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Embryonic Stem Cell-Derived Multipotent Mesenchymal Stromal Cell Therapy Following Focal Ischemia in the Rat

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1. Introduction

Stroke is a major public health concern, with ~795,000 strokes occurring in the United States each year, resulting in over 130,000 deaths annually, and 4.8 million stroke survivors (CDC Risk Survey 2008). The estimated direct and indirect cost of stroke for 2010 is $73.7 billion (CDC Risk Survey 2008). Clinical treatment options for ischemic stroke (which accounts for ~87% of stroke cases) are limited to chemical or mechanical clot-busting interventions during a short time-window following stroke. Therefore, the development of post-ischemic therapies to reduce the mortality and disability associated with stroke would have clear public health benefits.

Multipotent mesenchymal stromal cell (MSC; also sometimes called mesenchymal stem cell) transplantation has shown protection against stroke in animal models, reducing infarct volumes and improving behavioral function (Chen et al. 2001; Li et al. 2001); transplanted MSCs, however, often do not show show long-term survival and integration into the brain. This has led many investigators to believe that paracrine mechanisms underlie the benefits of MSC transplantation. Indeed, neural stem cell derivatives, such as neuroepithelial cells, may be better suited than MSCs to promote neuronal replacement due to their neuroectodermal developmental origin (Kelly et al. 2004; Jiang et al. 2006; Darsalia et al. 2007; Fong et al. 2007; Daadi et al. 2008). Transplanted cells face a hostile, inflammatory environment in the near term post-ischemic cerebrum, which may contribute to the limited survival of the engrafted stem cells. MSC transplantation induces gene expression changes that suggest an altered inflammatory response following ischemia (Ohtaki et al. 2008). In addition to MSC promotion of endogenous repair and replacement processes, this inflammatory modulation (Aggarwal & Pittenger 2005) may provide for an ameliorated pro-survival and/or pro-differentiation milieu for subsequent transplantation of other stem cell types, such as neuroepithelial cells. Addressing the issues of potential allograft rejection and limited long-term cell survival/replacement will be important to the clinical application of stem cell therapies, regardless of which type of stem cell is used. The immunosuppressive attributes of MSCs put them under consideration for use in co-transplantation approaches to mitigate graft rejection concerns.

2. Differentiation of human ES cells into multipotent mesenchymal stromal cells

2.1 Origin and function of mesenchymal stromal cells

Mammalian bone marrow contains multiple cell types including a population of multipotent hematopoietic stem cells, which gives rise to erythroid, lymphoid and myeloid lineages of blood cells. Bone marrow stromal cells provide support to the hematopoietic components of the marrow through the production of extracellular matrix (ECM) components, cytokines and trophic factors (Yin & Li 2006; Méndez-Ferrer et al. 2010). They may also play a role in maintaining immunological memory by providing a survival niche in the marrow for plasma cells and memory T lymphocytes (Tokoyoda et al. 2010). Some of these marrow stromal cells are capable of giving rise to mesenchymal tissues such as bone, cartilage, connective tissue, muscle and fat, thus giving rise to the term "mesenchymal stem cell" (Caplan 1991). Such cells are not unique to the bone marrow, as cells with the characteristics of mesenchymal stem cells have been isolated from a variety of human and animal tissues, including umbilical cord blood (Bieback et al. 2004), adipose tissue (Zuk et al. 2001), skeletal muscle (Williams et al. 1999), and dental pulp (Gronthos et al. 2000). Indeed, MSCs may reside within the connective tissue of the majority of organs (Young et al. 1995).

Traditionally, MSCs have been obtained through gradient centrifugation of bone marrow aspirates, and subsequently plating the heterogeneous mononuclear cells on plastic dishes. Hematopoietic cells do not attach to the plastic substrate so that after approximately two weeks in culture, the adherent cells should be primarily MSCs. Flow cytometry is then used to either characterize or sort, depending upon the uniformity of the cells, the CD73+, CD34– population. Alternate cell surface markers are sometimes used in addition to or in place of CD73. The CD34 antigen is a marker of hematopoietic cells, so MSC preparations should be free of CD34+ cells. Differentiation in culture to osteoblasts, chrondrocytes and adipocytes is commonly assayed using staining with Alizaren Red, Alcian blue and Oil Red O, respectively (Pittenger et al. 1999).

Although "bone marrow stromal cell" and "mesenchymal stem cell" have been used somewhat interchangeably in the literature, neither name adequately reflects both the marrow stromal function and the multipotent differentiation capacity of the cells. Additionally, there has been question as to whether these cells fulfill the criteria for "stemness" at the individual cell level (Horwitz et al. 2005). The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) promotes the use of "multipotent mesenchymal stromal cell" as a replacement moniker that better encompasses this range of function, yet still allows for the use of the widespread MSC acronym (Horwitz et al. 2005; Dominici et al. 2006). The diverse nature of MSC sources has also led to concerns that heterogeneity among different cell preparations may complicate comparisons of their functions in experimental systems. The ISCT has therefore further proposed (Dominici et al. 2006) minimal criteria to define human MSCs as the following:

a. Cells must be plastic-adherent under standard culture conditions.
b. Greater that 95% of the cell population must express CD73, CD90, and CD105 but lack expression (<2% positive) of CD34, CD45, CD11B or CD14, CD19 or CD79a, and should be unstimulated (lack HLA class II antigen expression).
c. Cells should be capable of in vitro differentiation into adipocytes, chondrocytes, and osteoblasts.

2.2 Overview of hES-MSC differentiation and comparison to BM-MSC

Using bone marrow as a source of MSCs has the advantage of a clinically relevant tissue source due to the history of bone marrow transplantation as a treatment for variety of blood disorders and cancers. Nevertheless, the expansion capacity of such bone marrow-derived MSCs (BM-MSCs), while significant, is not limitless (Fehrer & Lepperdinger 2005) and obtaining consistent homogeneous cultures among multiple individual donors can be challenging. In vitro differentiation of embryonic stem cells represents an alternate source of mesenchymal stem-like cells. Embryonic stem (ES) cells may be maintained in culture indefinitely (Amit et al. 2000) and should allow for the production of homogenous MSC preparations that vary little from batch to batch and in greater numbers than can be easily achieved with BM-MSCs.

One common method of differentiating ES cells begins with the transfer of undifferentiated colonies of ES cells into culture medium lacking basic fibroblast growth factor (bFGF). After approximately 4 days under these conditions, ES cells form a cluster of cells known as an embryoid body, in which the endodermal, mesodermal and ectodermal tissue layers are present (Itskovitz-Eldor et al. 2000). The embryoid body is dissociated enzymatically and the cells plated onto plastic for several days, after which the CD73+ population is selected by flow cytometry for further growth. The putative MSCs are further analyzed for appropriate marker expression and their differentiation capacity tested in vitro, as indicated above. Alternatively, the embryoid body stage may be skipped; undifferentiated ES cells may be plated directly onto plastic and the adherent cells sorted for CD73+ expression prior to further growth and characterization (Trivedi & Hematti 2008).

Human ES cell-derived MSCs (hES-MSCs) show very similar cell surface marker expression patterns (see Figure 1) and trilineage in vitro differentiation abilities compared to BM-MSCs (Trivedi & Hematti 2008; Liu et al. 2009; Seda Tigli et al. 2009). As will be discussed below, bone marrow-derived MSCs have been considered immunoprivileged and immunosuppressive, and can avoid or reduce an alloreactive immune response after transplant (Bartholomew et al. 2002; Di Nicola et al. 2002; Tse et al. 2003; Aggarwal & Pittenger 2005). Similarly to BM-MSCs, hES-MSCs do not express class II major histocompatibility complex antigens at their surface. MSCs of either origin do not induce proliferation of T lymphocytes in vitro and indeed, both types of MSCs can suppress activation in mixed lymphocyte reaction assays (Trivedi & Hematti 2008). Therefore, MSCs from these sources are quite comparable, phenotypically and immunologically.

3. Mesenchymal stromal cell therapy in preclinical stroke models

3.1 Rat middle cerebral artery occlusion model of stroke

Multiple animal species, including primates, cats, dogs and rodents (such as mice, gerbils and rats) have historically be used in stroke research. The rat has become a widely used animal model for stroke, however, due to its relatively low animal husbandry costs and the similarity of its cranial circulation to that of humans (Yamori et al. 1976; Lee 1995). In humans, the middle cerebral artery (MCA) is most commonly affected in stroke syndromes (Bogousslavsky et al. 1988) and multiple methods of MCA occlusion (MCAO) have been described to mimic this clinical syndrome in animal models (Macrae 1992). Because recanalization commonly occurs following an acute stroke in the human (Saito et al. 1987), reperfusion after a period of occlusion has been included in many of these models.

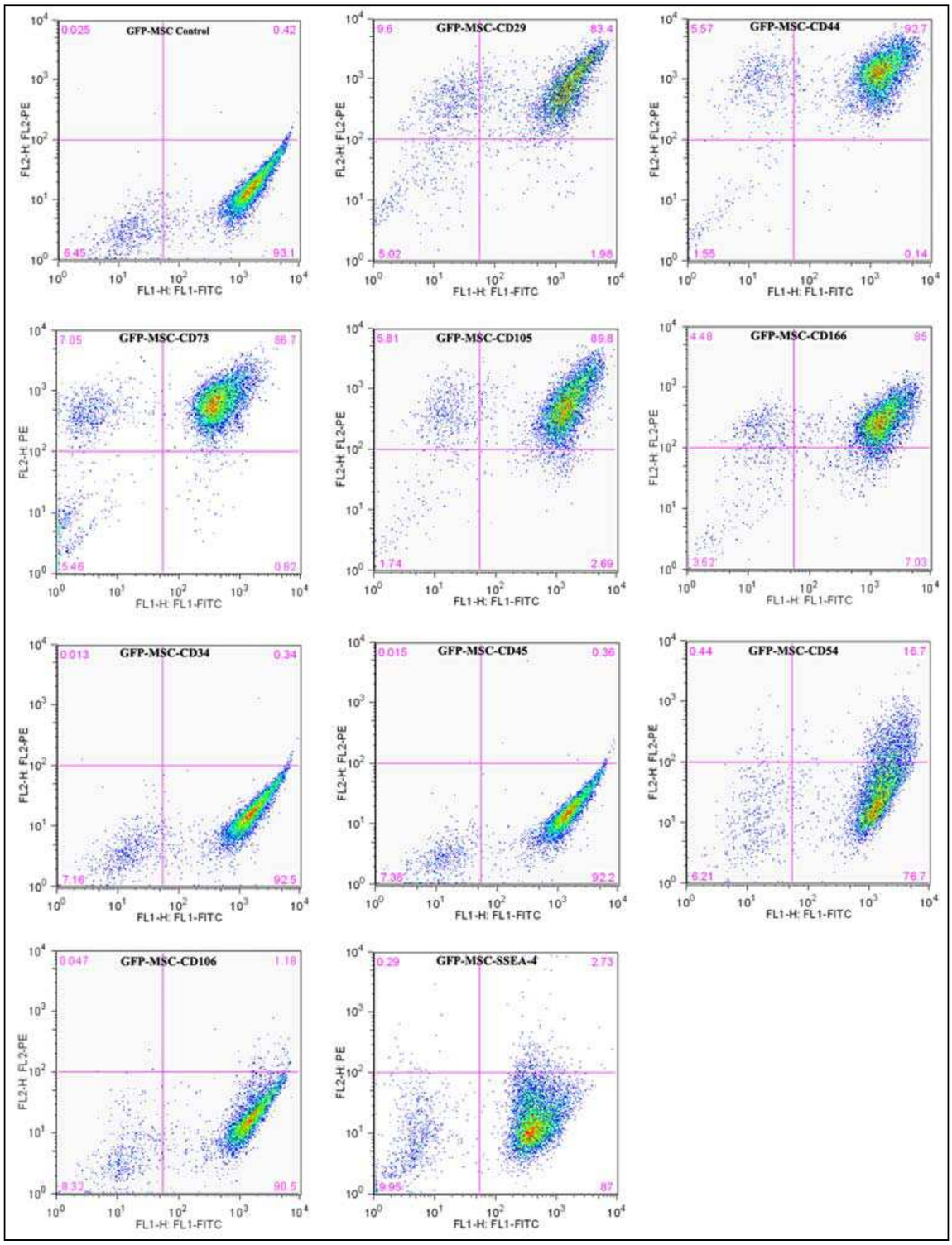

Fig. 1. Surface marker analysis of hES-MSCs expressing green fluorescent protein (GFP) using antibodies to the indicated antigens. GFP-MSC control cells represent unstained cells. The proportion of cells in each quadrant is indicated. Data taken from Liu et al. 2009.

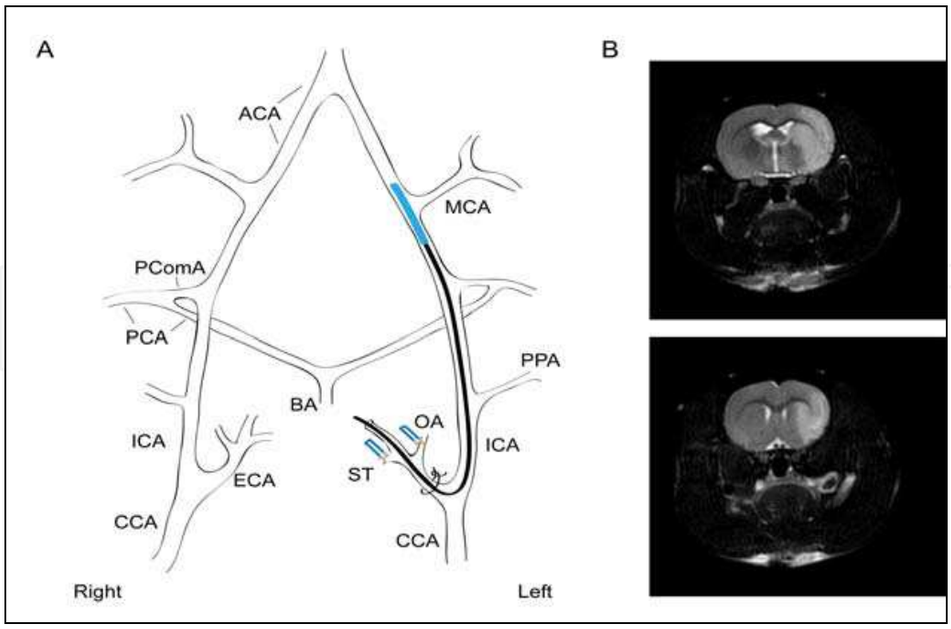

Fig. 2. Stroke induction in the rat. (A) Occlusion of the middle cerebral artery by endovascular silicon-coated suture in the rat. The OA and ST branches off of the left ECA are shown ligated and a suture tie around the ECA stump holds the intraluminal suture in place. ACA, anterior cerebral artery; BA, basilar artery; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; MCA, middle cerebral artery; OA, occipital artery; PCA, posterior cerebral artery; PComA, posterior communicating artery; PPA, pterygopalatine artery; ST, superior thyroid artery. (B) Representative T2-weighted magnetic resonance imaging of coronal rat brain 24 hours after 1 hour transient MCAO. The infarcted region appears hyperintense (bright).

One of the most widely used MCAO animal models (Figure 2) employs an intraluminal nylon suture, modified with a silicon-coated or flame-rounded end, which is advanced through the internal carotid artery to block the origin of the MCA (Koizumi et al. 1986; Longa et al. 1989). The period of occlusion may commonly range from 30 minutes to 2 hours, depending upon the rat strain utilized. Because the lenticulostriate arteries branching off of the MCA are end arteries that supply the basal ganglia without collateral branches, MCAO routinely causes a striatal infarct. The cortical territories of the MCA do receive collateral flow via leptomeningeal anastomoses and suffer a gradient of decreased blood flow from the periphery towards the center of the cortical MCA territory during MCAO. Severe reduction of cerebral blood flow disrupts both the functional and structural integrity of the brain, whereas more moderate blood flow impairment may result in loss of function without structural deterioration. This latter case underlies the concept of the "ischemic penumbra", as cells in such areas may recover function when reperfused. Variability in lesion size and location may occur due to surgical technique, physiological variables during surgery (including body temperature), rat weight, suture dimensions, occlusion period, etc.. The use of spontaneously hypertensive rat strains (which possess poor collateral circulation compared to other rat strains) in the hands of an experienced surgeon, however, generally provides reliable induction of cortical and subcortical infarction (Duverger & MacKenzie 1988; Macrae 1992; Dogan et al. 1998). Visualization of the infarct size in order to confirm a

similar extent of injury between study groups is, nevertheless, highly desireable. This is especially true in studies aimed at determining the efficacy of therapeutic interventions, e.g. stem cell transplantation. Although magnetic resonance imaging (MRI) of the infarct prior to stem cell delivery has not been universally employed, a growing number of studies are recognizing the importance of this approach for reducing the confounding effects of infarct induction variability when assessing potential neuroprotective regimens.

3.2 hES-MSC transplantation reduces infarct size and improves behavioral function

A number of investigators have evaluated the potential of MSCs to provide enhanced recovery from surgically-induced stroke in rodent animal models. Our laboratory has transplanted hES-MSCs intravenously into spontaneously hypertensive rats 24 hours after 1 hour MCAO (Liu et al. 2009). The cells were labelled with green fluorescent protein so that the survival, migration and differentiation patterns of the engrafted cells could be monitored. The hES-MSCs were found in the infarction region, ischemic penumbra and striatum of the ipsilateral hemisphere; hES-MSCs were not observed in the contralateral hemisphere, although transplanted cells have been reported to be present in both damaged and undamaged hemispheres in some other studies (Modo et al. 2002). Rats receiving hES-MSCs showed reduced sensory deficit during the first week following stroke, after which time the control animals had recovered to a similar extent (Figure 3); neither group returned

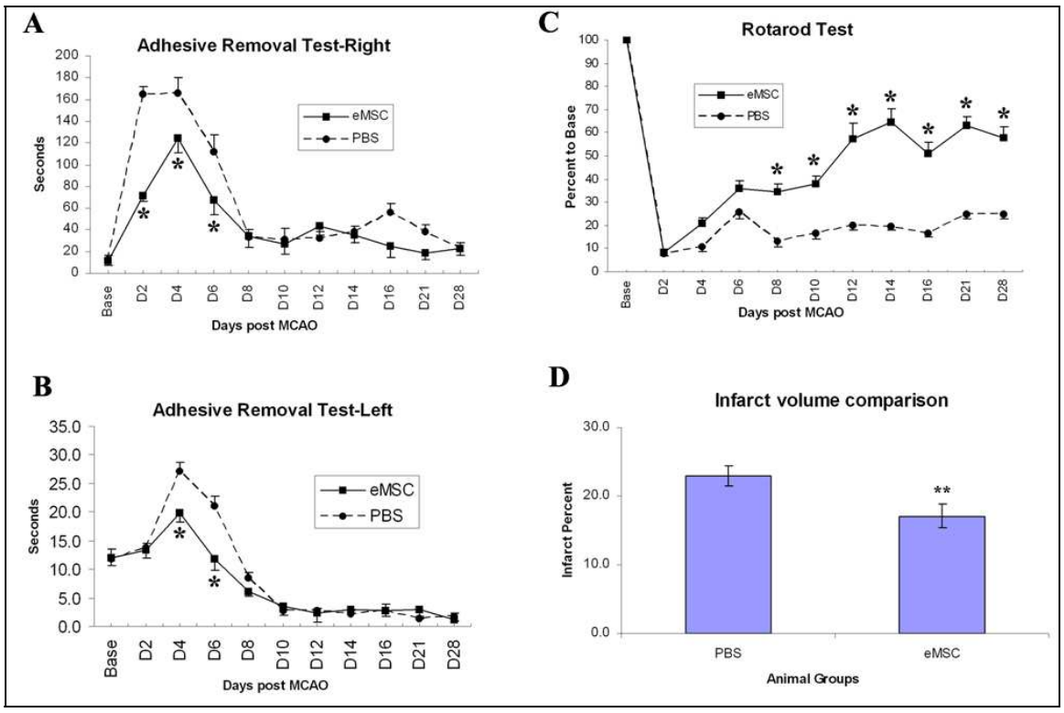

Fig. 3. Human ES cell-derived MSCs (eMSCs) improve sensory and motor function following MCAO. Rats received eMSC or PBS 24 hours after MCAO. Mean time to removal of a small adhesive label for the right (A) and left (B) forepaw. (C) Staying time on the rotarod is shown as a percentage of baseline pre-MCAO control. (D) Infarct volume calculated from cresyl violet-stained brain sections harvested 28 days after MCAO. * $P<0.05$, ** $P<0.01$. Data taken from Liu et al. 2009.

to baseline sensory performance, however. Transplant recipients also displayed improved motor function to approximately 60% of baseline performance by 8 days post-MCAO and this enhanced recovery was maintained through the study's end at 28 days. Infarct volume was ~25% smaller in the hES-MSC-recipient rats as determined by cresyl violet histological staining.

Many other reports have produced similar results using MSC-like cells from multiple sources, including bone marrow-derived stromal cells, human umbilical cord blood stem cells, adipocyte-derived stem cells, as well as other types of stem cells, such as neural progenitor cells [see (Locatelli et al. 2009) and (Bliss et al. 2007) for reviews]. Chopp's group, in particular, has performed a large number of studies with rat, mouse or human MSCs in young, adult or aged animals and observed significant improvement in neurological function following stroke (Li & Chopp 2009). The numbers of cells administered, the delivery route and the timing of cell administration vary with the investigating group and each method has its own advantages and disadvantages. For example, intracerebral injection can specifically target the peri-infarcted region but is invasive, risking further trauma to the already damaged brain. Intravenous injection is less invasive but may result in less discriminate delivery of stem cells to non-target organs. Certainly, these parameters will need to be optimized if this approached is ever to become broadly clinically applicable to stroke treatment.

3.3 Homing of MSCs to injured tissue

Several studies have internally labelled MSCs with radiolabelled, fluroescent or magnetic markers in order to follow the distribution of transplanted cells when administered intravenously. In the healthy animal, IV-infused MSCs home to the bone marrow with varying efficencies (Devine et al. 2001; Wynn et al. 2004) but are detected in multiple other tissues as well, including lung, liver and intestine (Gao et al. 2001; Devine et al. 2003). In the setting of injury or inflammation, however, MSC distribution is shifted preferentially to the site of inflammation (François et al. 2006; Jackson et al. 2010). The migration signals for stem cell homing include the stromal cell-derived factor 1 (SDF1)/chemokine CXC receptor 4 (CXCR4) pathway (Chapel et al. 2003; Dar et al. 2006; Shi et al. 2007; Ryu et al. 2010; Yu et al. 2010). SDF1/CXCR4 is the major signaling axis for homing of hematopoietic stem cells (Wright et al. 2002); MSCs, however, express receptors for and migrate in response to many growth factors and chemokines (Honczarenko et al. 2006; Ponte et al. 2007). In unstimulated human BM-MSCs, the growth factors IGF-1 and PDGF-AB had much stronger chemotactic activity than SDF1 in in vitro assays (Ponte et al. 2007). Upon pretreatment with the inflammatory cytokine, TNFα, however, migration of BM-MSCs in response to the chemokines RANTES, MDC and SDF1 increased dramatically (Ponte et al. 2007). In this study, the levels of CCR3 (one of the RANTES receptors) and CCR4 (receptor for MDC and RANTES) increased with TNFα pretreatment but CXCR4 (receptor for SDF1) levels did not change, leaving the authors to speculate that the downstream CXCR4 signal transduction pathway may have been modulated. Other studies reported that MSCs express low levels of CXCR4 at the cell surface (Rüster et al. 2006) but contain large intracellular pools of CXCR4 (Wynn et al. 2004). Fluid shear stress approximately doubled the number of MSCs with CXCR4 surface receptors (Rüster et al. 2006). Nitric oxide induced CXCR4 expression on mouse MSCs, and raised endogenous SDF1 levels in the ischemic brain (Cui et al. 2007). Co-treatment of mice with BM-MSCs and a nitric oxide donor following stroke improved homing to the ischemic brain, and enhanced functional recovery compared to BM-MSC

treatment alone (Cui et al. 2007). The added benefit of the combination therapy was not seen in the presence of a CXCR4 inhibitor. SDF1 can enhance nitric oxide synthase activity (Cherla & Ganju 2001) so SDF and NOS actions may be mutually reinforcing. The SDF1/CXCR4 signaling axis thus appears to be a major determinant of MSC homing to ischemic lesions in vivo.

At the cellular level, homing reflects the fluid shear stress-resistant interaction of cell surface homing receptors on the MSCs with surface receptors present on the vascular endothelial cells in the target organ. Following the model of leukocyte homing (Sackstein 2005), the initial tethering of the MSC to the vascular endothelium would be followed by further "rolling" and firm adhesion and, finally, extravasation (Rüster et al. 2006). BM-MSCs express multiple integrin proteins (De Ugarte et al. 2003; Rüster et al. 2006), including the integrin α4/β1 heterodimer involved in cell-cell and cell-ECM interactions with endothelial cell vascular cell adhesion molecule (VCAM)-1 and fibronectin, respectively (Guan & Hynes 1990). Blocking integrin β1 in the context of myocardial ischemia interfered with targeting of MSCs to the heart (Ip et al. 2007). MSC interaction with endothelial cells involves additional molecules, such as P-selectin, MMP-2 secretion, and cytokines (Rüster et al. 2006). Inflammation indirectly upregulates VCAM-1 expression in vascular endothelial cells in vitro (Stanimirovic et al. 1997) and in the intact ischemic brain (Frijns & Kappelle 2002; Hoyte et al. 2010). The binding of rat MSCs to vascular endothelial cells can be reduced in the presence of an anti-VCAM-1 antibody (Segers et al. 2006). Exit through the vasculature into the surrounding interstitia is reinforced via MSC integrin binding to ECM components, such as the V regions of fibronectin, which are increasingly exposed by fibronectin fragmentation occurring during tissue remodeling following injury (Valenick et al. 2005). Recent data from in vitro migration assays suggest that ECM collagens may also play a role in MSC chemotaxis (Mauney et al. 2010). Protease action on the ECM is an important component of cell migration. MSCs constitutively express a variety of matrix metalloproteases and a subset of these are induced by the proinflammatory cytokines TGF-β1, IL-1β and TNFα (Ponte et al. 2007; Ries et al. 2007) suggesting another means by which inflammation may direct MSC homing to the site of injury. Nitric oxide also induces expression of MMP9 and enhanced homing following stroke in mice (Cui et al. 2007).

3.4 Immunomodulation by MSCs

Allogeneic cells trigger an immune response by interaction of their cell surface MHC class I antigens in the presence of co-stimulatory molecules with the cognate receptors of host lymphocytes. MSCs express MHC class I, but not class II, markers on their cell surface. Intracellular pools of class II antigens can be brought to the surface by interferon-γ (IFN-γ) treatment of MSCs; IFN-γ fails to mobilize class II molecules, however, once MSCs are differentiated into adipocyte, chondrocyte or osteoblast lineages. Note that whether IFN-γ-stimulated or not, MSCs do not express co-stimulatory molecules such as CD40, CD80, or CD86. MSCs fail to invoke a proliferative response in allogeneic lymphocytes in vitro, including when antigen-presenting cells or costimulatory signals are provided. MSCs also fail to induce other indicators of lymphocyte activation, such as IFN-γ production or expression of activation-associated markers (e.g., CD25, CD38, or CD69). Furthermore, MSCs evade natural killer cell or cytotoxic lymphocyte-mediated cell lysis, despite lysis of other cell types from the same donor (Rasmusson et al. 2003). Not only do they avoid stimulating lymphocyte proliferation, MSCs can suppress T cell activation in mixed lymphocyte reaction assays (Aggarwal & Pittenger 2005; Trivedi & Hematti 2008)

Inflammation is a component of the acute phase response to ischemic injury. One means by which MSC transplantation might influence tissue injury and recovery is through the modulation of the extent of inflammation. A key mediator of inflammation is TNFα, which binds to the cell surface TNF receptor and initiates signaling through the NF-kB pathway. Activation of NF-kB reporter expression in rat hepatoma cells exposed to proinflammatory culture conditions (growth in serum from lipopolysaccharide-stimulated rats) was abrogated when the reporter cells were co-cultured with human BM-MSCs (Yagi et al. 2010). The MSCs in this study themselves showed upregulation of NF-kB signaling under proinflammatory conditions, which resulted in secretion of the soluble form of TNF receptor 1 (sTNFR1) as an anti-inflammatory measure. Production of sTNFR1 by transplanted MSCs in response to inflammation lowered the levels of inflammatory cytokines TNFα, IFN-γ and IL-6 and reduced organ injury in a rat endotoxemia model (Yagi et al. 2010); these effects were at least partly reduced in the presence of a neutralizing antibody to sTNFR1.

Administration of human MSCs into mouse hippocampus one day after transient global ischemia improved neurological function and reduced hippocampal neuronal degeneration (Ohtaki et al. 2008). Microarray gene expression analysis revealed that ~14% of the ischemia up-regulated mouse genes were reduced in mice receiving the MSCs, including many inflammatory and immune response genes. The MSCs, which showed altered gene expression patterns in response to the cerebral ischemic environment as well, elicited local expression of neuroprotective factors, such as insulin-like growth factor 1 and neuropeptide Ym, by microglial/macrophages. Furthermore, although only small scale changes in mouse TNFα, IFN-γ, and IL-4 occurred after MSC delivery, the investigators argued that the ratios of cytokines (e.g., increased IL-4/ IFN-γ and IL-4/ TNFα ratios) were altered in a manner suggestive of a shift from a proinflammatory type 1 helper T cell "Th1" directed immune response to a type 2 helper T cell "Th2" dominant response traditionally associated with lower inflammation and improved xenograft tolerance. This interpretation was supported by the increased presence of galectin-3-expressing microglia/macrophages, signifying alternate (i.e., Th2-directed) activation of these cells, in the MSC-recipient mice.

Transplantation of MSCs in non-human primate stroke models has also been performed. MSC administration was associated with increased IL-10 (an anti-inflammatory cytokine) levels, reduced neural apoptosis, and enhanced proliferation in the subventricular zone of the macaque hippocampus (Li et al. 2010). Such in vivo results are consistent with in vitro co-culture experiments in which human BM-MSCs decreased TNFα and increased IL-10 secretion in dendritic cells, reduced IFN-γ in Th1 cells, increased IL-4 secretion in Th2 cells, increased the proportion of immunosuppressive regulatory T cells and decreased IFN-γ in natural killer cells (Aggarwal & Pittenger 2005). The MSCs displayed enhanced secretion of IL-6, IL-8, vascular endothelial growth factor and prostaglandin E2 (PGE_2) in the presence of the immune cells or when MSCs were exposed to TNFα or IFN-γ. The alterations in cytokine production by the immune cells were mitigated by inhibition of MSC-mediated PGE_2 production (Aggarwal & Pittenger 2005). Thus, dampening of inflammatory responses by factors secreted by MSCs is likely to be one mechanism by which MSCs promote graft tolerance and reduce tissue injury.

3.5 Limited cell replacement

A common feature of many MSC transplant studies is limited term survival of the engrafted cells in the ischemic brain (Bliss et al. 2007). For example, we observed a dramatic reduction

in GFP-labelled cells between two and four weeks after post-stroke intravenous hES-MSC injection (Liu et al. 2009). Human MSCs transplanted into ischemic mouse hippocampus survived for fewer than seven days (Ohtaki et al. 2008). Interestingly, a report of survival of neural stem cells for as long as 540 days (Chu et al. 2004) may suggest a difference in the ability of this stem cell type relative to MSCs to integrate into the post-stroke cerebral environment (perhaps due to differing sensitivites to apoptosis, efficiencies of neural, glial, or oligodendritic differentiation, synaptogenic potential, detection by the immune system, etc.). Nevertheless, the bulk of the reports of stem cell transplantation, whether with MSCs or neural stem/progenitor cells, indicates more limited graft duration.

3.6 Do MSCs differentiate into neurons?

Controversy exists as to the potential for MSCs to differentiate into neuronal, glial or oligodendritic cell types. Because these latter cells are derived from a neuroectodermal embryonic origin, there would be no *prima facie* expectation that mesodermal MSCs would do so. Nevertheless, multiple groups reported neuronal-like or glial cells developing from bone marrow cells (studies used a mix of unfractionated marrow cells, HSCs or MSCs), either in culture (Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Black & Woodbury 2001; Deng et al. 2001; Kohyama et al. 2001; Kabos et al. 2002; Kim et al. 2002; Jiang et al. 2003) or in vivo following cell transplantation (Eglitis & Mezey 1997; Eglitis et al. 1999; Kopen et al. 1999; Brazelton et al. 2000; Chopp et al. 2000; Mezey et al. 2000; Nakano et al. 2001; Hofstetter et al. 2002; Keene et al. 2003; Mezey et al. 2003; Weimann et al. 2003a). Some of these in vitro neural induction protocols were quite rapid and used chemicals such as β-mercaptoethanol, dimethylsulfoxide (DMSO), butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), either alone or in combination (Woodbury et al. 2000; Hung et al. 2002). Other neural induction protocols have employed cytokines, growth factors, retinoic acid, neurotrophins and Noggin, among other agents. These multistage processes take days or weeks to produce neural cells (Sanchez-Ramos et al. 2000; Kohyama et al. 2001; Kabos et al. 2002; Jiang et al. 2003), which is more akin to the time frame required for hES cell or neural stem cell differentiation into neurons.

Some later reports have failed to duplicate earlier studies (Castro et al. 2002), calling the "transdifferentiation" potential of MSCs into question (Krabbe et al. 2005; Prockop 2007). Because classification of MSC-derived neural cells has often relied upon expression of neural marker genes, criticisms of some studies have included: (i) the failure to use confocal microscopy to establish true three-dimensional colocalization of markers in the same cell, suggesting that the observed colocalization was artefactual due to signals from cells in overlapping focal planes; (ii) suggestion that cell fusion may account for apparent marker colocalization (Terada et al. 2002; Alvarez-Dolado et al. 2003; Weimann et al. 2003b); (iii) the observation of "neuron-like" morphology and neural marker expression in multiple cell types after simple rapid chemical "induction" protocols in vitro that may be the result of cell toxicity or shrinkage occurring as part of a stress response (Lu et al. 2004; Neuhuber et al. 2004); and (iv) the rapid reversibility of the neural phenotype after withdrawal of the inducing agent(s) (Rismanchi et al. 2003; Lu et al. 2004). It should be noted that BM-MSCs can express immature neural markers even before "differentiation" and at later passages, can express more mature neuronal markers (Tondreau et al. 2004).

Our laboratory has observed neuronal marker induction in transplanted hES-MSCs following MCAO in rats, although we cannot say whether the marker-positive cells

possessed any functional attributes of neurons, including electrophysiology. The development of induced pluripotent stem cells (Takahashi et al. 2007; Yu et al. 2007) clearly demonstrates that reprogramming cell development is feasible. Regardless of whether the purported MSC-derived "neurons" can be considered true neuronal cells, the number of any such cells generated following stroke is minimal. Therefore, engrafted cell integration and replacement of damaged neurons is unlikely to be a major mechanism driving the beneficial effects of MSC therapy.

3.7 Multiple processes underlie beneficial action of MSCs

If the long-term survival, neuronal differentiation and integration of engrafted MSCs is minimal, then by what potential mechanisms do MSCs evoke a beneficial response? There are multiple processes by which ischemia/reperfusion generates tissue injury; these include: energetic failure; acidosis-induced toxicity and neurotransmitter excitotoxicity, both of which can result in calcium dysregulation leading to endoplasmic reticulum stress and inhibition of protein synthesis; inflammation; free radical generation during reperfusion; mitochondrial perturbations leading to release of apoptogenic molecules; edema; and, spreading depolarization injury in non-infarcted regions (Hossmann 2009). Thus, multiple pathways are likely to be involved in stem cell-mediated repair.

We have already alluded to the potential for MSCs to reduce inflammatory damage. Enhancement of endogenous neurogenesis and/or migration of newly formed neurons, which occurs after brain injury, is another potential avenue for MSC-mediated tissue repair. Increased incorporation of 5-bromodeoxyuridine (BrdU) into endogenous neural cells in the subventricular zone and in the dentate gyrus has been observed in rats and mice receiving BM-MSCs following cerebral ischemia (Chen et al. 2003a; Munoz et al. 2005). Rats receiving MSCs after stroke exhibited significantly fewer apoptotic cells, especially along the ischemic boundary (Chen et al. 2003a; Wu et al. 2008; Deng et al. 2010). Protection from apoptosis of cerebellar granular neurons in culture could be achieved using conditioned medium from adipose-derived MSCs, and this was partially attributable to IGF-1 modulation of Akt signaling (Wei et al. 2009b). MSC-conditioned medium also conferred protection in vivo against hypoxia-ischemia in neonatal rats (Wei et al. 2009a).

Astrocyte function is important for neuronal survival and recovery after stroke (Chen & Swanson 2003), partly because astrocytes produce trophic factors such as VEGF, bFGF, and brain-derived neurotrophic factor (BDNF). Co-culture with rat MSCs enhanced astrocyte trophic factor production, reduced hypoxia-induced rat cortical astrocyte apoptosis and increased cell proliferation (Gao et al. 2005). These effects were accompanied by activation of the Akt/phosphoinositde 3-kinase and mitogen activated protein kinase kinase (MAPKK)/extracellular signal-regulated kinases 1/2 (Erk1/2) signaling pathways in the astrocytes (Gao et al. 2005).

Vascularization of the infarcted area is another important component of functional recovery. Patients with the highest microvascular density in the ischemic penumbra have the best survival (Krupinski et al. 1994). Angiogenesis in response to ischemia begins shortly after the onset of ischemia and pro-angiogenic gene expression changes can be observed within 1 hour (Hayashi et al. 2003). MSC administration has been associated with stimulation of angiogenesis (Zhang et al. 2002; Chopp et al. 2008) resulting in greater vascularization in the ischemic boundary zone (Chen et al. 2003b). This was accompanied by increased VEGF production by reactive astrocytes (Chen et al. 2003b; Gao et al. 2005).

4. MSCs in clinical trials

Bone marrow-derived cell transplation has been widely used in a clinical setting for the treatment of hematopoietic disorders and cancers, as well as for some autoimmune disorders and graft versus host disease. To date, however, just a handful of small scale Phase I and Phase II clinical trials (summarized in Locatelli et al. 2009) have been performed to test the safety of stem cell transplantation (of any type) in stroke patients, and of these, only one study used MSCs. This randomized Phase I/II study (Bang et al. 2005) consisted of 30 patients with cerebral infarcts within the middle cerebral artery territory (as assessed by diffusion-weighted MRI) and severe neurological deficits (e.g., hemiparesis and agnosia; NIH Stroke Scale score of 7 or higher at 7 days after admission). Autologous bone marrow was collected 1 week after the onset of symptoms, and adherent mononuclear cells were expanded in culture and assessed for surface marker expression (CD73+, CD105+, CD34–, CD45–, HLA I–, leukocyte antigen D–). The treatment group (5 individuals) received two intravenous infusions of 5 x 10^7 autologous MSCs each (1 x 10^8 cells total) at 4–5 weeks and 7–9 weeks post-stroke. This dose was chosen because, once corrected for mean body mass, it is within the range that has been effective in several studies using the rat stroke model. The control group (n = 25), which was similarly matched with respect to infarct size, stroke etiology, and risk factors but had a slightly younger median age, received no MSCs. A follow-up MRI at 52 weeks was performed on 5 control and on all MSC-treated patients.

No cell-related adverse events were reported immediately following MSC administration or within the one year follow-up period, suggesting that the intravenous delivery of the cells was well tolerated. The Barthel Index (assessing functional recovery) of the MSC recipients was higher at 3 months and 6 months, although the improvement did not achieve statistical significance at 12 months. Scores in the modified Rankin Scale, another index of functional recovery, were not statistically different between groups, although the MSC-treated group consistently trended lower (better recovery) at each timepoint. The MRI analysis revealed that the evolution of the infarct was no different among the two groups but MSC recipients were reported to have less prominent secondary ventricular dilation than controls after one year.

In two clinical trials employing either hematopoietic stem cells or an immortalized neuroteratocarcinoma cell line following stroke, no adverse effects were reported (Kondziolka et al. 2000; Suárez-Monteagudo et al. 2009), whereas a seizure and a hematoma (1 patient each) were reported in a third study, which classified these as non cell-related events (Kondziolka et al. 2005). The U.S. Food and Drug Administration terminated a study of fetal porcine cell transplantation due to the development of seizures in one patient and aggravation of motor deficits in another within 1 week and 3 weeks of transplantation, respectively (Savitz et al. 2005). Human fetal cell delivery into the subarachnoid space was performed in 10 patients after ischemic or hemorrhagic stroke and some patients exhibited fever and meningism within two days of transplantation (Rabinovich et al. 2005). There are many differences in cell type, delivery method, timing and study population among these trials that render comparisons difficult and only small numbers of patients were involved in these studies. Although these studies indicate that stem cell transplants may be generally well tolerated as a proof of concept, it is fair to say that safety concerns remain a high priority and that many of the details regarding stem cell therapy remain to be optimized.

Tumor development is also a safety concern with stem cell populations. There are reports of oncogenic transformation of human and murine BM-MSCs or adipose-derived MSCs after

long-term culture (Rubio et al. 2005; Wang et al. 2005; Miura et al. 2006; Tolar et al. 2007). Furthermore, co-mixing of murine BM-MSCs with melanoma or weakly tumorigenic breast carcinoma cell lines enhanced the tumorigenic or metastatic potential of the transformed cell lines in animal studies (Djouad et al. 2003; Karnoub et al. 2007). Therefore, it will be desireable to limit the time in culture and to screen any cell populations to be used in a clinical setting for normal karyotype.

At this early stage, it is premature to judge the safety, much less the efficacy of stem cell therapy. Much larger scale trials designed with sufficient statistical power to test efficacy will be required once safety issues have been further understood. In 2010, two additional clinical trials involving multipotent mesenchymal stromal cell interventions against stroke are in the recruiting or planning stages (www.clinicaltrials.gov, NCT00875654 and NCT01091701). In addition, at least six trials employing other stem cell preparations targeted to ischemic stroke are also recruiting participants. Thus, the next several years should provide emerging data on the potential of stem cell therapy to treat this major human health issue.

5. Conclusion

The relative ease of obtaining MSCs from patients, coupled with the reports of differentiation of MSCs into cells of all three germ layers, has generated enthusiasm for the use of these cells in autologous transplantation. While transdifferentiation has generated controversy, the immunomodulatory properties and tissue repair promotion via paracrine action keeps MSCs as viable candidates for therapeutic evaluation. The exciting development of induced pluripotent stem (iPS) cells (Takahashi et al. 2007; Yu et al. 2007) may eventually make the production of autologous nervous system cells practical. While iPS-derived neural cells might perhaps prove to be a more effective cell source for neural repair and replacement following stroke, MSC co-transplantation may theoretically be a useful adjuvant for neural stem cell therapy applications.

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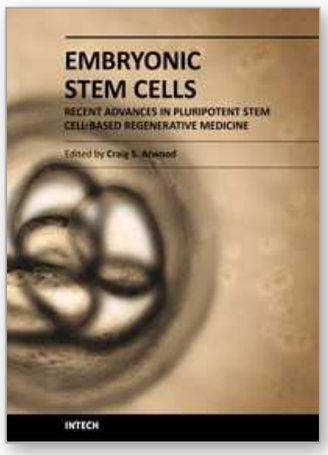

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Pluripotent stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases and conditions that currently lack therapies or cures. This book describes recent advances in the generation of tissue specific cell types for regenerative applications, as well as the obstacles that need to be overcome in order to recognize the potential of these cells.

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