been shown that the VCAM-1-VLA4 interaction is functionally involved in MSC homing[72,73].

In the final step of diapedesis or transmigration through the endothelial cell layer and the underlying basement membrane, lytic enzymes, such as the matrix metalloproteinases (MMP), are required to cleave the components of the basement membrane. In particular, the gelatinases MMP-2 and MMP-9 have important roles in this step because they preferentially degrade collagen and gelatin, two of the major components of the basement membrane[74,75]. We have shown that MSC migration is regulated by MMP-2 and tissue inhibitor of metalloproteinases 3 (TIMP-3)[76]. Membrane type 1 MMP (MT1-MMP) has also been reported to play a role in MSC migration[63]. MMPs are secreted as pro-enzymes. ProMMP-2 is activated by interactions with MT1-MMP and TIMP-2 and is inhibited by TIMP-1. This explains why the MMP-2, MT1-MMP or TIMP-2 knock-down decreased the invasive capacity of MSCs, and why TIMP-1 knock-down resulted in increased invasion in the study of Ries *et al*[77].

Table 1 gives an overview all of the migration and

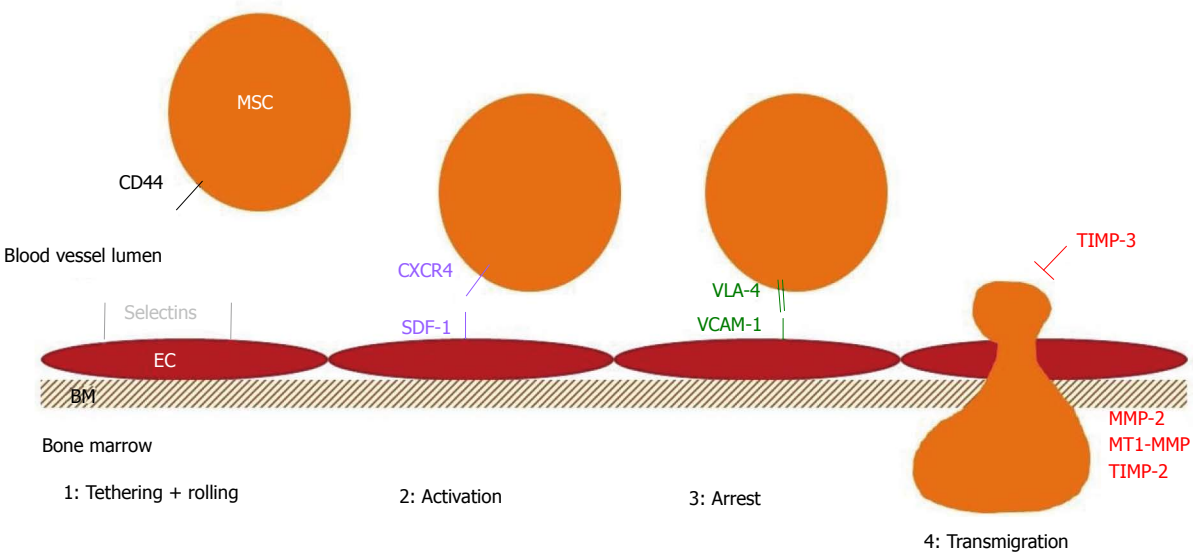

Figure 1 Overview of the homing molecules that are expressed on human mesenchymal stromal cells and known to be involved in the different steps of the bone marrow homing of mesenchymal stromal cells. EC: Endothelial cell; BM: Basement membrane; CD: Cluster of differentiation; SDF-1: Stromal cell derived factor 1; VLA-4: Very late antigen 4; VCAM-1: Vascular cell adhesion molecule 1; TIMP: Tissue inhibitor of metalloproteinases; MMP: Matrix metalloproteinase; MT1-MMP: Membrane type 1 matrix metalloproteinase; MSC: Mesenchymal stromal cell.

homing molecules that are reported to be expressed on human MSCs. Figure 1 shows a schematic overview of the molecules involved in human MSC bone marrow homing.

In addition to the expression of classic homing molecules, different groups have also described the expression of growth factor receptors on MSCs. Several studies have shown that growth factors can also induce MSC migration. For example, platelet-derived growth factor (PDGF) AB and BB can induce MSC migration *in vitro*[68,80,91]. Another growth factor involved in MSC migration is hepatocyte growth factor (HGF), which binds to c-met[63,68,80]. Both PDGF-BB and HGF have been loaded on gels or scaffolds as a means to improve the *in vitro* migration of MSCs[92,93].

HOW CAN WE IMPROVE THE HOMING EFFICIENCY OF MSCs?

Several groups have demonstrated MSC homing and migration, but only a small proportion of systemically administered MSCs actually reaches and remains in the target tissue[30]. Several factors are assumed to be involved. First, the expression of homing molecules on MSCs is limited. For example, the membrane expression of CXCR4, a critical receptor for homing to bone marrow, is very low, and some groups even claim there is no CXCR4 expression at all[58-70]. Another concern is that the MSCs appear to lose the expression of homing molecules during *in vitro* expansion[70,94]. Additionally, there is also heterogeneous expression of homing molecules in MSC cultures and in MSCs derived from different tissues (adipose tissue *vs* bone marrow), which show a different expression profile of homing molecules[95].

Because improving the homing efficiency to and retention of MSCs in a target tissue after systemic administration would improve their therapeutic effects, many groups are investigating methods to achieve this goal. Different strategies have been developed: the mode of administration could be modified, the MSC culture conditions can be adapted to optimize the expression of homing molecules, the cell surface receptors could be engineered to improve homing or the target tissue could be modified to better attract the MSCs. Again, we will mainly focus on the strategies that might improve the bone marrow homing of MSCs. The homing molecules involved in homing to bone marrow can also be of importance in homing to other organs or sites of injury, such as the CXCR4-SDF-1 interaction for homing to the injured myocardium[96]. However, we believe that methods that can upregulate or induce the expression of the homing molecules that are involved in bone marrow homing of MSCs are valuable. They show a potential means for improving bone marrow homing, even though the data supporting/proving this are not yet available. Figure 2 provides an overview of the methods that could be used to improve the bone marrow homing of MSCs.

Modification of the mode of administration

In vivo studies have repeatedly shown that MSCs are trapped in the lung after intravenous injection. When mice were treated with a vasodilator prior to MSC infusion, there was a clear decrease in the number of trapped MSCs in the lungs and a significant increase in MSC homing to the marrow of the long bones[44]. Yukawa *et al*[97] transplanted MSCs in combination with heparin treatment and found that this strategy also significantly decreased MSC trapping

Table 1 Overview of the homing molecules expressed on human mesenchymal stromal cells

Group	Molecule	Source	Transcript	Protein	Functional assay
Chemokine receptors	CCR1[70,77-82]	BM[70,77-79,81] WJ[79] AT[80] PB[82]	Yes[70,77,79,80]	Yes[70,77-82]	*In vitro* migration[70,77,78,80], *in vivo* tail vein injection in mice for tissue distribution[77]
	CCR2[68,78,81,82]	BM[68,78,81,82]	Yes[68,82]	Yes[68,78,81,82]	*In vitro* migration[68,78,82]
	CCR3[68,78,81-83]	BM[68,78,81,83] PB[82]	Yes[68]	Yes[68,78,81,82,83]	*In vitro* migration[68,78]
	CCR4[68,77,78,82]	BM[68,77,78,82]	Yes[68,77,82]	Yes[68,77,78] No[82]	*In vitro* migration[68,77,78,82], *in vivo* tail vein injection in mice for tissue distribution[77]
	CCR5[68,78,81-83]	BM[68,78,81,83] PB[82]	Yes[68]	Yes[68,78,81,82]	*In vitro* migration[68,78]
	CCR6[78,81,83]	BM[78,81,83]	Yes[82]	Yes[78,82]	*In vitro* migration[78]
	CCR7[70,78,80-83]	BM[70,78,81,83] AT[80] PB[82]	Yes[70,80,83]	Yes[70,78,80-83]	*In vitro* migration[70,78,83]
	CCR8[78,82,83]	BM[78,83] PB[82]	Yes[82]	Yes[78,82,83]	*In vitro* migration[78]
	CCR9[70,78,81-83]	BM[70,78,81,83] PB[82]	Yes[70,83]	Yes[70,78,81-83]	*In vitro* migration[70,78]
	CCR10[77,78,81,83]	BM[77,78,81,83]	Yes[77,83]	Yes[77,78,81]	*In vitro* migration[77,78], *in vivo* tail vein injection in mice for tissue distribution[77]
	CXCR1[78,81,82,84]	CB[84] BM[78,81,82] PB[82]	Yes[83,84]	Yes[78,81,82,84]	*In vitro* migration[78,83,84], *in vivo* injection in brain[84]
	CXCR2[62,78,81-83]	BM[62,78,81,83] PB[82]	Yes[62,83]	Yes[62,78,81-83]	*In vitro* migration[62,78,83], *in vivo* lung metastasis model[62]
	CXCR3[78,81-83]	BM[78,81,83] PB[82]	Yes[83]	Yes[78,81-83]	*In vitro* migration[78]
	CXCR4[60,62,65,66,68,70,76,78,80-83,85,90]	BM[60,62,68,70,76,78,81,83,85] CB[65,85,90] Foetal BM[66] AT[80] PB[82]	Yes[60,62,66,68,70,76,80,83,85]	Yes[62,65,66,68,70,76,78,80-83,85,90]	*In vitro* migration[60,62,65,66,68,70,76,78,80,83,85,90], *in vivo* lung metastasis model[62], tail vein injection in sublethally irradiated mice[66]
	CXCR5[68,70,77-83]	BM[68,70,77,78,81,83] WJ[79] AT[80] PB[82]	Yes[68,70,77,79,80,83]	Yes[68,70,77-83]	*In vitro* migration[68,70,77,78,80], *in vivo* tail vein injection in mice for tissue distribution[77]
	CXCR6[70,78,80-83]	BM[70,78,81,83] AT[80] PB[82]	Yes[70,80,83]	Yes[70,78,80-83]	*In vitro* migration[70,78,80]
	CXCR7[60,82]	BM[60] PB[82]	Yes[60]	Yes[82]	*In vitro* migration[60]
	CX3CR[82]	BM[82] PB[82]	Yes[82]	Yes[82]	
	XCR[82,82]	BM[82] PB[82]	Yes[82]	Yes[82]	
Adhesion molecules	VCAM-1[74,85,86]	BM[74,85,86] CB[86] AT[86]	Yes[85]	Yes[85,86]	*In vitro* migration[74]
	ICAM-2[85]	BM[85]	Yes[85]	Yes[85]	
	CD62[11,17,54,86-89]	BM[11,54,86-89] CB[17,86,87,89] AT[86-89] Skin[87]		Yes[11,17,54,86-89]	*In vivo* homing in a mouse model[54]
	LFA-3[85]	BM[85]	Yes[85]	Yes[85]	
	Integrin α1[11,85,87]	BM[11,85,87] CB[87] AT[87] Skin[87]	Yes[85]	Yes[11,85,87]	
	Integrin α2[85]	BM[85]	Yes[85]	Yes[85]	
	Integrin α3[11,85]	BM[11,85]	Yes[85]	Yes[11,85]	
	Integrin α5[11,85]	BM[11,85]	Yes[85]	Yes[11,85]	
	Integrin α6[85]	BM[85]	Yes[85]	Yes[85]	

	Integrin αv[85]	BM[85]	Yes[85]	Yes[85]	
	Integrin β1[11,86-88]	BM[11,86-88] CB[86,87] AT[86-88] Skin[87]		Yes[11,86-88]	
	Integrin β3[85]	BM[85]	Yes[85]	Yes[85]	
	Integrin β4[85]	BM[85]	Yes[85]	Yes[85]	
	ALCAM[17,87]	BM[87] AT[87] CB[17,87]		Yes[17]	
Proteases	MMP-1[90]	BM[90]	Yes[90]	Yes[90]	*In vitro* migration[90]
	MMP-2[65,68,74,76,77,85,90]	BM[68,76,77,85,90] CB[85]	Yes[68,76,77,85,90]	Yes[65,68,76,77,85,90]	*In vitro* migration[65,68,74,76,77,85,90]
	MMP-13[68,90]	BM[68,90]	Yes[68,90]	Yes[68,90]	*In vitro* migration[68,90]
	MT1-MMP[68,77,85]	BM[68,77,85] CB[85]	Yes[68,77, 85]	Yes[68,77,85]	*In vitro* migration[68,77,85]
	TIMP-1[68,77,90]	BM[68,77,90]	Yes[68,77,90]	Yes[68,77,90]	*In vitro* migration[68,77,90]
	TIMP-2[68,90]	BM[68,77,90]	Yes[68,77,90]	Yes[68,77,90]	*In vitro* migration[68,77,90]
	TIMP-3[76]	BM[76]	Yes[76]	Yes[76]	*In vitro* migration[76]
Growth factor receptors	c-met (HGF-R)[68,80,85]	BM[68,85] CB[85] AT[80]	Yes[68,80, 85]	Yes[68,85] No[80]	*In vitro* migration[85,68]
	PDGFRα[68,80,87]	BM[68,87] AT[80,87] CB[87] Skin[87]	Yes[68,80]	Yes[68,80,87]	*In vitro* migration[68,80]
	PDGFRβ[68,80,87]	BM[68,87] AT[80,87] CB[87] Skin[87]	Yes[68,80]	Yes[68,80,87]	*In vitro* migration[68,80]
	FGF-R1[80]	AT[80]	Yes[80]	Yes[80]	*In vitro* migration[80]
	FGF-R2[68]	BM[68]	Yes[68]	Yes[68]	*In vitro* migration[68]
	EGF-R[68,78]	BM[68,78] AT[80]	Yes[68,80]	Yes[68,80]	*In vitro* migration[68,80]
	IGF-R1[68]	BM[68]	Yes[68]	Yes[68]	*In vitro* migration[68]
	TIE-2[68]	BM[68]	Yes[68]	Yes[68]	*In vitro* migration[68]
	TGFRB2[80]	AT[80]	Yes[80]	Yes[80]	*In vitro* migration[80]
	TNFRSF1A[80]	AT[80]	Yes[80]	Yes[80]	*In vitro* migration[80]

BM: Bone marrow; CB: Cord blood; AT: Adipose tissue; WJ: Wharton's Jelly; VCAM: Vascular cell adhesion molecule; ICAM: Intercellular adhesion molecule; CD: Cluster of differentiation; LFA: Lymphocyte function associated antigen; ALCAM: Activated leukocyte cell adhesion molecule; MMP: Matrix metalloproteinase; TIMP: Tissue inhibitor of metalloproteinase; HGF: Hepatocyte growth factor; PDGFR: Platelet-derived growth factor receptor; FGF-R: Fibroblast growth factor receptor; EGF-R: Epidermal growth factor receptor; IGF-R: Insulin-like growth factor receptor; TIE: Tyrosine kinase with immunoglobulin-like and EGF-like domains; TGFR: Transforming growth factor receptor; TNFRSF: Tumour necrosis factor receptor superfamily.

in the lungs.

Pretreatment or priming of MSCs in culture or modifying the MSC culture conditions

Because MSCs appear to downregulate homing molecule expression during expansion, many groups are investigating different ways to induce or upregulate the expression of important homing molecules.

Much effort has been focused on increasing CXCR4 expression on the membrane. One way to achieve this is by adding cytokines or cytokine cocktails to the culture medium during expansion. Shi *et al*[66] showed that exposure to a combination of flt3 ligand, stem cell factor (SCF), IL 3, IL 6 and hepatocyte growth factor (HGF) increased both the intracellular and membrane expression of CXCR4 on cultured MSCs. More of the pretreated cells migrated towards an SDF-1 gradient, and there was no effect of the pretreatment on the function of the MSCs in supporting haematopoiesis. *In vivo* homing experiments where MSCs were intravenously injected into sublethally irradiated mice revealed a significant increase in bone marrow homing after the cytokine treatment[66]. Other molecules that have been shown to increase CXCR4 expression are insulin-like growth factor 1 (IGF-1), tumour necrosis factor α (TNFα), IL 1β, interferon γ (IFNγ)[68,98-100]. CXCR4 expression could also be upregulated by treating cultured MSCs with glycogen synthase kinase 3β (GSK-3β) inhibitors, resulting in an improved *in vitro* migration capacity, without affecting cell viability[101]. Exposure to complement 1q (C1q) has been shown to increase MSC migration towards SDF-1, although there was no significant increase in CXCR4 expression. Therefore, it was postulated that C1q exposure increases the MSCs' ability to sense SDF-1 gradients[65].

Treatments with GSK-3β inhibitors and C1q also increase MMP expression in MSCs, which are important for the degradation of the basement membrane during extravasation[60,101]. A combination of the haematopoietic growth factors erythropoietin (EPO) and granulocyte colony stimulating factor (G-CSF) has also been reported

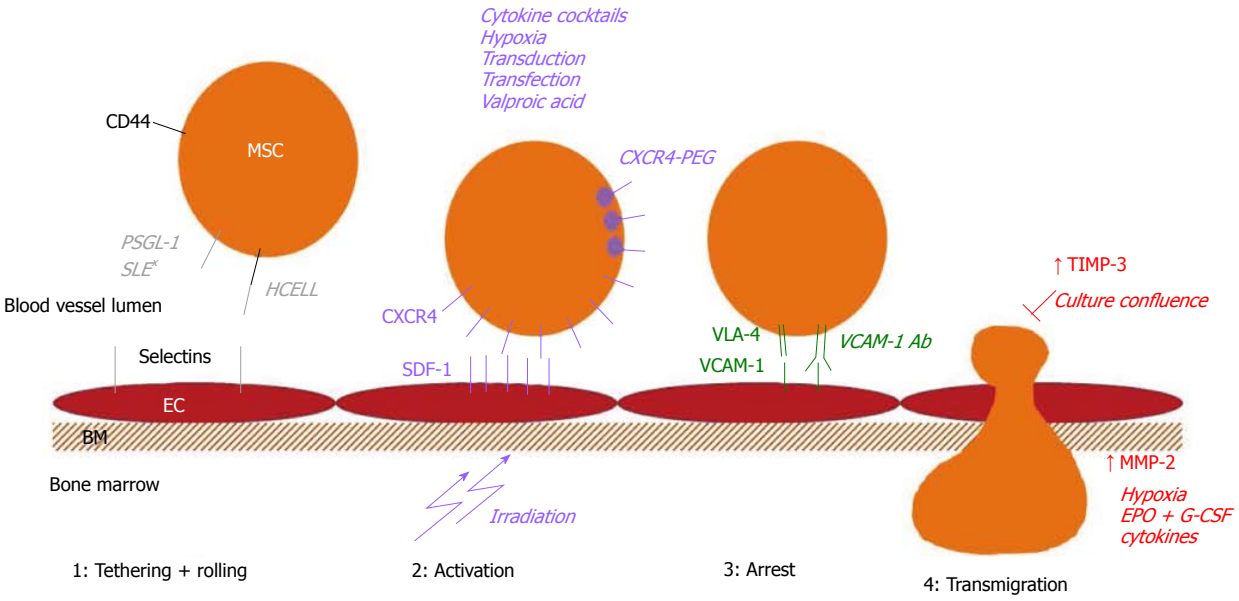

Figure 2 Schematic overview of the different strategies that can be used to improve homing in the different steps of mesenchymal stromal cell migration. CD: Cluster of differentiation; EC: Endothelial cell; BM: Basement membrane; HCELL: Hematopoietic cell E-/L-selectin ligand; PSGL-1: P-selectin glycoprotein ligand-1; SLEX: Sialyl Lewis X; SDF-1: Stromal cell derived factor 1; VLA-4: Very late antigen 4; VCAM-1: Vascular cell adhesion molecule 1; Ab: Antibody; TIMP: Tissue inhibitor of metalloproteinases; MMP: Matrix metalloproteinase; EPO: Erythropoietin; G-CSF: Granulocyte colony stimulating factor; MSC: Mesenchymal stromal cell.

to increase MMP-2 expression in MSCs and improve their motility[102].

There is also evidence that the epigenetic modulation induced by a short-term exposure to valproic acid results in increased expression of CXCR4 and MMP-2 in cultured MSCs and an increase in their migration towards SDF-1. There was no impact of this priming on the differentiation capacity of the cells[103].

Another approach that is under investigation is culturing MSCs under hypoxic conditions. Several groups have shown that these conditions result in increased CXCR4 expression and an improvement in MSC migration both *in vitro* and *in vivo*. This effect of hypoxia not only appears after short-term exposure but also in response to continuous culture in hypoxic conditions[104-108]. The increase in CXCR4 expression is reported to be regulated by an increase in hypoxia inducible factor (HIF) 1α[108]. Hypoxia also leads to differential expression of MMPs. For example, a decrease in MMP-2 secretion and an increase in MT1-MMP secretion and activity has been described in MSCs cultured under hypoxic conditions[104]. However, one could be concerned that culturing MSCs under hypoxia might change their behaviour. Valorani *et al*[109] reported that adipose tissue-derived MSCs cultured under hypoxic conditions exhibited an increased adipogenic or osteogenic differentiation capacity[109]. Crowder *et al*[110] reported that concurrent exposure to extreme hypoxia (0.5%) and a carcinogenic metal (nickel) induces carcinogenic changes in late passage MSCs. They did not observe these changes in early passage control cells[110].

A simpler modification of culture conditions is to maintain lower confluence. Our group found that MSCs that were cultured to complete confluence had a lower migration capacity than MSCs maintained at a low confluence. The cells cultured at higher confluence secrete more TIMP-3, an inhibitor of MMPs, which decreases migration compared to the MSCs cultured at low confluence[76].

Finally, MSCs are a heterogeneous cell population, and a particular subset of MSCs might have better homing abilities. MSCs were separated based on their expression of Stro1 and cultured further; these cells exhibited different migration capacities in NOD/SCID transplantation experiments. The amount of Stro1- MSCs was higher than the amount of Stro1+ MSCs in the target tissues of the mice, such as the bone marrow and spleen, after systemic administration *via* the retro-orbital plexus[111].

Genetic modifications

As already mentioned, MSCs express low levels of CXCR4, if any at all[58,59]. Because the CXCR4-SDF1 axis is important for bone marrow homing[20,112], many groups have designed transfection or transduction experiments in which CXCR4 expression plasmids are either nonvirally or virally introduced into the cells. Viral transduction is the most efficient method for obtaining high and stable expression levels in the target cells. CXCR4 overexpression resulted in improved MSC homing to the bone marrow after intracardiac injection into a NOD/SCID transplant model[112]. In a similar model, the overexpression of integrin α4, a subunit of VLA4 that interacts with VCAM-1, also resulted in increased bone marrow homing[113]. However, there are some draw-backs to this technique. Most importantly, there is the concern that the use of viral vectors to introduce the plasmid DNA poses a risk of insertional oncogenesis. Techniques for site-directed integration have been developed to circumvent

this problem[114]. Moreover, there is also a risk of adverse immune reactions and the production costs are high[115].

Different modes of non-viral transfection of plasmid DNA have been developed. One group overexpressed CXCR4 in MSCs using mRNA nucleofection. They obtained 90% expression of the surface receptor, but cell viability was only 62% and no increase in MSC homing could be observed[96]. Another group investigated the feasibility of inserting a short interfering RNA in MSCs using ultrasound and microbubbles to promote survival. A significant knock-down of the target (PTEN) could be obtained, but the cells were damaged after the manipulation[116].

Different modes of chemical, non-viral transfection have been studied, including the use of lipid agents. Although these techniques are easier to scale up and less expensive than viral transduction, they come with a price. The transfection efficiencies are significantly lower because approximately 35% of the MSCs express the transfected protein compared to over 90% of the cells after viral transduction[20].

Cell surface engineering

A method to improve homing efficiency of MSCs that has garnered interest in recent years is cell surface engineering, *i.e.*, a transient modification of the cell surface. Because transmigration through the activated endothelium takes 1-2 h, these transient alterations can be instrumental in improving MSC homing[117]. It has been shown that these modifications do not impact cell viability, proliferation, adhesion or differentiation[118-121]. For cell surface engineering, most groups focus on improving the first step of the homing process, tethering and rolling, by modulating the expression of adhesion molecules[54,118,120,121]. Since the first publications, many groups have developed different techniques for the cell surface modifications of MSCs.

A seminal paper in this field was published in 2008, when Sackstein *et al*[54] reported that they had converted the native CD44, which is readily expressed on MSCs, into the haematopoietic cell E-selectin/L-selectin ligand (HCELL) glycoform *ex vivo*[54]. E-selectin plays a key role in haematopoietic stem cell (HSC) homing to the bone marrow; however, MSCs do not express P-selectin glycoprotein ligand-1 (PSGL-1) or HCELL, the two E-selectin ligands that are required for HSC bone marrow homing, thus impairing their homing capacity to the bone marrow[54,122]. MSCs natively express CD44. In this study, Sackstein *et al*[54] were able to alter sialofucosylation *ex vivo* and transform CD44 into the HCELL glycoform. This treatment had no effect on the viability or phenotype of the cells. *In vivo* homing experiments that injected MSCs into the tail veins of NOD/SCID mice showed that the HCELL+ MSCs homed to the bone marrow, even in the absence of CXCR4, in contrast to the unmanipulated MSCs[54].

Sialyl Lewis X (SLE^X) is the active site of PSGL-1. Therefore, introducing this molecule into the MSC cell membrane should also lead to improved MSC homing. Sarkar *et al*[118] used biotinylated microvesicles to modify the MSCs. When the vesicles were brought into contact with the MSCs, they integrated into the cell membrane, thus generating biotinylated MSCs. Using a streptavidin linker, biotinylated SLE^X could be immobilized on the cell surface. The accessibility of the lipids integrated in the cell membrane was assessed and the researchers found they could still be detected after 4 h, but the intensity had already decreased to 50% compared with that at 0 h. After 8 h, all signals were lost, confirming that the modification is indeed transient. *In vitro* tests showed that the SLE^X-expressing MSCs exhibited improved adhesion under shear stress compared to the sham-treated MSCs[118].

Cheng *et al*[120] described a rapid (30 min) procedure to conjugate peptide K, an E-selectin binding peptide, to the MSC membrane. The MSC viability and proliferation rates were normal after engineering and their differentiation capacity was also maintained. In an *in vitro* model of inflamed endothelium, they subsequently demonstrated that the engineered MSCs adhered better than the control MSCs under shear stress[120].

Lo *et al*[121] described yet another engineering method to improve MSC binding to selectins and facilitate tethering and rolling. The first 19 amino acids of PSGL-1 (Fc19) were combined with an IgG tail and with an SLE^X glycan to engineer a pan-selectin-binding ligand. Tests in flow chambers showed that these MSCs were indeed capable of adhesion under shear stresses[121].

However, adhesion molecules are not the sole targets of the cell surface engineers. There is also interest in conjugating antibodies to the cell surface. Protein painting is a technique that binds antibodies to the cell surface. First, the palmitated proteins acting as docking stations for the antibodies are integrated into the cell membrane, and, subsequently, antibodies can be bound to the cell without losing affinity and with no impact on the viability and differentiation potential of the engineered cells[123]. One example using this technique is the binding of intercellular adhesion molecule (ICAM)-1 antibodies to MSCs, which increased the binding of these cells to endothelial cells[124]. This same protein painting technique has been applied to express VCAM-1 antibodies on MSCs, resulting in improved homing. In this study, the target tissues were the mesenteric lymph nodes and the colon. However, this technique might also be applied to improve homing to other organs, such as the bone marrow, because VCAM-1 is implicated in the bone marrow homing of MSCs[125].

Recently, a method was also described in which recombinant CXCR4 is bound to the cell surface of MSCs using lipid-PEG. In a one-step mixture procedure, recombinant CXCR4 could be transiently expressed on MSCs, leading to migration towards SDF-1 in a concentration-dependent manner[119].

Modification of the target tissue

Finally, MSC migration and homing can be influenced by modifying the target tissue. In early homing studies, it was

already shown that altering the target tissue by irradiation increases MSC homing[7,8]. After chemo- and radio-therapy, there are increased levels of SDF-1 in the bone marrow, thus increasing its attraction for HSCs and MSCs[126]. There are also reports of manipulating MSC migration with ultrasound or magnetic or electric fields[127-129]. However, these techniques do not appear to be very practical and they need adequate expression of homing molecules. For example, application of electrical fields could induce heat and electrochemical products near the electrodes. On the other hand, ultrasound-guided delivery might be more challenging in deep organs. Finally, homing directed by a magnetic field might require the implantation of a magnet in or near the tissue of interest[127-129].

Caveats in modifying homing molecules

In animal models and clinical studies, only limited engraftment or no engraftment at all is often observed, raising the question of whether tissue-specific homing is required for the therapeutic effect of MSCs[30,42]. A study on the use of systemically administered MSCs for the treatment of stroke in an animal model also showed very limited migration of MSCs to the tissue of interest, the brain. However, the researchers found that MSC homing to the spleen was important and correlated with a reduced infarct size and peri-infarct inflammation. They propose that MSCs exert a beneficial effect by abrogating secondary, inflammation-related cell death[130]. These data show that tissue-specific MSC homing is important, even though the target tissue is not the brain, as one would expect in a stroke model. Fernández-García *et al*[131] performed cotransplantation studies with MSCs and HSCs and found that cotransplantation improves short- and long-term haematopoietic reconstitution. This was the result of MSC and HSC interactions, and they propose that MSCs act as carriers that facilitate HSC homing to the bone marrow[131].

Manipulating stem cells, such as MSCs, to improve their homing capacities might not only change their migratory capacities but also have other consequences. For example, Liu *et al*[132] claim that the CXCR4-SDF-1 axis plays an important role in MSC survival because MSCs pretreated with SDF-1 exhibited significantly improved survival and proliferation. These effects could be partially inhibited by AMD3100, an inhibitor of CXCR4[132]. The pretreatment of MSCs with cytokines also revealed some conflicting observations. In a recently published paper, Kavanagh *et al*[133] report that licensing murine MSCs with inflammatory cytokines does not improve homing to the injured gut in an ischaemia/reperfusion model in their hands. More importantly, they found that while the untreated MSCs improved tissue perfusion, this effect was abrogated with the pretreated MSCs[133]. However, another group reported positive effects of pretreatment on the biological functions of the MSCs. Szabó *et al*[134] found that licensing murine MSCs with pro-inflammatory cytokines resulted in a significant reduction in the variability in immunosuppressive capacities of these MSCs. This reduction in variability was due to an increased immunosuppression of clones that were poor inhibitors of T-cell proliferation prior to licensing[134].

The pretreatment of MSCs with different factors or conditions, *e.g.*, hypoxia and inflammatory cytokines, could also modify their response to these treatments. Naaldijk *et al*[135] found that the oxygen concentration (normoxia *vs* hypoxia) alters the response of rat and human AT MSCs. They also found that the migration of MSCs isolated from older donors (rat and human) was not significantly impaired compared with the MSCs from young donors[135]. In contrast to this last finding, Choudery described that MSCs from aged mice exhibit diminished effectiveness and increased expression of apoptotic and senescent genes[136].

In this review, we have described different techniques for improving MSC homing and the expression of homing molecules on MSCs. Importantly, however, the expression of homing molecules and the resulting migration, homing and biological functions of MSCs might easily be altered unintentionally. Currently, many different protocols are used to expand MSCs for *in vitro*, animal and clinical studies. These variables can have a major impact on the expression of the homing molecules and the biological functions of MSCs; we will briefly discuss this below.

MSCs were first isolated from bone marrow. Since then, MSCs have been isolated from a wide variety of tissues, including adipose tissue (AT), umbilical cord blood (CB), Wharton's jelly (WJ), *etc*.[59,79,80,82]. Several groups have reported differences in the expression of homing molecules in human MSCs isolated from different sources; these are listed in Table 1. Additionally, the MSCs derived from different sources also exhibit differences in their biological functions. For example, AT MSCs might have better immunosuppressive capacities than bone marrow MSCs[95]. On the other hand, bone marrow MSCs appear to be the only MSCs that are capable of forming a haematopoietic niche that can support human haematopoietic tissue in an *in vivo* model[87].

When using MSCs for organ-specific treatments, one might choose to induce differentiation *in vitro* before transplantation. However, *in vitro* differentiation might not always result in a clinical benefit during MSC therapy. In a study using human CB MSCs in a mouse model for liver disease, the researchers found that hepatic differentiated MSCs performed worse than the undifferentiated MSCs. The differentiated MSCs showed decreased expression of the homing molecules and decreased *in vivo* migration after IV infusion. Additionally, their immunosuppressive capacity was decreased and the expression of HLA DR was increased, thus increasing their immunogenicity[137]. Ullah *et al*[138] also found that chondrogenic differentiated human MSCs exhibited a significantly reduced *in vitro* migration capacity than undifferentiated MSCs. However, CCR9 expression and *in vitro* migration to its ligand, CCL25, were retained in the differentiated MSCs[138].

Many parameters in MSC cultures vary between different research groups, including seeding density, number of passages, basal medium, and growth supplements [foetal bovine serum (FBS) *vs* platelet lysate (PL)]. All of these

factors might have an important impact on MSC function and migration. For example, Cholewa *et al*[139] found that PL increased MSC proliferation and increased the number of population doublings before senescence compared to FBS. However, they also showed that seeding MSCs at lower densities selected a highly migratory MSC population[139]. There are also reports of MSCs losing their migratory capacity and/or expression of homing molecules after *ex vivo* expansion[48,94]. After culture, MSCs are harvested with trypsin to detach them for passaging. Chamberlain *et al*[140] reported that the cell surface expression of chemokine receptors was decreased when the cells were detached with trypsin.

Future research directions

As described above, there is currently substantial variability in the isolation and expansion protocols for MSCs. Research on MSC homing and migration would clearly benefit from standardized MSC expansion protocols. What appears to be a rather minor aspect of the expansion protocol might have a significant impact on MSC function and/or migration. Thus, standardizing MSC expansion protocols would minimize unintentional modifications of the homing molecules. Of course, different culture conditions should be compared to create an optimal expansion protocol. Once this protocol is defined, it will also be easier to evaluate therapeutic efficacy of MSCs in clinical settings. It may be that different clinical applications require different expansion protocols to obtain the desired therapeutic effect.

We summarized the strategies for improving MSC homing. Many of these methods have not yet been validated *in vivo*. Before they can be translated to the clinic, the techniques with the most promising results should be first validated using *in vivo* homing models. In these experiments, the migration of engineered MSCs should be compared with the migration of untreated cells, and the therapeutic efficacy of the treated MSCs can also be assessed in animal disease models.

Although MSCs are widely studied and used in many clinical trials in a variety of clinical domains, little is known about the exact mechanisms by which MSCs exert certain therapeutic effects and their homing to certain tissues. Further studies would benefit from a better understanding of MSC biology. Understanding whether and where MSC migration or homing is necessary can help to define the optimal expansion protocols.

Finally, when transitioning to clinical trials, all conditions should be strictly defined, and, ideally, randomized controlled trials would be designed.

CONCLUSION

MSCs are interesting effector cells that can be used in a variety of therapeutic applications. Systemic administration is often the preferred route of delivery. However, this approach requires that adequate numbers of MSCs migrate and home to the target tissue(s). MSCs do not express many homing receptors, which impairs their migration capacity and hampers their therapeutic efficacy. Studies are ongoing and are needed to further elucidate the MSC homing mechanisms. A better understanding of MSC homing, as well as the factors influencing this process, will allow researchers to optimize the migration capacities of these stem cells and their therapeutic effects in a target tissue.

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REVIEW

Malaria modeling: *In vitro* stem cells *vs in vivo* models

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Abstract

The recent development of stem cell research and the possibility of generating cells that can be stably and permanently modified in their genome open a broad horizon in the world of *in vitro* modeling. The malaria field is gaining new opportunities from this important breakthrough and novel tools were adapted and opened new frontiers for malaria research. In addition to the new *in vitro* systems, in recent years there were also significant advances in the development of new animal models that allows studying the entire cell cycle of human malaria. In this paper, we review the different protocols available to study human Plasmodium species either by using stem cell or alternative animal models.

Key words: Malaria; Stem cells; *In vitro* models; Animal models; Humanized mice

Core tip: To better understand Plasmodium biology, researchers can whether proceed to *in vitro* studies or use *in vivo* models. Thanks to recent progresses, stem cells have been extensively employed to study Plasmodium liver and blood cycle *in vitro*. In parallel, the development of animal models opened new opportunities to study parasite biology *in vivo*. In this review, I go through and discuss the different available protocols using stem cells for modeling malaria *in vitro* as well as available animal models. This review has for goal to decipher which system would be the more suitable to study the parasite biology.

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INTRODUCTION

Despite many years of eradication efforts, Malaria remains a major threat to humans living in endemic area, particularly in sub-Saharan Africa (WHO report 2014). In the last two decades, the knowledge on many aspects of *Plasmodium* biology advanced significantly

including mechanisms of motility and cell invasion[1], modification of the host cell such as cytoadherence[2], immune evasion[3] establishment of liver infections[4] and hypnozoites dormancy[5]. These achievements would not be possible unless Trager *et al*[6] were able to establish *Plasmodium* culturing *in vitro*. The ability to successfully freeze *Plasmodium* isolates[7,8] and routinely culture laboratory-adapted strains (*i.e.,* DD2, 3D7, W2) was one of the most important steps that allowed more researchers to study malaria outside endemic areas.

In recent years, breakthroughs in stem cell research provided additional opportunities to study new aspects of the parasite biology, primarily of stages in the cell cycle in which culturing had been thus far challenging or impossible. In addition, the development of novel animal models completes the study of the entire cell cycle of human *Plasmodium* spp. and represents an appealing alternative to study host-parasite interactions with no need of human infection.

In this current paper we review and discuss the recent advances of novel procedures used to study human *Plasmodium* infection *in vitro* and *in vivo*.

STEM CELL DERIVED CELLS

Stem cell derived-erythrocytes

The lack of blood supply in blood banks that rely on constant blood donations, lead many researchers to look for alternative solutions to produce erythrocytes for transfusion[9]. The first report of the production of human erythrocytes from hematopoietic stem cells using a liquid system was described by Fibach *et al*[10]. These authors isolated mononuclear cells (MNC) from peripheral blood of a patient with -thalassemia, in which a defect in the chain of hemoglobin, cause an erythropoiesis increase. Using a two-step protocol, they could observe erythroid cells when cultured in the presence of erythropoietin (EPO). However, ethical concerns of using blood from a β-thalassemia patient presenting a defect in hemoglobin still remain (Figure 1).

Following this study, many protocols have been developed in order to generate erythrocytes from HSC (reviewed by Migliaccio *et al*[11]). In 2005, Giarratana *et al*[12] published what could be considered as the reference protocol to generate erythrocytes from HSC. Briefly, after isolation of HSC from diverse origins (peripheral blood, umbilical cord blood, bone marrow and leukaferesis product) through a magnetic assorted cell sorting (MACS) selection based on the $CD34^+$ expression, cells were co-cultured with mouse stromal cells (MS5). The cells were cultured in the presence of a cocktail of specific growth factors to allow a correct differentiation toward erythroid commitment: interleukin 3 (IL-3), hydrocortisone (HDS), stem cells factor (SCF) and EPO. After 20 d in culture, pure population of erythrocytes could be isolated from the supernatant. Nonetheless, production of erythrocytes from HSC faced some difficulties that limited the amount of cells which are produced as well as the ability to produce mature red blood cells (RBCs) (as the hemoglobin isoforms remain at fetal state).

The stem cell-derived erythrocytes have recently been intensively used in the malaria field to try to solve the challenging *in vitro* culture of *Plasmodium vivax* (*P. vivax*)[13]. Unlike *P. falciparum* that can invade erythrocytes of all ages, *P. vivax* shows a preference for invading immature erythrocytes (named reticulocytes)[14]. This preference for reticulocyte invasion makes use of peripheral blood as a source of cells to culture parasites *in vitro* nearly impossible as reticulocyte are only 0.5%-1% of erythrocytes in the blood stream and their lifespan prior to maturation is only 24 h. Thus a reticulocyte-enriched source of blood is needed in order to grow *P. vivax in vitro*. Early studies used several methodologies to concentrate reticulocytes from blood by ultracentrifugation[15], centrifugation on Percoll layer[8,16] or lysis buffer[17]. However, more recent studies demonstrated the preference of *P. vivax* for $CD71^{high}$ cells (reticulocytes)[18-20] revealing the possibility of using stem cell-derived reticulocytes. The first report attempting to establish an *in vitro* culture of *P. vivax* using HSPC-derived reticulocytes showed that parasites could be maintained in culture for more than 50 d using stem cell-derived reticulocytes[21]. This important study confirmed that stem cells could be used as a source of reticulocytes for *P. vivax in vitro* culture. However conditions still needed to be optimized as reticulocyte production were only 0.5% (after 14 d) and the parasitemia reached very low levels (below 0.0013%). In a more recent study, Noulin *et al*[22] were able to generate, after 14 d of culture, up to 18% of reticulocytes which were permissive to *P. vivax* invasion. They were also able to successfully cryopreserve reticulocytes in order to create a stock of cells to provide to *P. vivax* at each schizogony cycle. Nevertheless, the amount of reticulocytes generated remained extremely low and the parasite could still not multiply *in vitro*.

Before HSPC-derived reticulocytes can be used for successful *P. vivax in vitro* culture, the problems of low reticulocyte yield and the lack of intra-erythrocyte development of the parasite must be addressed. Very recently, Roobsoong *et al*[23] proposed optimized *P. vivax* culture conditions in order to better maintain the parasite *in vitro*. As a source of reticulocytes, they differentiated $CD34^+$ cells into reticulocytes using the previously described protocol and interestingly purified the reticulocyte population passing the cells through leukocyte reduction filters to get rid of nucleated cells. Alternatively, they also concentrated reticulocytes from peripheral blood (PB) and umbilical cord blood (UCB) on a 19% Nycodenz layer. They also tested different culture media (McCoy's 5A, RPMI or Waymouth) supplemented with different serum concentrations. The authors claimed they could maintain the parasite *in vitro* for 26 mo, though the parasite density dramatically dropped from the first day to an almost undetectable level after the second day. We could conclude from these observations that *P. vivax* did not grow *in vitro* and thus more improvements are needed to reach a viable *in vitro* system.

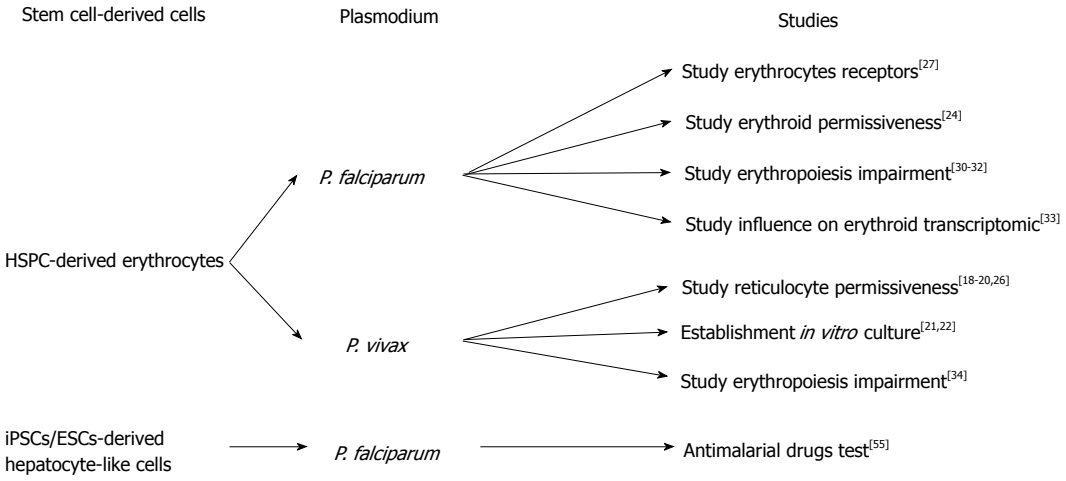

Figure 1 Chart of the different stem cells used for *Plasmodium in vitro* modeling and their applications. Scheme of the different sources of stem cells used for liver and blood cycles of *Plasmodium falciparum in vitro* studies. The main aims of each study are indicated on the right.

The parasite's ability to invade and replicate within reticulocytes generated from HSPC is a precondition for the establishment of *in vitro* culture that relies on stem cells as a source.

Tamez *et al*[24] were interested in identifying the earliest erythroid stage, which is permissive to *P. falciparum* invasion. They differentiated HSPC according to a previously published protocol[25]. Briefly, after $CD34^+$ isolation, HSPCs were cultured for 8 d in the presence of IL-3, EPO and SCF with medium refreshment at day 3 and 6 without IL-3 and decreased SCF concentrations. A selection was done after 7 d by FACS sorting based on $CD71^+$ expression and the cells were cultured with only EPO supplementation for an additional 10 d. They found that the polychromatic erythroblasts were poorly invaded while ortho-erythroblasts could be invaded and allowed for parasite intra-cellular maturation, indicating the permissiveness of erythroid progenitors to *P. falciparum*.

Fernandez-Becerra *et al*[26] generated reticulocytes starting from HSPC isolated from adult PB, umbilical cord blood UCB and bone marrow (BM). Notably, they used 3T3 cells instead of the more-commonly used MS5 cells as a layer for differentiating erythroid progenitors. They could reach a significant yield of reticulocytes (up to 83.5%) and observed the presence of adult hemoglobin in reticulocytes derived from PB and BM. However, no information was given about the level of parasitemia post-invasion. A year later, Noulin *et al*[20] investigated different sources of HSPC. Remarkably, after $CD34^+$ isolation and before differentiation, they applied an expansion step to increase their HSPC population. They could dramatically increase the HSPC population up to 10 fold for UCB source, 3 fold for BM source and 1 fold for PB source. They also observed better enucleation in PB source (32%) *vs* BM (20.5%) and UCB (18%). All three sources tested had similar permissiveness and better invasion rates compared to reticulocyte-enriched blood leading to the hypothesis that *P. vivax* prefers immature reticulocytes.

Recently, Egan *et al*[27] used a reverse genetics approach to investigate the role of RBC receptors that are involved in *P. falciparum* invasion. Using lentiviral shRNA delivery, they performed gene knockdown (kd) of different genes encoding for potential receptors in erythroid cells (starting from PB/HSPC isolated from Granulocyte-colony stimulating factor-stimulated patient or BM). They differentiated those that were genetically modified erythroid progenitors on stromal cell layer to obtain enucleated cells used further for *P. vivax* invasion assays. The authors observed a dramatic invasion decrease in $CD55^{kd}$ as well as in $CD44^{kd}$ RBCs. This work was the first to highlight the possibility of using genetically modified erythrocytes to study *Plasmodium* biology. Nevertheless, since it is impossible to maintain HSPC as stem cells it is essential to repeat the kd procedure every time, which causes some variability between kd experiments.

Significant blood hemolysis was reported during malaria infection[28] and thus, HSPC are also of particular interest to study erythropoiesis impairment that leads to anemia during malaria episodes[29]. Several studies tried to investigate the mechanism by which *Plasmodium* infection causes erythropoiesis impairment. In this scope Hemozoin (Hz) attracted particular interest. Hz is produced by the parasite when it metabolizes heme in its food vacuole[30]. Casals-Pascual *et al*[31] and Skorokhod *et al*[32] investigated the influence of Hz on erythroid development. They isolated $CD34^+$ cells by MACS followed by a well-established differentiation protocol[12] and noticed a marked decrease in erythroid production in presence of Hz.

The influence of Hz on erythropoiesis was investigated by Malleret *et al*[19] using a different protocol. Starting from UCB, they differentiated $CD34^+$ cells based on a 3-step process[20]: 7 d in presence of Fms-like tyrosine kinase 3 (FLT-3) and thrombopoietin (TPO), 7 d with an addition of insulin growth factor-1 (IGF-1), SCF and EPO, and finally 2 to 7 d without SCF. They found that the main reasons for hemolysis are soluble mediators

from Hz-stimulated PBMC rather than erythropoiesis impairment due to Hz itself.

It appears that *P. falciparum* infection significantly influences transcription in erythroid progenitors as shown by Tamez *et al*[33]. Following the erythroid development protocol they previously developed[24], they observed an up-regulation of 35 genes in polychromatophilic erythroblasts and 609 regulated genes in ortho-erythroblasts. These results may indicate a negative effect (direct or indirect) of *P. falciparum* on erythropoiesis.

P. vivax infection has a similar effect on erythroid development[34]. Using a modified protocol previously developed by Giarratana *et al*[12] (without stromal feeder cells) the authors co-culture erythroid cells with *P. vivax*-infected reticulocytes (intact or lysed), uninfected erythrocytes, in presence of tumor necrosis factor alpha (TNF-α) or interferon gamma (IFN-γ). They observed a decrease of the erythroid multiplication and development in the presence of infected reticulocytes lending support to the idea that that *P. vivax* might have a negative effect on erythropoiesis.

The recent important development of stem cell research contributed to the production of stem cell-derived erythrocytes, and enabled testing the use of human embryonic stem cells (hESCs) or human induced pluripotent stem cells (hiPSC). Indeed, recent findings have demonstrated that those pluripotent cells can be maintained and expanded *in vitro* prior to differentiation into specific lineage[35,36]. To date, several protocols have been developed to generate mature erythrocytes from hESC or hiPSCs with partial success. Lu *et al*[37] developed a protocol to produce enucleated red blood cells from ESC. The differentiation was initiated by dispensing hESC as erythroid bodies (EBs) in presence of bone morphogenetic protein 4 (BMP4), vascular endothelial growth factor 165 ($VEGF_{165}$), beta fibroblast growth factor (β-FGF), TPO, FLT-3 and SCF to induce mesodermal commitment. The second step, which is the erythropoiesis leading to the last stages of the blood production, was performed in co-culture with OP9 cells or human mesenchymal stem cells (huMSC) in presence of IL-3, HDS, EPO and SCF. Using this technique, they could get up to 40% of enucleated cells. In 2010 Lapillonne *et al*[38] reported that by starting from hESC and hiPSCs they could get up to 66% of enucleated erythrocytes. To achieve this amount, they used a two-step protocol where the cells were initially cultivated as EBs in the presence of 5% human plasma with BMP-4, $VEGF_{165}$, TPO, FLT3, SCF, IL-3, EPO and IL-6 for 20 d. After dissociation into single cells with collagenase B, cells were cultured in feeder-free condition with 10% human serum together with growth factor cocktail as previously described[12] for 25 d.

Although discussed in many papers, to the best of our knowledge, there is no record for the application of ESCs/iPSCs in malaria research thus far. The development of such techniques and the possibility to permanently edit the genome of erythroid cells will make a great contribution for a deeper study of intra-erythrocyte parasite biology.

Stem cell derived-hepatocytes

Studying the liver stage of *Plasmodium* parasites is of great importance for understanding the establishment of infection, and for immunogenic and therapeutic purposes[39,40]. In addition, *P. vivax* can produce dormant forms known as hypnozoites in the liver[41]. These hypnozoites are responsible for its ability to maintain long term infections and relapsing episodes that contribute to the difficulties of eradicating *P. vivax*[42].

An immortalized HepG2 cell line[43] has been extensively used as starting material to investigate the exo-erythrocytic (E.E) cycle of *P. berghei*[44], *P. vivax*[45], *P. falciparum*[46] and *P. gallinaceum*[47]. However, even though they were able to infect liver cells with *P. falciparum* sporozoites, it is still difficult to get these parasites to successfully complete the cycle and infect RBCs. This difficulty was overcome by Sattabongkot *et al*[48] that generated a hepatocyte cell line (HC04) that enabled the full development of both *P. vivax* and *P. falciparum*. Briefly, hepatocytes isolated from a hepatoma patient were cultivated with insulin, epidermal growth factor, thyrotropin releasing factor, HDS, glucagon, nicotinamide, linoleic acid, L-glutamine, pyruvic acid and MEM essential amino acids at 37 ℃ and 5% CO_2. Interestingly, the levels of enzyme activities and protein secretions were higher than the ones observed in HepG2 cell line. These cells were infected with *P. falciparum* and *P. vivax* sporozoites and when RBCs were added to the culture, blood stage parasites were observed after 7 and 10 d for *P. falciparum* and *P. vivax,* respectively.

One of the drawbacks of using immortalized cell lines is that the metabolism of those cells might differ from the *in vivo* ones. To solve this problem Mazier *et al*[49] used primary rodent hepatocytes to try and mimic the *in vivo* conditions. They found that *P. vivax* parasites were able to develop and after 10 d, rings could be observed in reticulocytes added in co-culture. Recently, using human and primate primary hepatocytes, Dembélé *et al*[50] successfully cultured *in vitro* E.E stages of *P. falciparum* as well as *P. cynomolgi,* for which they able to get the hypnozoite forms.

Using primary cells for parasite cultures requires that fresh cells be constantly available. To bypass this obstacle, March *et al*[51] were able to culture previously frozen primary hepatocytes that remained permissive to *Plasmodium* sporozoites, in a microsystem surrounded by fibroblast stromal cells.

Primary simian hepatocytes were used to evaluate the effect of a drug (KAI407) on *P. cynomolgi* liver stage[52]. Primary hepatocytes isolated from rhesus macaques were infected with *P. cynomolgi* sporozoites in the presence or absence of the KAI407 compound. The development of E.E stage was well established *in vitro*, but the incubation with KAI470 was shown to inhibit formation of liver schizonts as well as hypnozoites. Similarly, *P. berghei* liver stages could develop within murine primary hepatocytes *in vitro* but their ability to infect RBCs remain unexplored in this study[53].

Immortalized murine hepatocytes (Hepa1-6) were

used to test malaria vaccine candidates on *P. berghei* E.E stage[54]. It appears that the TRAP-based vaccine in the presence of $CD8^+$ enriched splenocytes inhibits the parasite development in the liver. This method was proposed as an *in vitro* system to screen possible vaccine candidates but its suitability to human *Plasmodium* vaccines needs further investigation.

Surprisingly, stem cell-derived hepatocytes have not been used widely used for malaria research. Nonetheless, Ng *et al*[55] generated hepatocyte-like cells (HLCs) from ESC or iPSCs originating from human foreskin fibroblasts, which were permissive to different *Plasmodium* species including *P. falciparum*, *P. vivax*, *P. berghei* and *P. yoelii*. The hepatocyte differentiation protocol was adapted from the one previously described[56], in which the ESCs/ iPSCs were cultivated for the first 5 d in presence of activin A (100 ng/L) to induce endodermal commitment. The following 10 d led to hepatoblast formation through hypoxia culture condition and was divided into two steps; the first 5 d in presence of BMP-4 and FGF-2 and the last 5 d in presence of hepatocyte growth factor (HGF). During the last 5 d, the cells were maintained with oncostatin to generate mature hepatocyte-like cells. Their HLCs allowed them to test different antimalarial drugs such as Atovaquon or Primaquine on liver stages. However, iPSC-derived HLCs have low levels of enzymes that metabolize drugs as they remain immature hepatocytes and thus are not optimal for antimalarial drug screen.

Many protocols to generate HLCs from ESCs/iPSCs (reviewed in Schwartz *et al*[57]) or adult stem cells (reviewed in Zhang *et al*[58]) are available. However, one should note that different protocols seem to create a variety of HLCs with different characteristics. A general scheme can be drawn with a 4-steps protocol: Mesodermal differentiation (in presence of activin A), hepatic specification, hepatoblast expansion and hepatic maturation. For each of these 4 steps, growth factor concentrations as well as the time of exposure remain variable between different studies.

Optimizing the generation of stem cell-derived hepatocytes, which are more similar to the adult hepatocyte, would have great impact on understanding the biology of *Plasmodium* E.E stages and lead to improved testing of potential antimalarial drugs and vaccine candidates.

ANIMAL MODELS

Murine models

Besides the use of *in vitro* modeling to study *Plasmodium* biology, there are several *in vivo* models that aim to mimic human infections. Several animal models are available for diverse *Plasmodium* species. Among those, the most common one remains the mouse model, which is less costly, more available and much more convenient to maintain than primates or other large models. *P. berghei* and *P. yoelii* (both rodent *Plasmodia*) are commonly used for *in vivo* studies as these species share important similarities with primate and human parasites[59]. *P. yoelii* shares common features with *P. vivax* [*i.e., P. yoelii* virulence genes (yir genes) homolog of *P. vivax* vir genes] that make this parasite ideal for *in vivo* studies[60]. *P. berghei* seems to be a better model for *P. falciparum in vivo* studies, especially concerning blood stage vaccine studies[61] (Figure 2).

Nevertheless, even in murine *Plasmodium* species that share some features with human *Plasmodium*, the ability to interpret and draw conclusion from phenotypic observations from murine to human species remains questionable.

Recent developments has partially resolved this problem, through the use of humanized mice (reviewed in Kaushansky *et al*[62]) .This system represented an important breakthrough in the field of laboratory modeling[63] and the application of this model through infections of humanized mice with *P. falciparum* begins to make an impact the malaria field. Infection of humanized mice with *P. falciparum* infected RBCs was done by directly injecting human red blood cells (huRBCs) into the mouse blood stream *via* the intra-peritoneal route[64] or intravenous route[65]. The main problem using intra-peritoneal injections is the difference in migration of the injected huRBCs into the blood stream between experiments and the lack of reproducibility from one mouse to another. Intravenous delivery as proposed by Arnold *et al*[65] allows a more stable and long-lasting presence of huRBCs within mouse host. Notably, they also injected parasitized huRBCs and could reach significant parasitemia (up to 10%) by adding new huRBCs intravenously every 2-3 d.

An alternative method that is now more commonly used is the engraftment of HSPCs into immune-deficient mice[66]. This method allows for continuous production of human RBC in the mouse blood stream. The main obstacles of this methodology are the short lifespan of those cells within mouse bone marrow and the variability in the engraftment success[67]. Technically speaking, many protocols have been investigated with different combination of mice and HSC sources. Generally, $CD34^+$ cells mainly isolated from umbilical cord blood were isolated *via* MACS selection and injected intrahepatic or intravenously within immune-depressed mice. Interestingly, TPO was shown to increase engraftment of $CD34^+$ cells in mouse host[68].

Recently, Amalados *et al*[69] were able to generate huRBC permissive to different *P. falciparum* strains by transfecting the HSC with a plasmid that expresses IL-3 and EPO. Using this procedure they were able to produce only low amounts of huRBC (1.5%-2.8%) and parasite density was decreasing constantly and thus will require further optimization. Unfortunately, to the best of our knowledge there is no report on using similar systems of humanized mice to study the *P. vivax* asexual cycle *in vivo*.

To date, the major contribution of using humanized mice was to study liver stage parasites which remain the main target for vaccine development[70].

To engraft human hepatocytes (huHep) within immune-

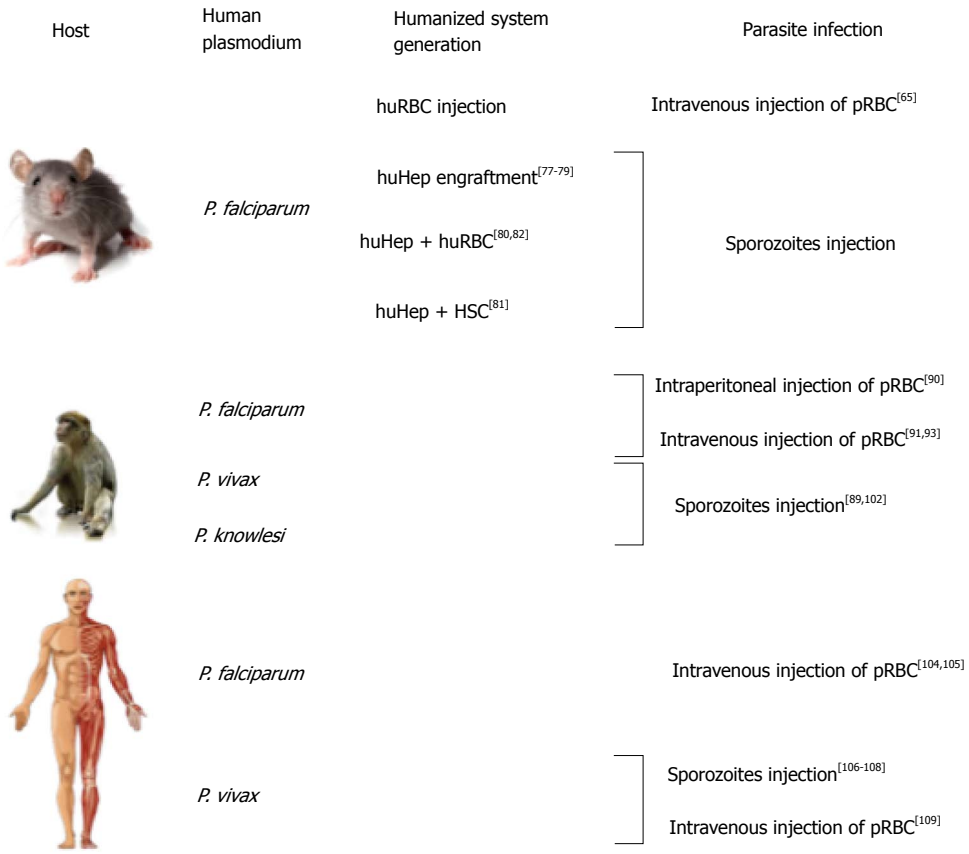

Figure 2 Chart of the different combinations animal model/human *Plasmodia* for *in vivo* studies. Scheme of the different animal models coupled with human *Plasmodium* studies. The different cell types injected within humanized mice are indicated in the column "Humanized system generation" and the *Plasmodium* injection mode under the column "Parasite infection".

depressed mice, it is essential to initially generate damage in order to activate the liver cell repopulation to allow integration of delivered human cells. Several options are available to manipulate mouse hepatocytes: (1) use urokinase plasminogen activator (uPA) toxin[71]; (2) use fumarylacetoacetate hydrolase knockout mice, dependent of the protection of the 2-(2-nitro-4-trifluomethylbenzoyl)-1,3-cyclohexanedione (NTBC) drug[72]; (3) use herpes simplex virus type 1 thymidine kinase transgene dependent of ganciclovir (GGV) activation[73]; (4) use caspase 8 oligomerization activated with AP20187 drug[74]; and (5) use diphtheria toxin receptor transgenic mice[75]. According to the different reports the success rate of colonization of the mouse liver by human hepatocytes range between nearly 50%[76] to 60%[75]. A higher ratio of 90% remains so far untenable as murine hepatocytes are needed for metabolic functions.

P. falciparum was shown to be able to invade and develop within hepatocytes of several humanized mice models. The first successful attempt was reported by Morozan *et al*[77]. Using uPA mice, they were able to engraft human hepatocytes (up to 26%) and after 3 mo, injected them with *P. falciparum* sporozoites. This methodology allowed them to complete the EE development of the parasite to the final schizogony stage. One of the applications of humanized mice is the investigation of potential hepatocyte receptor for *Plasmodium* sporozoites invasion. Foquet *et al*[78] investigated the possibility that CD81 receptor and scavenger receptor type B class Ⅰ (SR-BI) are the entry gates for *P. falciparum* into the liver. They showed that in presence of anti-CD81, the hepatocyte invasion was prevented while anti-SR-B1 did not alter the infection process. The humanized mice used for this study were uPA mice but no indication on the level of human hepatocytes engrafted was indicated. Drug tests can be performed as well in humanized mice as demonstrated by Douglass *et al*[79]. They monitored the clearance of GFP-luc transgenic *P. falciparum* from the liver after treatment with different antimalarial drugs and could observe a complete clearance of the parasite using Atovaquone (inhibitor of mitochondrial electron transport chain), Serdemetan (p-53 activator) and Obatoclax (BCL-2 family inhibitor). This work shed light on the use of humanized mice to test potential antimalarial drug effect in the human hepatocyte niche.

Recently, humanized mice have been described as a perfect environment to genetically cross *Plasmodium* strains in order to study genetic determinants[80]. Sporozoites of two different *Plasmodium* strains were injected intravenously into FRG NOD HuHep mice and the EE stage monitored by bioluminescence. The injected sporozoites could maturate and invade huRBC injected within the same mice. Unfortunately, the mice were rapidly euthanized and thus no information on the parasite

development in the huRBC could be documented.

Combining human liver and blood stages to get a whole vertebrate cycle within humanized mice remains the ultimate goal of those models. Wijayalath *et al*[81] reported this full cycle in a humanized mouse. To create the mouse model, they injected $CD34^+$ cells that can be the origin of many cell types (*i.e.,* cardiomyocytes, endothelial cells or hepatocytes) in order to be able to recreate a whole human system suitable for the parasite. They could observe an engraftment of 0.023% of huHep, 11% of human Kupffer cells and only 0.2%-1% of huRBC due to poor erythroid differentiation. Sporozoites were injected intravenously, developed within the liver (as shown by immunohistochemistry) and then reached the blood circulation with a very low parasite density of 2-5 parasite/L (parasite density 0.0001%). The asexual stages were then cultivated *in vitro* to obtain gametocytes that could develop into oocytes and sporozoites within mosquitoes. The possibility of getting the whole *P. falciparum* cycle in humanized mice would be a great achievement. Nevertheless, in this study, the low levels of engraftments of human cells, the low parasite density as well as the obligation to generate gametocytes *in vitro* indicate that this system needs further optimization for studying the complete cycle of *Plasmodium* parasites. More recently, Soulard *et al*[82] achieved the complete *P. falciparum* cycle in humanized mice, from liver stages to sexual forms in the blood. They got up to 80% of huHep and above 80% of huRBC that can be maintained for 5 wk in mice with daily injections. *P. falciparum* sporozoites could migrate into huHEP in the mice liver and schizonts were observed 7 d post-infection. The parasite asexual stages could be detected in the blood from 8 d post-infection and sexual stages after 21 d. The parasitemia reached up to 1.52%. In addition, they could infect hepatocytes with *P. ovale* sporozoites and observe the formation of several hypnozoites, but there was no indication for *P. ovale* asexual stages in the blood.

These mice open new frontiers for studying human *Plasmodium in vivo*. Nevertheless, the variations between mice and experiments should be taken into account when designing experiments and analyzing the results. It would be extremely beneficial to expand the use of these mice and apply it to get a mouse model for *P. vivax* (using reticulocyte-enriched huRBC).

While the use of humanized mice obviously offers a wide range of new possibilities to study the biology of human *Plasmodium* spp. *in vivo,* the need to work with immune-depressed mice makes them unsuitable for vaccine development.

Primate models

Beside the use of mice as animal model, primates appear to be a very suitable model to study malaria as they are evolutionary close to humans[83] and they are natural hosts of human *Plasmodium spp.*[84,85]. Studies on host-parasite interactions benefit from of this *in vivo* system that allows collecting samples and data regularly. A complete overview of the use of primates for malaria modeling has already been reviewed by Beignon *et al*[86].

Nowadays, the uses of non-human primates (NHP) are preferred for *in vivo* research despite ethical reasons that restrict experimenting on primates. Therefore, NHP allows larger sample size and more reagents are available for these models[87].

To increase parasite density and maintain long-term *Plasmodium* infections within the host, primates need to be splenectomized[88]. Parasite infection is done either by injecting *Plasmodium* sporozoites[89] or by direct injection of parasitized RBC (pRBC)[90,91].

The use of these primate models for *P. falciparum* studies has been restricted mainly to *Aotus* monkeys[92] that could be infected with several *P. falciparum* strains. The first report of *Aotus* infection with *P. falciparum* was described by Geiman *et al*[90]. They injected intra-peritoneally pRBC from a *P. falciparum* infected woman into a splenectomized *Aotus* monkey. They were able to detect asexual forms of the parasite in the primate blood 54 d post-injection. These primate models were used to test potential blood stage antigens for vaccine development, *i.e.,* MSP-1[93] or PfEBA-175[91]. Briefly, *Aotus* primates pre-treated with potential vaccine-candidates were challenged by the injection of pRBC and the parasite density was monitored in order to analyze the protection provided by the initial challenge of the potential vaccines.

Many *P. vivax* isolates have been adapted to several NHP models, among those: The Chesson strain, Salvador I strain and others, which allowed getting an important source of study material. In 1966, Young *et al*[94] were able to infect *Aotus* primate with pRBC isolated from a *P. vivax* infected patient. Interestingly, they also infected two human volunteers as well as one primate through infected mosquito bites and after 11 d they could only identify *P. vivax* infection in the human volunteers while parasitemia in the monkeys could be observed only after 41 d followed by his death 5 d later.

Primate models have been intensively used to study *P. vivax* liver stages[87]. The development of an *in vivo* system to study the ability of *P. vivax* to generate dormant forms (hypnozoites) in the liver, which cause relapses of the infective forms[41] would aid in understanding the dynamics of this process. Collins *et al*[89] tested different primate species infected with the *P. vivax* Salvador I strain and identified *Saimiri boliviensis* as the most suitable primate species to study *P. vivax* liver stages.

As an alternative to human *Plasmodium* studies in monkeys, researchers recently focused on close simian *Plasmodium* spp in NHP: *P. knowlesi* as a model for *P. falciparum* and *P. vivax*[95,96] and *P. cynomolgi* for *P. vivax*[97]. Indeed, these simian *Plasmodium* spp share important features with their human orthologues and can be used to better understand parasite biology or test potential vaccines[98]. The primate infection remains identical to the process used for *P. falciparum* and *P. vivax*. Krotoski *et al*[99] were the first to identify the *P. cynomolgi* hypnozoite stage after inoculation of

sporozoites into rhesus monkeys. Akinyi *et al*[100] were able to create a *P. cynomolgi* transgenic line expressing a red fluorescent protein, which was used to track the parasite *in vivo*.

P. knowlesi can also infect humans can be used as model for both *P. falciparum* and *P. vivax* infections and also to study its own infection traits in human[101]. Irradiated *P. knowlesi* sporozoites injected into rhesus monkeys achieved a relative protection against further *P. knowlesi* infections, demonstrating the use of *P. knowlesi* as a model for vaccine development[102].

P. knowlesi can also be applied as a model to study severe malaria usually caused by *P. falciparum*. Barnwell *et al*[103] could observe a link between the expression of schizont-infected cell agglutination and the severity of the infection in rhesus monkeys, linking between pathogenicity and antigenic variation caused by variant surface antigens in *P. knowlesi* and *P. falciparum*.

One of the major drawbacks of using primate as a model to investigate *Plasmodium in vivo* remains their availability and the significant cost of the colony maintenance that limits the development of this research line.

Human model

Perhaps the most relevant model to study malaria, in such cases that allows experimenting, is the human host itself. Naturally, potential candidate vaccine candidates have to be tested in humans during clinical trial. For example, to test the RTS/S vaccine, healthy volunteers that were prime-boosted immunized with candidate vaccines were infected with *P. falciparum* sporozoites to test the efficacy of these vaccine candidates[104,105]. We can also cite the control human malaria infection program that allows inoculating parasites in human volunteers in order to test potential vaccines or anti-malarial drugs[106,107].

Interestingly, the lack of an *in vitro* model for *P. vivax*[13] pushed researchers to infect human volunteers with *P. vivax* sporozoites in order to develop a model for *P. vivax* drug screening and vaccine development. Herrera *et al*[108] let *P. vivax*- infected Anopheles mosquitoes feed on different groups of volunteers, each exposed to increasing number of mosquito bites. They observed that malaria symptoms appeared after 9 d and a total clearance of the parasites was observed 48 h post treatment at the latest. No record of *P. vivax* relapsing after the end of the study was reported and thus, the system is claimed to be safe to test antimalarial drugs *in vivo*.

More recently, McCarthy *et al*[109] infected human volunteers *via* intra-venous injection of pRBC isolated from a *P. vivax* positive woman. The first symptoms appeared 11 d post-inoculation and disappeared 24 h post-antimalarial drug treatment. The advantage of using pRBC instead of sporozoites is to avoid the formation of hypnozoites and thus re-infection. Their goal was to establish a *P. vivax in vivo* system similar to the work described earlier by Herrera *et al*[108] and only the inoculation method (sporozoites *vs* pRBC) and the number of volunteers (18 *vs* 2) was different. Although biologically, humans are the most relevant models, there are important ethical issues that prevent wide use of human volunteers in *in vivo* experiments out of phase Ⅱ clinical trials.

CONCLUDING REMARKS

Establishing good experimental models for malaria research has great importance in understanding fundamental aspects of the parasites' biology, the course of infection and disease establishment and progression. It is an important tool for laboratories located in non-endemic areas that have more facilities to perform state of the art research to help fighting malaria.

The development of stem cell research has opened many new options to study parasite interactions with human host. Combining these novel *in vitro* systems with animal models offered a wide range of new avenues to study aspect of the parasite biology, which were not possible before.

Each technique has its advantages and weakness depending on the parasite species and the stage in the cell cycle being investigated.

Thus far, despite their great potential, the use of stem cells for malaria *in vitro* studies is limited. The establishment of good *in vitro* culture of *P. vivax* in reticulocytes originated from HSC has not been successful even though there is a great interest in such a model. On the other hand, the use of HSC to study erythroid impairment during malaria episodes was shown to be a great tool, which is expected to have a significant contribution to the field in coming years. Very surprisingly, although understanding the biology of *Plasmodium* liver stages is of major importance for drug and vaccine development, the use of hepatocyte-derived stem cells is poorly developed and there is a great need for a better cell line that differentiates into mature hepatocytes.

Animal model have been intensively developed to gain an understanding that will be able to be rapidly translated to the clinic. Monkeys appear to be the most suitable models, especially for *P. vivax*, but the cost of colony maintenance limits the use of this model. Nevertheless, *P. vivax* primate model are used successfully and are currently the best option for research since an *in vitro* culture of this parasite remains challenging. The development of *in vitro* stem cell techniques would offer an important tool to study *P. vivax* biology, especially for the intra-erythrocytic cycle. Recent protocol improvements give great hope that with additional optimization these systems will be available in coming years.

The use of humanized mice to study *Plasmodium* biology through an *in vivo* system offers new opportunities, however, the short-term life span of engraftment and the low levels of chimeric systems eventually obtained have to

be taken into account in the process of data analyses.

The use of simian *Plasmodium* species that could infect humans to overcome the difficulties in maintaining cultures seems like an attractive option, however, the use of the human *Plasmodium* spp. will yield the most relevant observations that could be directly translated to human malaria.

Additional tools such as mathematical and bio-informatics modeling could also become valuable as recently shown by MacDonald *et al*[110] that used computational methods to investigate potential antimalarial drugs.

The combination of stem cell research and animal modeling such as humanized mice could be the key to move a step forward in the study of *Plasmodium* biology. Optimization of those techniques and generation of new animal/human stem cell combinations could bring malaria modeling to the next level.

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MINIREVIEWS

Role of adipose-derived stromal cells in pedicle skin flap survival in experimental animal models

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Abstract

The use of skin flaps in reconstructive surgery is the first-line surgical treatment for the reconstruction of skin defects and is essentially considered the starting point of plastic surgery. Despite their excellent usability, their application includes general surgical risks or possible complications, the primary and most common is necrosis of the flap. To improve flap survival, researchers have used different methods, including the use of adipose-derived stem cells, with significant positive results. In our research we will report the use of adipose-derived stem cells in pedicle skin flap survival based on current literature on various experimental models in animals.

Key words: Pedicle skin flap; Adipose stromal cells; Flap survival; Stem cell; Skin defect; Reconstructive surgery

Core tip: The use of skin flaps in reconstructive surgery is the first-line surgical treatment for the reconstruction of skin defects and is essentially considered the starting point of plastic surgery. Our work, summarizing the current literature, presents the role of adipose-derived stromal cells in pedicle skin flap survival in experimental animal models.

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INTRODUCTION

Flaps are used in plastic surgery for wound coverage

when insufficient blood supply impedes the viability of skin grafts. Examples of such applications include large wounds over a flexion crease or wounds with exposed bone, tendon, or other vital structures. Flaps are also preferred in plastic surgery over free grafts because they have a better aesthetic and functional result[1]. A first distinction of cutaneous flaps was established in the 1970s. Skin flaps were classified depending on the blood irrigation into the axial pattern flaps, which have an anatomically recognized arteriovenous system running along their long axis, and random pattern flaps, which lack any significant bias in their vascular patterns[2].

Since then, there has been a rapid development of reconstructive surgery, which has kept pace with the goal of understanding, improving, and developing methods to avoid partial or total flap necrosis, the main complication of the use of skin flaps. Although the cause of skin flap necrosis has not been fully resolved yet, the lack of adequate nutrient blood supply certainly plays a significant role in the pathophysiology of necrosis. To reverse this phenomenon and strengthen vascular reserves, various therapeutic approaches have been pursued. For example, the administration of exogenous agents such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) has been shown to enhance revascularization and improve survival of ischemic flaps[3-5]. However, the beneficial effect of such exogenous factors is reduced due to their short half-life[6] and the limited number of existing endothelial cells. Hence, the abovementioned factors are not enough to control the complex cascade of wound healing.

In recent years the rapid development of cell biology and genetics has helped to highlight the ability of somatic stem cells, especially bone marrow-derived stem cells (BSCs) and adipose-derived stem cells (ADSCs), to promote neovascularization[7-12]. Various studies conducted to compare the forms of stem cells derived from bone marrow, umbilical cord, or adipose tissue showed no significant differences in terms of morphology, immunogenicity, and pluripotent differentiation[13]. The proangiogenic effect of ADSCs und BSCs has been well established, however the two groups seem to have different promoting angiogenesis mechanisms[14]. This fact, combined with the minimally invasive techniques in extraction, isolation, and culture from ADSCs[15-17], places them in the first line of research for various therapeutic purposes in medical science[18].

This review presents the therapeutic benefits of ADSCs in pedicle skin flap survival based on current literature on various experimental models in animals.

EFFECT OF ADSCS ON VIABILITY OF RANDOM PEDICLE SKIN FLAPS

The first time adipose stem cells were used as an antinecrotic treatment in random pedicle flaps was by Lu *et al*[19] in 2008. Intracutaneous injection of (DiI)-labeled (*i.e.*, chemical used for labeling cell membranes and hydrophobic structures) adipose-derived stem cells in ICR mice (*i.e.*, mice originating from a Swiss mice strain from Institute for Cancer Research in Philadelphia) led to a statistically significant increase in survival of the flaps with considerable improvement in capillary density. Furthermore, the immunohistochemical test showed that on some occasions there was *in vivo* differentiation of ADSCs in endothelial cells. Uysal *et al*[20] examined the behavior and properties of adipose-derived stem cells in an ischemia-reperfusion model in ICR mice. They established that ADSCs could prevent ischemia-reperfusion injury, mainly by regulating growth factors, especially VEGF, bFGF, and transforming growth factor-beta (TGF-β). Gao *et al*[21] showed that topical use of ADSCs could improve viability of ischemic random pedicle skin flap in streptozotocin-induced diabetic mice *via* expression of hypoxia-inducible factor-1α. Sheng *et al*[22] implicated the beneficial effect of BSCs *vs* stromal vascular factor (SVF), which contains a group of heterogeneous cells in the adipose tissue, including ADSCs. No statistically significant difference in promoting vascularization and survival of pedicle skin flaps in Wistar rats could be observed.

In 2013, Karathanasis *et al*[23] examined whether genetically modified autologous ADSCs increase graft survival. They conducted an experimental study in which autologous green fluorescent protein (GFP)-producing ADSCs were injected intracutaneously into random-pattern skin flaps in Wistar rats. The results indicated that transplantation of modified GFP-ADSCs improves the survival of the flaps. GFP-ADSCs were detected in the endothelium of blood vessels co-expressing the endothelial marker von Willebrand factor, suggesting that they promoted blood vessel regeneration *in vivo*[23]. The same year, Yue *et al*[24], using a hypoxic preconditioning experimental flap model, showed that preoperative transplantation of ADSCs, combined with hypoxic preconditioning, effectively improves the survival of ischemic skin flaps in Lewis rats by enhancing neovascularization associated with the production and activation of hypoxia-inducible factor 1 alpha (HIF-1α), together with an increase in VEGF. Comparing the effectiveness of different administration routes of ADSCs in improving the viability of random-pattern skin flaps, Lee *et al*[25] indicated that the collagen sponge method delivers ADSCs most effectively within the flap, increasing flap vascularity. Nevertheless, the intravascular administration of ADSCs also positively affects the skin-flap survival, as shown in experiments established by Suartz *et al*[26] in Wistar rats.

Recently, Park *et al*[27] investigated the effects of low-level light therapy (LLLT) on transplanted human adipose-derived mesenchymal stromal cells in the skin flaps of mice. The results indicated that LLLT is an effective biostimulator of ADSCs in vascular regeneration, which enhances the survival of ADSCs and stimulates the secretion of growth factors in skin flaps. Therefore, although the use of ADSCs led to improved viability

Table 1 The most relevant studies on the effect of adipose-derived stem cells on viability of pedicle skin flaps in experimental animal models

Ref.	Year	Contribution
Lu *et al*[19]	2008	Intracutaneous injection of (DiI)-labeled ADSCs improves capillary density
Uysal *et al*[20]	2009	ADSCs prevent ischemia-reperfusion injury by regulating growth factors, especially VEGF, bFGF, TGF-β
Gao *et al*[21]	2011	Human-ADSCs improve viability of ischemic random pedicle skin flap in mice *via* expression of hypoxia-inducible factor-1α
Sheng *et al*[22]	2011	BSCs vs SVF promotes vascularization
Karathanasis *et al*[23]	2013	Transplantation of modified GFP-ADSCs promotes blood vessel regeneration *in vivo*
Yue *et al*[24]	2013	Transplantation of ADSCs, combined with hypoxic preconditioning, enhances neovascularization associated with the production and activation of HIF-1α, together with an increase in VEGF
Lee *et al*[25]	2014	ADSCs delivered *via* sponge method increase flap vascularity
Suartz *et al*[26]	2014	Administration of ADSCs affects positively in skin-flap survival
Derby *et al*[28]	2014	Genetic modified GFP-ADSC improves overlying skin composition and appearance after fat graft transplantation
Park *et al*[27]	2015	LLLT on transplanted human-ADSCs in the skin flaps of mice stimulates the secretion of growth factors in skin flaps
Reichenberger *et al*[29]	2012	Topical application of ADSCs embedded in a fibrin matrix, increases ischemic tissue survival, blood flow and expression of pro-angioactive genes in an animal epigastric skin flap model
Reichenberger *et al*[30]	2012	ADSCs in an extended inferior epigastric artery skin flap enhance blood supply and tissue regeneration
Feng *et al*[31]	2014	Heterologous transplantation of human ADSCs in axial pedicle skin flaps improves viability of axial skin flap in mice
Xu *et al*[32]	2015	Transplantation of ADSCs promotes capillary formation
Tomita *et al*[33]	2013	Utilization of ADSCs in Lewis rats improved the sensory capability of skin flaps *via* the production of neurotrophic factors and nerve growth factors
Uysal *et al*[36]	2010	ADSCs and BSCs increased the vascular density, and the VEGF
Li *et al*[37]	2010	ADSCs increase the vascular density and the survival percentage of the flaps producing high cytokine levels such as VEGF-A

ADSCs: Adipose-derived stem cells; HIF-1α: Hypoxia-inducible factor 1 alpha; SVF: Stromal vascular factor; VEGF-A: Vascular endothelial growth factor A; VEGF: Vascular endothelial growth factor; bFGF: Basic fibroblast growth factor; TGF-β: Transforming growth factor-beta; BSCs: Bone marrow-derived stem cells; GFP: Green fluorescent protein; LLLT: Low-level light therapy.

of skin flaps, their combination with LLLT significantly enhanced their action.

Derby *et al*[28] used the well-documented epithelial stem cell marker p63 to identify *in vivo* transdifferentiation of genetic modified GFP-ADSC in epithelial cells, and therefore show, their contribution to the improvement of overlying skin composition and appearance after fat graft transplantation.

EFFECT OF ADSCS ON VIABILITY OF AXIAL PEDICLE FLAPS

To the best of our knowledge, the first attempt to examine the effect of ADSCs in axial pedicle skin flap survival took place in 2012[29]. Reichenberger *et al*[29] indicated that the topical application of ADSCs embedded in a fibrin matrix increases ischemic tissue survival, blood flow, and expression of pro-angioactive genes in an animal epigastric skin flap model. In the same year it was also shown that the administration of ADSCs in an extended inferior epigastric artery skin flap-which was used as a flap ischemia reperfusion injury (IRI) model-may protect axial skin flaps from IRI by enhancing blood supply and tissue regeneration[30]. The heterologous transplantation of ADSCs in axial pedicle skin flaps was examined by Feng *et al*[31], in which an increase in the viability of human adipose-derived stem cells was observed after local intra-arterial injection in the superficial epigastric arteria of axial skin flaps in mice. A further study was conducted by Xu *et al*[32] in which stem cells were shown to contribute positively to the survival of axial flaps. Xu and his team established a rabbit ear venous-congested skin flap model, where they transplanted ADSCs. After histological and immunofluorescence evaluation, it was indicated that ADSCs not only increase the survival of venous-congested skin flaps but also promote capillary formation.

Tomita *et al*[33] investigated the phenomenon of flap reinnervation through the utilization of ADSCs. They indicated that the use of the aforementioned cells improved the sensory capability of skin flaps in Lewis rats *via* the production of neurotrophic factors and nerve growth factors[33].

EFFECT OF ADSCS ON VIABILITY OF PREFABRICATED PEDICLE FLAPS

The concept of flap prefabrication is relatively new to the field of reconstructive surgery and was first introduced by Yao[34] in the 1980s. In the procedure of flap prefabrication, a vascular pedicle is introduced in a donor area that lacks any axial vascularization, improving the blood supply and enhancing the viability of the surrounding tissues. Although the above flaps can be used for wound coverage in almost any part of the body, their use in head and neck regions has prevailed, especially after extensive burns in which the available reconstructive options are scarce[35].

Despite the undeniable utility of prefabricated flaps in plastic surgery, the risk of total or partial necrosis after flap transplantation remains a problem for further investigation. Among the concepts employed to resolve this potential complication is the application of ADSCs.

There are two studies in the literature in which ADSCs have been used in prefabricated flaps as an anti-necrosis therapy. Uysal *et al*[36] used the femoral artery, vein, and fascia of Wistar rats as a vascular crane for a prefabrication model in which they introduced ADSCs and BSC. Their experiments showed that both of the aforementioned cells increased the vascular density, and the VEGF indicated that mesenchymal stem cells could be useful in any prefabrication procedure in which neovascularization is necessary. Li *et al*[37] applied a prefabricated abdominal island flap model in rats, also using the right femoral artery, in which ADSCs were injected. The post-operative control demonstrated that ADSCs increased the vascular density and the survival percentage of the flaps producing high cytokine levels such as vascular endothelial growth factor A. Table 1 summarizes the most relevant studies on the effect of ADSCs on viability of pedicle skin flaps in experimental animal models.

CONCLUSION

The current literature shows that in all cases where ADSCs were applied to investigate their effect on pedicle skin flap survival, they led to improved viability of the flaps. This was established through the increase of skin flap vascularity *via* the production of growth factors and/or ADSCs' direct transformation into epithelial cells with neoangiogenesis. Although the number of experimental studies on the application of stem cells as an anti-necrosis therapy is limited, an increasing number of researchers have been focusing on this field. This tendency, combined with the already successful clinical application of adipose stem cells in other fields of medical science, might show that their future use in the field of reconstructive surgery - where skin flaps are widely used-is no longer utopian.

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ORIGINAL ARTICLE

Basic Study

Updates in the pathophysiological mechanisms of Parkinson's disease: Emerging role of bone marrow mesenchymal stem cells

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Author contributions: Ahmed HH designed and coordinated the research as well as wrote the paper; Salem AM analyzed the data; Atta HM performed the isolation and preparation steps of bone marrow mesenchymal stem cells from rats; Eskandar EF participated in the designation of the research; Farrag AH performed the immunohistochemical examination and histopathological investigations; Ghazy MA performed the molecular investigations; Salem NA and Aglan HA participated in the induction of Parkinson's disease in rats and treatment as well as performed the biochemical measurements.

Institutional review board statement: The study was reviewed and approved by the National Research Centre Institutional Review Board.

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Data sharing statement: Technical appendix, statistical code, and dataset available from the corresponding author at hanaaomr@yahoo.com.

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Abstract

AIM: To explore the approaches exerted by mesenchymal stem cells (MSCs) to improve Parkinson's disease (PD) pathophysiology.

METHODS: MSCs were harvested from bone marrow

of femoral bones of male rats, grown and propagated in culture. Twenty four ovariectomized animals were classified into 3 groups: Group (1) was control, Groups (2) and (3) were subcutaneously administered with rotenone for 14 d after one month of ovariectomy for induction of PD. Then, Group (2) was left untreated, while Group (3) was treated with single intravenous dose of bone marrow derived MSCs (BM-MSCs). *SRY* gene was assessed by PCR in brain tissue of the female rats. Serum transforming growth factor beta-1 (TGF-β1), monocyte chemoattractant protein-1 (MCP-1) and brain derived neurotrophic factor (BDNF) levels were assayed by ELISA. Brain dopamine DA level was assayed fluorometrically, while brain tyrosine hydroxylase (TH) and nestin gene expression were detected by semi-quantitative real time PCR. Brain survivin expression was determined by immunohistochemical procedure. Histopathological investigation of brain tissues was also done.

RESULTS: BM-MSCs were able to home at the injured brains and elicited significant decrease in serum TGF-β1 (489.7 ± 13.0 *vs* 691.2 ± 8.0, $P < 0.05$) and MCP-1 (89.6 ± 2.0 *vs* 112.1 ± 1.9, $P < 0.05$) levels associated with significant increase in serum BDNF (3663 ± 17.8 *vs* 2905 ± 72.9, $P < 0.05$) and brain DA (874 ± 15.0 *vs* 599 ± 9.8, $P < 0.05$) levels as well as brain TH (1.18 ± 0.004 *vs* 0.54 ± 0.009, $P < 0.05$) and nestin (1.29 ± 0.005 *vs* 0.67 ± 0.006, $P < 0.05$) genes expression levels. In addition to, producing insignificant increase in the number of positive cells for survivin (293.2 ± 15.9 *vs* 271.5 ± 15.9, $P > 0.05$) expression. Finally, the brain sections showed intact histological structure of the striatum as a result of treatment with BM-MSCs.

CONCLUSION: The current study sheds light on the therapeutic potential of BM-MSCs against PD pathophysiology *via* multi-mechanistic actions.

Key words: Parkinson's disease; Pathophysiology; Bone marrow derived mesenchymal stem cells; Rotenone; Anti-inflammatory action; Ovariectomy; Anti-apoptotic effect; Neurogenic potential

Core tip: The current study was planned to clarify the mode of action of mesenchymal stem cells (MSCs) in targeting multiple systems implicated in the pathophysiology of Parkinson's disease (PD) in the rat model. For this purpose, the MSCs were isolated from bone marrow (BM) of rat femur bone and PD was induced in ovariectomized rats by rotenone administration for 14 d. Our results provided clear evidences for the therapeutic role of BM-derived MSCs against PD pathophysiology through their immunomodulatory properties, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials.

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INTRODUCTION

Parkinson's disease (PD) is one of the most common neurodegenerative diseases, associated with extrapyramidal motor dysfunction[1] due to the progressive and specific loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine (DA) in the striatum[2]. It affects approximately seven million people globally[3]. The commonness of PD raises with age, as 1% of people over 60 years of age, 3.4% of those over 70, and 4% of those over 80 were affected by the disease[1]. Epidemiological studies and pathological investigations exhibit a mean period of onset of 70 in sporadic PD, which represents about 95% of patients[4,5]; but familial forms of the disease linked to transformation in a limited number of genes account for 4% and these patients suffer from early-onset disease before the age of 50[6].

Growing body of evidences have demonstrated that environmental factors play a critical role in the etiology of PD[7]. For example, the environmental toxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was identified as one the causative agents of Parkinsonism[8]. Also, herbicides or pesticides usage increase the risk of PD[9,10]. As, the pesticide rotenone and the herbicide paraquat reproduce the PD phenotype in animals[11]. Additionally, it has been suggested that exposure to organic solvents, carbon monoxide and carbon disulfide[12] play roles in the etiology of PD. Epidemiological studies have proposed a potential link between pesticide exposure and increased risk of PD. For example, agrarian laborers, particularly individuals who work with pesticides, are at increased risk for suffering from PD[13].

At present, there is no therapy clinically accessible to postpone neurodegeneration, thusly modulation of the disease course is an imperative unmet clinical need. Along these lines, understanding of the pathophysiology and etiology of the disease at cellular and molecular levels to find new targets against which neuroprotective/disease-modifying therapy may be developed is the pivotal issue in the field of PD research[7].

Mesenchymal stem cells (MSCs) are a heterogeneous subset of stromal stem cells that have the capacity of self-renewal and differentiation into mesodermal lineage cells and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells, epithelial cells, *etc*.[14]. Additionally, these cells have several advantages, such as easy availability as well as few ethical concerns and low immunogenicity. An expanding number of data has demonstrated that MSCs not only depend on their differentiation capacity to repair damaged tissue, but also rely on their ability to modify local environment, activate endogenous progenitor

cells, and secrete several factors[15]. The aforementioned properties make MSCs perfect candidate cell type for tissue engineering, regenerative medicine and autoimmune disease treatment[14].

The focus of our interest was to clarify the mode of action of bone marrow derived MSCs (BM-MSCs) in targeting multiple systems implicated in the pathophysiology of PD in the rat model.

MATERIALS AND METHODS

Preparation of BM-MSCs

BM was harvested by flushing the tibiae and femurs of 6-wk-old male Sprague Dawley rats with Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL, Grand Island, New York, United States, Cat. #42430-082) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, Cat. #16000-044). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin–streptomycin (GIBCO/BRL, Cat. #10378-016). Cells were incubated at 37 ℃ in 5% humidified CO_2 for 12-14 d as primary culture or upon formation of large colonies. When large colonies developed (80%-90% confluence), cultures were washed twice with phosphate buffer saline (PBS; Gibco/BRL, Cat. #10010056) and the cells were trypsinized with 0.025% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) (Gibco/BRL, Cat. #R-001-100) for 5 min at 37 ℃. After centrifugation, cells were resuspended with serum-supplemented medium and incubated in 50 mL falcon tube. The resulting cultures were referred to as first-passage cultures. MSCs in cultures were characterized by their adhesiveness and fusiform shape[16].

Experimental set up

Twenty four adult female Sprague-Dawley rats weighing 130-150 g were obtained from the Animal House Colony of the National Research Centre, Giza, Egypt and acclimated in a specific area where temperature (25 ℃ ± 1 ℃) and humidity (55%). Rats were controlled constantly with a 12 h light/dark cycles at National Research Centre Animal Facility Breeding Colony. Rats were individually housed with *ad libitum* access to standard laboratory diet consisted of casein 10%, salt mixture 4%, vitamin mixture 1%, corn oil 10%, cellulose 5% and completed to 100 g with corn starch and tap water. Rats were cared for according to the guidelines for animal experiments which were approved by the Ethical Committee of Medical Research at National Research Centre, Giza, Egypt.

After the acclimatization period (2 wk), the female rats were ovariectomized surgically in Hormones Department, Medical Research Division at the National Research Centre. Then, after one month from ovariectomy the animals were classified into 3 different groups (8 rats/group). The first group (Ovariectomized control group) was untreated ovariectomized control group. While, the second and third groups were subcutaneously injected with rotenone (Sigma, United States, Cat. #R8875) in a dose of 12 mg/kg b. wt.[17] daily for 14 d for induction of PD. Thereafter, the second group (PD untreated group) was left untreated for 4 mo while, the third group (PD + BM-MSCs group) was infused intravenously with a single dose (3×10^6 cells/rat) of BM-MSCs[18]. For MSCs infusion, the PD induced rats were deeply anaesthetized *via* diethyl ether and MSCs were suspended in 100 μL PBS before transplantation and then slowly injected into the tail vein in 5 min with a 27G needle. The needle was kept in the tail vein for another 5 min to avoid regurgitation and then withdrawn.

At the end of the experimental period (4 mo), all animals were fasted for 12 h and the blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia. The blood samples were left to clot and the sera were separated by cooling centrifugation (4 ℃) at 1800 × *g* for 10 min and then stored immediately at -20 ℃ in clean plastic Eppendorf until analyzed. Moreover, the whole brain of each rat was rapidly and carefully dissected. Then, each brain was sagittally divided into two portions. The first portion was immediately frozen in liquid nitrogen and stored at -80 ℃ prior to extraction for molecular study and DA level determination. While, the second portion was fixed in formalin buffer (10%) for histological investigation and immunohistochemical study.

Detection of male-derived MSCs in the brain of females

The genomic DNA was isolated from the brain tissues of female rats which were treated with BM-MSCs using phenol/chloroform extraction and ethanol precipitation method according to Sambrook *et al*[19] with minor modifications. The presence or absence of the sex determination region on the Y chromosome male (*SRY*) gene in recipient female rats was assessed by PCR. Primer sequences for *SRY* gene (forward 5′-CATCGAAGGGTTAAA-GTGCCA-3′, reverse 5′-ATAGTGTGTAGGTTGTTGTCC-3′, Invitrogen) were obtained from published sequences[20] and amplified to a product of 104 bp. The PCR conditions were as follows: Incubation at 94 ℃ for 4 min; 35 cycles of incubation at 94 ℃ for 50 s, 60 ℃ for 30 s, and 72 ℃ for 1 min; with a final incubation at 72 ℃ for 10 min. PCR products were separated using 2% agarose gel electrophoresis and stained with ethidium bromide.

Biochemical analyses

Serum transforming growth factor beta-1 (TGF-β1) level was assayed by enzyme linked immunosorbent assay (ELISA) using kit purchased from DRG Diagnostics Co., Germany (Cat. #EIA-1864), according to the method described by Kropf *et al*[21]. While, serum monocyte chemoattractant protein-1 (MCP-1) level was determined by ELISA method using kit purchased from Bender MedSystems GmbH, Europe (Cat. #BMS631INST), according to the method described by Baggiolini *et al*[22]. Moreover, serum brain derived neurotrophic factor (BDNF) level was evaluated by ELISA method using kit purchased

from Millipore Corporation, United States (Cat. #CYT306), according to the method described by Laske *et al*[23]. Finally, the quantitative determination of brain DA level was carried out according to the method described by Ciarlone[24] using a fluorometric method.

Detection of tyrosine hydroxylase and nestin genes expression level

Total RNA was isolated from brain tissues of female rats by the standard TRIzol® reagent extraction method (Invitrogen, Cat. #15596-026). Then, the complete Poly(A)$^+$ RNA was reverse transcribed into cDNA in a total volume of 20 μL using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Germany, Cat. #K1631). An amount of total RNA (5 μg) was used with a reaction mixture, termed as master mix. The MM was consisted of 50 mmol/L $MgCl_2$, 5 × reverse transcription (RT) buffer (50 mmol/L KCl; 10 mmol/L Tris-HCl; pH 8.3; 10 mmol/L of each dNTP, 50 μmol/L oligo-deoxyribonucleotide primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25 ℃ for 10 min, followed by 1 h at 42 ℃, and the reaction was stopped by heating for 5 min at 99 ℃. Afterwards the reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for DNA amplification through semi-quantitative real time PCR (sqRT-PCR). An iQ5-BIO-RAD Cycler (Cepheid, United States) was used to determine the rat cDNA copy number. PCR reactions were set up in 25 μL reaction mixtures containing 12.5 μL 1 × SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd., Germany, Cat. #RR820A), 0.5 μL 0.2 μmol/L forward primer, 0.5 μL 0.2 μmol/L reverse primer (Invitrogen), 6.5 μL distilled water, and 5 μL of cDNA template. Primer sequences were F: 5′-ACTGTGGAATTCGGGCTATG-3′, R: 5′-GACCTCAGGCTCCTCTGACA-3′ for tyrosine hydroxylase (TH)[25]; F: 5′-TGGAGCGGGAGTTAG-AGGCT-3′, R: 5′-ACCTCTAAGCGACACTCCCGA-3′ for nestin[26] and F: 5′-CTGTCTGGCGGCACCACCAT-3′, R: 5′-GCAACTAAGTCATAGTCCGC-3′ for β-actin[27]. The reaction program was allocated to 3 steps. First step was at 95.0 ℃ for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 steps: (1) denaturation at 95.0 ℃ for 15 s; (2) annealing at 58.0 ℃ for 30 s, 55.0 ℃ for 5 s and 60 ℃ for 30 s for TH, *nestin* and *β-actin* genes respectively; and (3) extension at 72.0 ℃ for 30 s. The third step consisted of 71 cycles started at 60.0 ℃ and then increased about 0.5 ℃ every 10 s up to 95.0 ℃ for melting curve analysis which was performed at the end of each sqRT-PCR to check the quality of the used primers. Each experiment included a distilled water control.

Immunohistochemical examination of brain survivin expression

Samples were taken from brain of rats of the different groups and fixed in 10% formalin buffer for 24 h. Washing was done in tap water then ascending grade of ethyl alcohol (30%, 50%, 70%, 90% and absolute) was used for dehydration. Specimens were cleared in xylene and embedded in paraffin (melting point 58 ℃-60 ℃) for 24 h. Sections were cut into 4 μ thick by sledge microtome then fixed on positive slides in a 65 ℃ oven for 1 h. Slides were placed in a coplin jar filled with 200 mL of triology working solution (Cell Marque, CA-United States, Cat. #920P-04) which combines the three pretreatment steps: Deparaffinization, rehydration and antigen unmasking. Then, the jar is securely positioned in the autoclave which was adjusted so that temperature reached 120 ℃ and maintained stable for 15 min after which pressure is released. Thereafter, the coplin jar is removed to allow slides to cool for 30 min. Sections were then washed and immersed in Tris-buffer saline to adjust the pH and these were repeated between each step of the immunohistochemical procedure. Quenching endogenous peroxidase activity was performed by immersing slides in 3% hydrogen peroxide for 10 min. Broad spectrum LAB-SA detection system (Invitrogen, Cat. #85-8943) was used to visualize any antigen-antibody reaction in the tissue. Background staining was blocked by putting 3 drops of 10% goat non immune serum blocker on each slide and incubating them in a humidity chamber for 10 min. Without washing, excess serum was drained and the working solution (1:100) of survivin mouse monoclonal (Thermo Scientific, United States, Cat. #RB-9245-P1) was prepared. Three drops of the working solution were applied and slides were incubated in the humidity chamber overnight at 4 ℃. Henceforward, biotinylated secondary antibody from ultravision detection system anti-polyvalent HRP/3,3′-diaminobenzidine (DAB) (Thermo Scientific, Cat. #TP-015-HD) was applied on each slide for 20 min followed by 20 min incubation with the streptavidin HRP enzyme conjugate (Thermo Scientific, Cat. #TP-015-HD). Then, DAB chromogen (Thermo Scientific, Cat. #TP-015-HD) was prepared and 3 drops were applied on each slide for 2 min. DAB was rinsed, after which counterstaining with Mayer hematoxylin and cover slipping were performed as the final steps before slides were examined under the light microscope (Olympus Cx21 with attached digital camera)[28]. Image analysis was performed using the image J, 1.41a NIH, United States analyzer.

Histopathological investigation of brain tissue of rats

Samples were taken from brain of rats in different groups and fixed in 10% formalin buffer for 24 h. Washing was done in tap water then ascending grade of ethyl alcohol (30%, 50%, 70%, 90% and absolute) was used for dehydration. Specimens were cleared in xylene and embedded in paraffin (melting point 58 ℃-60 ℃) for 24 h. Paraffin wax tissue blocks were prepared for sectioning at 4 μ by sledge microtome. The obtained tissue sections were collected on glass slides, deparffinized and stained by hematoxylin and eosin (H and E) stain[29] for histopathological examination through the electric light microscope.

Statistical analysis

In the present study, all results were expressed as mean ± SE of the mean. Data were analyzed by one

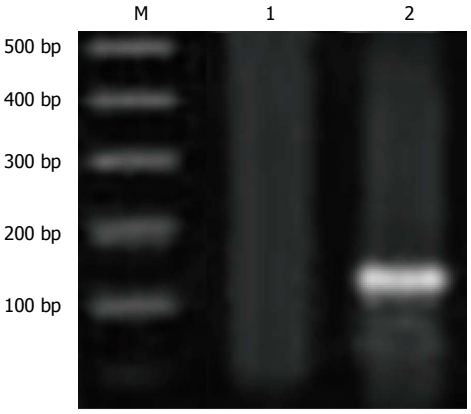

Figure 1 An agarose gel electrophoresis of DNA fragments showed *SRY* gene in recipient female rats for bone marrow derived mesenchymal stem cells in Parkinson's disease model. Lane (M) represents DNA ladder; Lane (1) represents ovariectomized control sample; Lane (2) represents sample from PD group treated with BM-MSCs. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences (SPSS) program, version 14 followed by least significant difference (LSD) to compare significance between groups. Difference was considered significant when *P* value was < 0.05.

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort to the animals. The animals were acclimatized to laboratory conditions (25 ℃, 12 h/12 h light/dark, 55% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. The animals were deeply anaesthetized *via* diethyl ether for intravenous infusion of MSCs. Also, blood samples were collected from retro-orbital venous plexus under diethyl ether anaesthesia.

RESULTS

BM-MSCs homing

To confirm that the intravenously transplanted MSCs derived from male bone marrow migrate and home to the female injured brain, DNA was isolated from the brain tissues of female rats and the presence or absence of the responsible region for sex determination on Y chromosome (*SRY* gene) was assessed by PCR. The agarose gel demonstrated that *SRY* gene was present in the brain tissues obtained from the group of rats treated with BM-MSCs. While, *SRY* gene was absent in the brain tissues obtained from the ovariectomized control rats (Figure 1).

Effect of treatment with BM-MSCs on inflammatory markers

Since, TGF-β1 has a pivotal role in the control of the transition between pro-inflammatory and anti-inflammatory response[30] and MCP-1 has a vital role in the migration of inflammatory cells across the blood-brain barrier as well as forms chemotactic gradients within the CNS to control the local inflammatory response[31]. Serum TGF-β1 and MCP-1 levels were determined by ELISA to evaluate the anti-inflammatory and immunomodulatory effects of the injected BM-MSCs in PD model.

Table 1 Effect of treatment with bone marrow derived mesenchymal stem cells on serum transforming growth factor beta-1 and monocyte chemoattractant protein-1 levels in Parkinson's disease model

	TGF-β1 (pg/mL)	MCP-1 (pg/mL)
Ovariectomized control	481.5 ± 7.5	88.1 ± 0.9
PD untreated	691.2 ± 8.0[a]	112.1 ± 1.9[a]
PD + BM-MSCs	489.7 ± 13.0[c]	89.6 ± 2.0[c]

Data are represented as mean ± SE of 8 rats/group. [a]Significant change at $P < 0.05$ in comparison with the ovariectomized control group; [c]Significant change at $P < 0.05$ in comparison with the untreated PD group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells; TGF-β1: Transforming growth factor beta-1; MCP-1: Monocyte chemoattractant protein-1.

Our data revealed that rotenone administration causes significant ($P < 0.05$) elevation in serum TGF-β1 (43.6%) and MCP-1 (27.2%) levels *vs* the ovariectomized control group (Table 1). While, treatment with BM-MSCs elicits a significant ($P < 0.05$) reduction in both serum TGF-β1 and MCP-1 levels by 29.2% and 20.1% respectively relative to the group of rats left untreated.

Effect of treatment with BM-MSCs on neurotrophic and neurogenic markers

Brain derived neurotrophic factor plays an important role in supporting the survival of existing neurons and encouraging the growth as well as differentiation of new neurons and synapses[32]. Thusly, serum BDNF level was estimated by ELISA to evaluate the neurotrophic capacity of the injected BM-MSCs in PD model. In view of the data of the current work, rotenone administration experiences significant ($P < 0.05$) decline in serum BDNF level by 21.5% (Table 2) as compared to the ovariectomized control group. In contrast, treatment with BM-MSCs elevates serum BDNF level significantly ($P < 0.05$) by 26.1% (Table 2) relative to the group of rats left untreated.

Brain DA level was determined by a fluorometric method, while brain TH and nestin genes expression level was detected by sqRT-PCR to evaluate the neurogenic potential of the injected BM-MSCs in PD model. It is well known that DA is a neurotransmitter released by nerve cells to play crucial role in motor control, motivation, arousal, cognition and reward[33]. Furthermore, TH enzyme catalyzes the conversion of L-tyrosine to L-3,4-dihydroxy-phenylalanine[34]. While, nestin is one of the markers of neural precursors[35]. The data of our work revealed that rotenone administration leads to significant ($P < 0.05$) depletion of brain DA level (32.1%) and significant ($P < 0.05$) down-regulation in the expression level of brain TH and nestin genes by 54.6% and 48.5% respectively (Table 2) as compared to the ovariectomized control group.

Table 2 Effect of treatment with bone marrow derived mesenchymal stem cells on serum brain derived neurotrophic factor and brain dopamine levels as well as brain tyrosine hydroxylase and nestin genes expression level in Parkinson's disease model

	BDNF (pg/mL)	DA (μg/g tissue)	Relative expression of TH gene (TH/β-actin)	Relative expression of nestin gene (nestin/β-actin)
Ovariectomized control	3700 ± 26.4	882 ± 20.3	1.19 ± 0.004	1.30 ± 0.004
PD untreated	2905 ± 72.9[a]	599 ± 9.8[a]	0.54 ± 0.009[a]	0.67 ± 0.006[a]
PD + BM-MSCs	3663 ± 17.8[c]	874 ± 15.0[c]	1.18 ± 0.004[c]	1.29 ± 0.005[c]

Data are represented as mean ± SE of 8 rats/group. [a]Significant change at $P < 0.05$ in comparison with the ovariectomized control group; [c]Significant change at $P < 0.05$ in comparison with the untreated PD group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells; BDNF: Brain derived neurotrophic factor; DA: Dopamine; TH: Tyrosine hydroxylase.

Table 3 Effect of treatment with bone marrow derived mesenchymal stem cells on brain survivin expression in Parkinson's disease model

	Survivin (cell number)
Ovariectomized control	288 ± 16.5
PD untreated	271.5 ± 13.9
PD + BM-MSCs	293.2 ± 15.9

Data are represented as mean ± SE of 8 rats/group. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

However, treatment with BM-MSCs produces significant ($P < 0.05$) elevation in brain DA level by 45.9% and significant ($P < 0.05$) up-regulation in brain TH and nestin genes expression level by 122.2% and 92.5% respectively (Table 2) *vs* the group of rats left untreated.

Effect of treatment with BM-MSCs on anti-apoptotic marker

The anti-apoptotic action of the single intravenous dose of BM-MSCs in PD model was evaluated through the detection of brain survivin expression using immuno-histochemical technique. As, survivin belongs to a family of endogenous cellular inhibitors of caspases that directly repress apoptotic cell death through interactions with pro-apoptotic caspases[36]. In view of the current data, rotenone administration causes insignificant ($P > 0.05$) decrease in the number of positive cells for survivin expression by 5.7% (Table 3 and Figure 2B) relative to the ovariectomized control group. While, treatment with BM-MSCs produces insignificant ($P > 0.05$) increase in the number of positive cells for survivin expression by 8.0% (Table 3 and Figure 2C) in comparison with the group of rats left untreated.

Effect of treatment with BM-MSCs on brain structure

The brain section photomicrograph of ovariectomized control rat shows congestion in the blood vessels in striatum area (Figure 3A). While, brain section photomicrographs of untreated rotenone administered rat show congestion in the blood vessels and capillaries (Figure 3B) in the striatum as well as hyalinization and plaques formation in the matrix of the striatum indicating the occurrence of neurodegeneration (Figure 3C). Finally, the brain section photomicrograph of rotenone administered rat treated with BM-MSCs shows intact histological structure of the striatum (Figure 3D).

DISCUSSION

MSCs have been considered as an effective tool for regenerative cell therapy. These cells could be isolated from both healthy and patient tissues and expanded *in vitro* on a therapeutic scale without posing significant ethical or procedural problems[37]. Furthermore, it has been proposed that stem cells may replace lost cells by differentiating into functional neural tissue; provide source of trophic support for the diseased nervous system or alter the immune system to prevent further neurodegeneration[38]. Therefore, the current study was planned to elucidate the mechanisms by which BM-MSCs could attenuate PD pathophysiology in the experimental model.

In consistent with Yoon *et al*[39] who found that intravenously transplanted BM-MSCs could migrate and home into the brain, the data presented in this work demonstrated that the intravenously transplanted MSCs were able to migrate to the site of injury (brain). The homing property afforded by MSCs was likely attributable to their broader expression of homing molecules[40]. Furthermore, it has been reported that, chemokines released from tissue or endothelial cells may contribute to the activation of adhesion ligands, transendothelial migration, chemotaxis, and/or subsequent retention in surrounding tissue[41].

In view of the data of the current work, rotenone administration for 14 d in ovariectomized rats elevated the level of serum TGF-β1 and MCP-1 significantly. This finding is greatly supported by those of Rota *et al*[42] and Reale *et al*[43] who stated that both TGF-β1 and MCP-1 levels are increased in several chronic neurodegenerative pathologies such as PD. It has been reported that the inflammatory response due to Parkinsonism is characterized by activation of microglia in the brain. The proposed explanation in regards to the reason of degeneration in dopaminergic neurons is that PD is caused by activation of microglial cells as a result of increased levels of cytokines[44]. Activated microglia release a wide array of pro-inflammatory and cytotoxic factors as well as eicosanoids and nitric oxide[45], which work in concert to develop neurodegeneration[46]. Moreover, Gao *et al*[47] reported that the dopaminergic neurodegeneration enhanced by rotenone might be attributed primarily to

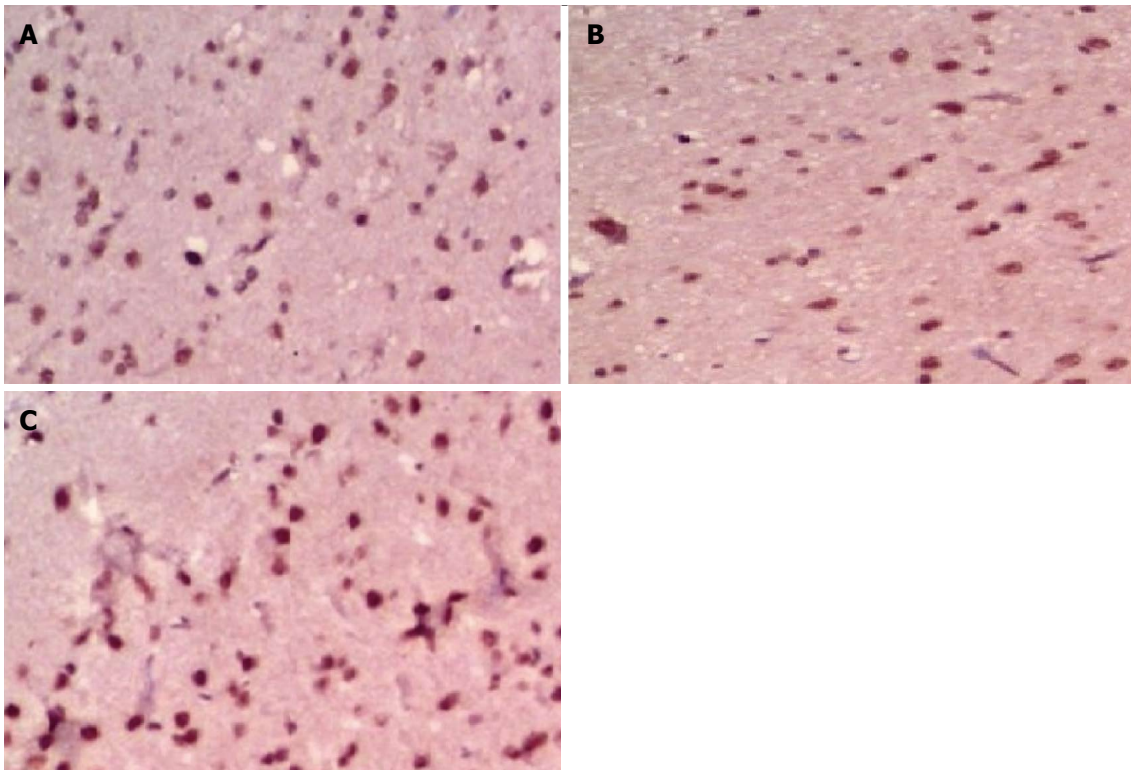

Figure 2 Immunohistochemical examination of survivin expression in Parkinson's disease model groups. A: Ovariectomized control; B: PD untreated; C: PD + BM-MSCs. PD: Parkinson's disease; BM-MSCs: Bone marrow derived mesenchymal stem cells.

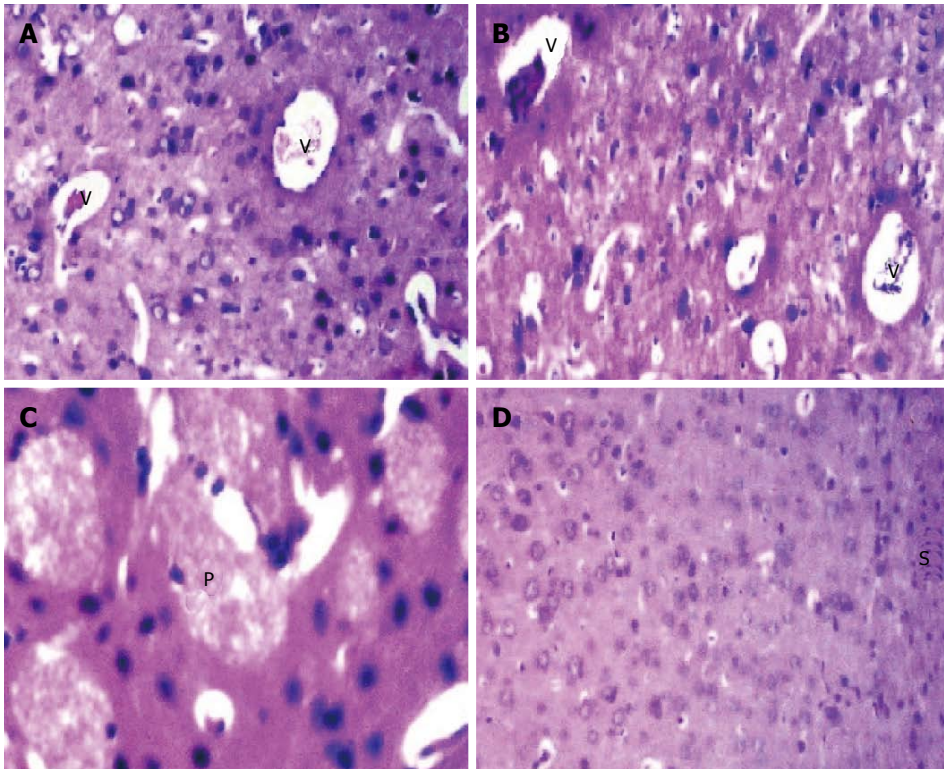

Figure 3 Photomicrograph of brain section of: A: Ovariectomized control group shows congestion in blood vessels of striatum (v) (H and E × 80); B: untreated Parkinson's disease (PD) group shows congestion in blood vessels and capillaries of striatum (v) (H and E × 80); C: Untreated PD: Parkinson's disease group shows hyalinization with plaques formation in the matrix of striatum (H and E × 160); and D: PD group treated with bone marrow derived mesenchymal stem cells shows intact histological structure of the striatum (H and E × 80).

the activation of microglia and consequently their release of superoxide free radicals that play an important role in

the inflammation mediated oxidative damage to neurons. This effect might be ascribed to the known susceptibility of dopaminergic neurons to oxidative stress as a result of reduced antioxidant capacity, high content of iron and DA, and possible defect in mitochondrial function[48]. The release of cytokines from the brain into the peripheral blood supply through the blood brain barrier[49] could explain the observed increase in serum TGF-β1 and MCP-1 levels.

The results of the current study manifested that treatment with BM-MSCs lessen the level of serum TGF-β1 and MCP-1 significantly. This finding is in great accordance with our previous work on adipose tissue derived MSC[50] that proved its anti-inflammatory and immunomodulatory activities which are implicated in mitigating neuroinflammation characterizing PD. Accordingly, the observed role of BM-MSCs in depleting serum TGF-β1 and MCP-1 levels could be allied to the ability of BM-MSCs to modulate microglia/macrophage activation including inflammatory responses as documented by Németh *et al*[51] and Choi *et al*[52].

Growing body of evidence indicates that there is a link between pro-inflammatory cytokines and neurotrophic factors in the CNS[53]. It has been postulated that there is a balance between cytokine and neurotrophin in the brain and disruption of this balance cause injurious changes in the CNS[54]. Moreover, Borchelt[55] observed that astrocytes stimulated by mediators released from microglia down-regulate neurotrophic factors expression and release additional inflammatory mediators that in turn activate microglia. Parallel to these evidences, our results indicated that rotenone administration elicited significant decrease in serum BDNF level. This finding could be allied to the diminished level of brain BDNF due to inflammation. As, Klein *et al*[56] reported that BDNF level in the blood correlates with alteration in the level of BDNF in the brain.

In view of the current data, treatment with BM-MSCs experienced significant increase in serum BDNF level. This preferable effect could be related to the ability of MSCs to secrete BDNF as observed by Lattanzi *et al*[57] and Han *et al*[58]. Blandini *et al*[59] documented that MSCs have the ability to differentiate into glial cells that release diverse neurotrophic factors to provide protection against neurotoxin after their grafting into Parkinsonian rat brains. Additionally, there is an evidence that MSCs may modulate the expression of neurotrophic factors according to the environment in which they exist[60,61].

The data presented in this work revealed that rotenone administration led to significant down-regulation in brain *TH* gene expression level in concomitant with significant decline in brain DA level. This observation could be ascribed to the dopaminergic degeneration[62] due to elevated sensitivity of dopaminergic neurons to oxidative damage[47] as well as inhibition of complex Ⅰ activity and decrement of the mitochondrial membrane potential as a result of rotenone administration[47,63].

Our previous findings indicated the neurotrophic and neuroprotective potentials of adipose tissue derived MSC against neurodegenerative insult of PD[50]. Similarly, the data of the present work demonstrated that treatment with BM-MSCs elicited significant increase in brain DA level as well as brain TH gene expression level. This finding comes in line with the study of Shetty *et al*[64] who demonstrated that BM-MSCs can be transdifferentiated efficiently into functional dopaminergic neurons capable of secreting DA and alleviating behavioral deficiencies. Moreover, the results of Bouchez *et al*[25] study showed that grafting of BM-MSCs caused an increase in the immunostaining of TH in striatum associated with elevation in the number of TH^+ neurons in the substantia nigra pars compacta. Also, Blondheim *et al*[65] and Offen *et al*[66] stated that the transplantation of BM-MSCs into the animal model induced with 6-hydroxydopamine resulting in an increase in the level of TH in the striatal region thus improving motor behavior in a mouse model of PD. Since, TH is the rate-limiting enzyme in DA synthesis, the increase in the level of TH would increase the production of DA. Additionally, the observed increase in brain DA content and TH expression level as a result of treatment with BM-MSCs could be explained by the ability of MSCs to secrete a wide array of cytokines and growth factors, including BDNF[57] which exert neurotrophic and neuroprotective effects on DA neurons[67]. Furthermore, Trzaska *et al*[68] reported that BDNF has a crucial role in the functional maturation of MSC-derived DA progenitors.

In line with previous studies reported by Höglinger *et al*[69] and Abdipranoto *et al*[70], the current study manifested that rotenone administration caused significant down-regulation in brain nestin gene expression level. This finding could be imputed to the depletion in DA level due to degeneration of dopaminergic neurons as documented by Crews *et al*[71]. In contrast, treatment with BM-MSCs induced significant up-regulation in nestin gene expression level. Bouchez *et al*[25] found that rat MSCs express neuronal proteins such as nestin at the RNA and protein levels. Moreover, the study of Ye *et al*[72] indicated the presence of nestin positive cells in brain tissue of PD rat after transplantation of undifferentiated BM-MSCs. The suggested mechanism by which BM-MSCs treat PD rat model could be related to that transplanted BM-MSCs might become nestin-positive stem cells that differentiate into astrocytes or other non-dopaminergic neurons and participate in the reconstruction of dopaminergic neurons circuits[72].

The data of this work revealed that rotenone administration produced slight decrease in the number of positive cells for survivin expression. This finding harmonizes with that of Zhang *et al*[73] who reported that degenerating neurons lacked survivin expression. Jiang *et al*[74] results showed that survivin is critically required for the survival of developing CNS neurons. Moreover, Zhang *et al*[75] suggested that there is a connection between the expression of survivin and adult neurogenesis. Thus, the observed decrement in survivin expression might be attributed to the decreased neurogenesis due to DA depletion[71]. Another possible mechanism by which rotenone could decrease survivin expression might be related to its effect on p53

which was shown to be over expressed by rotenone[76]. Under normal conditions, p53 protein levels are low and regulated by IκB kinase (IKK) and prominently by mouse double minute 2 (Mdm2), an ubiquitin ligase responsible for p53 degradation. Cellular stress reduces the interaction between p53 and Mdm2 leading to accumulation of the former[77]. Wu *et al*[76] reported that the degeneration of dopaminergic neurons by rotenone was accompanied by an increase in p53 protein level which in turn induces p21 expression. Then, the increased level of p21 suppresses the expression of cycline dependent kinases leading to accumulation of hypophosphorylated retinoblastoma that interact with E2F (a transcriptional activator) to repress survivin expression[78].

In the light of our results, treatment with BM-MSCs caused insignificant increase in the number of positive cells for survivin expression. This increment is in agreement with Okazaki *et al*[79] and it could be imputed to the ability of MSCs to enhance neurogenesis and inhibit apoptosis through their secreted BDNF as documented by Ye *et al*[72]. Moreover, Kim *et al*[80] reported that grafted MSCs attenuate dopaminergic neuronal loss through their anti-apoptotic effects. Also, the increase in survivin expression by MSCs treatment might be related to their inhibitory action on P53 through the inactivation of ERK1/2[81].

In view of the histopathological investigations of brain tissues section of the current work, rotenone administration resulted in congestion in the blood vessels and capillaries of striatum. Also, there were hyalinization and plaques formation in the matrix of striatum indicating the occurrence of neurodegeneration. Sai *et al*[82] demonstrated that rotenone causes dopaminergic neurons degeneration *in vivo* and substantia nigra pars compacta and striatum are the main targets of rotenone in the rat brain. These findings could be allied to the inhibition of neuronal mitochondrial complex I activity[47] and consequently oxidative damage[83] as a result of rotenone administration.

Brain tissue sections examination indicated that single infusion with BM-MSCs resulted in intact histological structure of the striatum. This finding coincides with Dezawa *et al*[84] who reported that nerve system recovery after BM-MSCs transplantation could be related to their secretion of neurotrophic factors that restore the function of nervous system, promotion of local angiogenesis and vascular reconstruction and neuronal regeneration through promotion of autologous neuronal regeneration and differentiation of transplanted cells into neural cells.

In conclusion, the current study provided experimental evidences for the ability of BM-MSCs to mitigate PD pathophysiology through multi-mechanistic approaches (immunomodulatory, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials). These promising results pave the way for the clinical trial application of MSCs in the treatment of neurodegenerative diseases particularly PD.

COMMENTS

Background

Parkinson's disease (PD) is one of the neurodegenerative diseases, accompanied by extrapyramidal motor dysfunction due to the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine in the striatum. So, it is very important to stop or halt neurodegeneration. However, to date, there is no therapy clinically available that delays the neurodegenerative process itself, therefore modification of the disease course is an important unmet clinical need. Transplantation of mesenchymal stem cells (MSCs) for treating neurodegenerative disorders has received growing attention recently because these cells are readily available, easily expanded in culture, and when transplanted survive for relatively long periods of time.

Research frontiers

MSCs are a heterogeneous subset of stromal stem cells that have the ability of self-renewal and multipotency. In the area of neurodegenerative disorders treatment, the current research hotspot is how to modify the disease course by specifically target the pathophysiologic cascade, hoping to delay the onset of the disease and slow its progression.

Innovations and breakthroughs

Modern research has focused on discovering effective disease-modifying therapies, which specifically target the pathophysiologic cascade, hoping to delay the onset of the disease and slow its progression. The study provided a non invasive approach for mitigating PD pathophysiology *via* bone marrow derived MSCs (BM-MSCs) transplantation which has immunomodulatory, anti-inflammatory and anti-apoptotic effects as well as neurotrophic and neurogenic potentials.

Applications

The study results shed light on the therapeutic potential of BM-MSCs against PD pathophysiology *via* multi-mechanistic actions.

Terminology

PD is the second most common neurodegenerative disease, accompanied by extrapyramidal motor dysfunction which resulting from the progressive and selective loss of dopaminergic neurons in the substantia nigra pars compacta and declining levels of dopamine in the striatum. MSCs are a heterogeneous subset of stromal stem cells that have the ability of self-renewal and multipotency, which could differentiate into cells of the mesodermal lineages and other embryonic lineages, including adipocytes, osteocytes, chondrocytes, hepatocytes, neurons, muscle cells, epithelial cells, *etc*.

Peer-review

This article is well written, clearly demonstrating the therapeutic effect of BM-MSCs for the treatment of PD. Authors also presented the molecular basis for the amelioration of PD pathology by showing decrements and increments in inflammatory mediators and neurotrophic factors in the serum, respectively. The overall data presented in this manuscript are sound.

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