

Research Advancements in Porcine Derived Mesenchymal Stem Cells

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Abstract: In the present era of stem cell biology, various animals such as Mouse, Bovine, Rabbit and Porcine have been tested for the efficiency of their mesenchymal stem cells (MSCs) before their actual use for stem cell based application in humans. Among them pigs have many similarities to humans in the form of organ size, physiology and their functioning, therefore they have been considered as a valuable model system for *in vitro* studies and preclinical assessments. Easy assessability, few ethical issues, successful MSC isolation from different origins like bone marrow, skin, umbilical cord blood, Wharton's jelly, endometrium, amniotic fluid and peripheral blood make porcine a good model for stem cell therapy. Porcine derived MSCs (pMSCs) have shown greater *in vitro* differentiation and transdifferentiation potential towards mesenchymal lineages and specialized lineages such as cardiomyocytes, neurons, hepatocytes and pancreatic beta cells. Immunomodulatory and low immunogenic profiles as shown by autologous and heterologous MSCs proves them safe and appropriate models for xenotransplantation purposes. Furthermore, tissue engineered stem cell constructs can be of immense importance in relation to various osteochondral defects which are difficult to treat otherwise. Using pMSCs successful treatment of various disorders like Parkinson's disease, cardiac ischemia, hepatic failure, has been reported by many studies. Here, in this review we highlight current research findings in the area of porcine mesenchymal stem cells dealing with their isolation methods, differentiation ability, transplantation applications and their therapeutic potential towards various diseases.

Keywords: Cell therapy, mesenchymal stem cells, multipotency, porcine.

1. INTRODUCTION

Human health and proceedings towards its welfare has been remained as the main focus of any organization since the beginning of civilization. Stem cells are the specialized cells with a potential to self-replicate and differentiate into other cells of the body. Easy isolation, self-renewability, high propagation rate and controlled *in vitro* culturing conditions demarcate them from other normal cells. Due to their marked biological properties and vast applications in regenerative therapy, stem cells have been focused globally. Recently the potential use of stem cells in the repair and replacement of the damaged organs is in full pace. On the basis of origin, stem cells can be broadly grouped into two categories: embryonic stem cells and adult stem cells.

Embryonic stem cells (ESCs) which are derived from inner cell mass of blastocyst stage embryos can be indefinitely maintained by continuous passages in cell cultures therefore they have been termed as continuous cell lines with immortal property [1]. Whereas post-natal adult tissues derived stem cells (ASCs) are referred to as finite cell lines because of

their limited passage capacity [2]. ESCs are pluripotent cells with the ability to differentiate into derivatives of all the three germ layers i.e. ectoderm, mesoderm and endoderm. Pluripotent ESC lines with long term propagation capacity and broad differentiation potential have been successfully isolated from inner cell mass (ICM) of mouse blastocyst [3] which can be transformed into tropo-ectodermal cells *in vitro* under culture conditions [4]. Establishment of potent ESC lines from genetically engineered valuable domestic species (i.e. pig, sheep, goat, cow or horse) can be preferentially used for agricultural traits, products, biomedicine, biopharming, and xenotransplantation [5]. The features of differentiation ability help scientists to generate efficient *in vitro* models for the study of biological processes and complex genetic diseases. Since two decades mostly mouse and, to lesser extent humans ESC lines have been most extensively used as models for evaluation of cell based therapies for various diseases. Whereas adult stem cells (ASCs) which are tissue or organ specific cells, can be propagated *in vitro* under defined culture conditions, exhibits self-renewability, express cell specific CD markers, pluripotent genes and finally with a potential to differentiate into same cell or into different cell lineage [6]. Present day ASCs have displayed greater plasticity and developmental potential as compared to the earlier reported studies. Pluripotency, higher proliferation rate, stable morphology and telomerase activity during prolonged *in vitro* culture, ability to differentiate into any lineage, makes

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ESCs more favorable than ASCs. However, difficulty in isolation, low cell number, ethical concerns, lack of understanding in molecular regulation of ESCs differentiation and widely reported tumorigenicity makes ASCs a better choice for stem cell therapy [7]. Moreover, it is possible to use host stem cell derivatives harvested from the same adult tissues, which can be utilized in autologous stem cell therapy without immune related complications.

Previously, many experimental animals such as mouse, rat, and rabbit have been tested as models for clinical applications, however the importance of porcine has been described briefly as best experimental model compared to other animals based on the similarities of porcine organ physiology with the human beings [8]. Recently, porcine has been considered as most suitable animal model in current time practice for evaluation of choice of cells in the development of stem cell based therapy, regenerative medicine and transplantation [9]. Generation of specific porcine cell lines will help in a variety of experimental research and understanding the stem cell xenotransplantation safety.

This review highlights the current research findings and their prospective related to porcine mesenchymal stem cells, isolation methods, *in vitro* differentiation ability, comparative analysis of gene expressions, preclinical evaluation, cryopreservation methods, limitations and the future of porcine mesenchymal stem cells (pMSCs).

2. CHARACTERISTICS OF MSCS

About four decades ago, multipotent MSCs were isolated from bone marrow, capable of differentiating into cells of mesenchymal lineages such as bone, fat and cartilage [10]. When it comes to MSCs characterization there was no single universally accepted definition nor standardized protocols for the isolation and culturing. Lack of cell specific markers for identification of MSCs population resulted in the hindered stem cell progress, therefore International Society for Cellular Therapy (ISCT) set up three basic parameters to define minimal characteristics of MSCs [11]:

- (1) when maintained in standard culture conditions, MSCs should display plastic adherence.
- (2) MSCs must express certain cell specific markers, CD105, CD73, CD90, but lacks the expression of other hematopoietic cell surface markers such as CD45, CD34, CD14 or CD11b, CD79 α or CD19, HLA-DR.
- (3) They should be capable of differentiation into osteoblasts, adipocytes, and chondroblasts *in vitro* under specific set of culture conditions.

These parameters have laid a milestone for the assessment of stem cell and their confirmation in any species including human, porcine, bovine, murine, and others. Earlier MSCs were thought to be constituted as small proportion of cellular component of the bone marrow, although they can be isolated from almost any tissue of the body [12]. Moreover, MSCs harbors immunoregulatory properties and can interact with immune related cells including B-cell, T-cell, natural killer (NK) and antigen presenting cells [13].

2.1. MSCs Origin

It is well understood that source of MSCs origin is not only restricted to fetal stage but also found throughout the other organs of adult body. Bone marrow, the most commonly studied source of MSC has been extensively reported since beginning [14-18]. Moreover, MSCs from other sources like umbilical cord blood [19], Wharton's jelly [20-23], peripheral blood [24], amniotic fluid [25], endometrium [26, 27], neural progenitors [28], retinal progenitors [29], skin [30-32], synovial fluid [33-35] and aorta tunica media [36] have also been isolated successfully. MSCs from these sources exhibit stem cell properties like plastic adherence, multilineage differentiation, expression of CD markers and pluripotent genes. It is clearly evident that post-natal organs and tissues served as good MSCs sources, however each source of MSCs have various extent of differentiation potential as well as the expression of stem cell related markers. Therefore, comparative analysis of mesenchymal stem cell potency from different sources has been demonstrated in various studies [37-39]. Comparing various porcine MSCs sources from same or different hosts, it has been found that not all the sources have same potential towards mesenchymal characteristics. Moreover, each individual MSC source can have comparable or better potency towards any lineage specific differentiation and other important features like high proliferation, immunomodulation and xenotransplantation. Therefore, suitable MSCs should be carefully validated for cell based therapies before clinical application.

2.2. Isolation and Culturing of MSCs

MSCs have properties which make them suitable for therapeutic use, but for clinical application, standardized isolation techniques are required. After researching appropriate stem cells source, isolation and homogenous primary culture in hygienic condition is an essential prerequisite for expansion of MSCs. Yet so far, bone marrow has been considered as best featured source of MSCs and is therefore most extensively used in differentiation studies. A highly specific nucleic acid probe called 'aptamer' has been established for rapid, efficient and direct isolation of adult MSCs from porcine bone marrow [15]. In this study, aptamer generation is done by targeting the porcine MSCs by combinatorial chemistry out of a huge random library with *in vitro* technology called systematic evolution of ligands by exponential enrichment (SELEX) [15]. Furthermore, binding affinity is detected by using a cell sorting assay with streptavidin-coated magnetic micro beads. Porcine derived MSCs isolation depends upon the type of source and its physiology, therefore various types of methods are employed for stem cell extraction from different tissue sources using variety of chemical treatments for varied time durations. Basically two major methods namely "attachment method" and "enzymatic digestion" are used for solid tissue specific MSCs isolation. Whereas other sources like body fluids, utilizes fractionation on density gradient solutions such as Percoll [40, 41], Ficoll [21, 42-45], Lymphoprep [46], Biocoll separating solution [47] and Histopaque [18, 48, 49] for successful MSCs isolation. Density gradient based centrifugations lead to centrally located buffy coat at gradient interface from where mononuclear cell fractions are obtained which are washed with phosphate buffered saline (PBS) to extract MSCs [9]. Het-

erogeneous mixture of cells such as endothelial cells, lymphocytes, hematopoietic cell fractions collectively called 'cell debris' are also observed along with the firmly attached MSCs showing fibroblastoid morphology. To avoid contamination due to cell debris, it is highly recommended to use antibiotics and fungicides followed by media change every alternative day. To obtain homogenous fibroblastoid stem cells and for further assessment of morphology and basic mesenchymal stem cell characteristics including expression of cell surface antigens and transcription factors, isolated cells should be sub-cultured at least two or three passages. *In vitro* expansion of MSCs requires different culture media with certain specific growth factors. Use of Dulbecco's Modified Eagle's Medium (DMEM) [9, 41, 42], Advanced DMEM [21, 43], alpha MEM [10, 50], DMEM F-12 (1:1) [30], TCM 199/DMEM (1:1) [43], EM6-F [47] have been well documented in various studies. Different cell lines need different types of media constituents along with different oxygen concentrations, therefore the proliferations of pMSCs with different oxygen tensions have been previously investigated [49]. Certain growth factors, whether present in media or provided additionally have been proved better for growth and proliferation of MSCs [18]. Different concentrations of fetal bovine serum (FBS) in media to assess *in vitro* MSC propagation and expansion have been reported. Bosch *et al.* (2006) showed the effect of different concentrations of FBS at low and high oxygen tensions, on the growth and proliferation of MSCs. It was observed that 10% FBS concentration with 5% oxygen tension displayed increased proliferation rate as compared to other parameters whereas lack of FBS in media had no visible colonies [49]. FBS contain unknown growth factors which are necessary for MSCs propagation but due to its xenogeneic behavior, it may lead to bovine prion, viral and zoonose contamination in stem cell product [51]. Interestingly, various attempts have been tried to find suitable alternatives of FBS to prevent xenogeneic side effects, alternatively supplementation of EGF for optimizing a serum free culture system has been reported for bone marrow stem cell proliferation in miniature pigs [18]. Moreover, desirable results including successful pMSCs isolation and their proliferation were observed by using certain growth factors like EGF and PDGF along with reduced serum concentration (FBS 2%) [45]. Therefore, for appropriate isolation and better expansion of pMSCs, growth factor enriched low serum media or certain supplement added serum free culture media can be used for same morphology and better stem cell potential. In regenerative medicine, MSCs plays an amenable role and are required in sufficient numbers to accomplish this goal. Like bone marrow, low number of isolated MSCs and lack of efficient protocols for their extraction and propagation, valuable MSC sources have been ignored in cellular transplantation studies. Solution to such problems can be the development of new isolation strategies with necessary modification. Recently, Mori and colleagues have demonstrated an improved explant isolation method using cellamigo- an autoclavable stainless steel mesh over minced human umbilical cord pieces to expose more tissue area in contact with plastic surface. Interestingly, more number of cells were isolated with attainment of more confluence within short duration as compared to MSCs isolated by conventional ex-plant method [52], this method can also be utilized for isolation of pMSCs. Other methods may in-

clude use of tissue specific digestion enzymes under low temperature conditions followed by mechanical agitation at regular intervals of time instead of longer enzymatic treatment at higher temperatures which causes over digestion of cells and decrease their potency. Cell to cell contacts are very much necessary for MSCs proliferation *in vitro*, therefore small culture dishes can be preferably used for primary cultures when sample size is not enough. In case of bulk sample size, gentle removal of unwanted tissue layers and other tissue debris are recommended to avoid longer durations of enzyme treatments under high temperature conditions. Age of donor is also one of the crucial factor in determining the fate of MSCs. Various studies have already shown that aged donor derived MSCs resulted in low cell numbers and proliferation rate, higher population doubling times, and reduced differentiation potential [53]. Therefore, while selecting MSC source, age of donor should also be considered to get desirable results.

2.3. Basic Criteria for Characterization of Stemness: Cluster of Differentiation Markers

Expression of cell surface markers is one of the defined criteria for confirmation of stemness of any cell type [11]. Absence of appropriate markers creates difficulty for exclusive isolation of MSCs, Wetzig and colleagues, while working to identify markers for mesenchymal stem cells and non-mesenchymal stem cells (fibroblasts), have demonstrated that irrespective of differentiation potential, both cell types displayed similar phenotypes with no discrimination of surface markers [54]. In addition, only three differentially expressed markers namely CD24, CD108 and CD40 were revealed distinct in the comparative study. Porcine fetal and adult tissues derived mesenchymal stem cells during *in vitro* culture condition, express various surface markers as assessed by immunocytochemistry and flow cytometry. Differential expression of CD markers by various sources derived porcine MSCs have been shown in Table 1.

2.4. *In Vitro* Expansion and Long-term Culture of MSCs

Mesenchymal stem cells have capacity to expand and self-renew under *in vitro* conditions which makes them exclusive as compared to other normal cells. Senescence, higher population doubling times and reduced differentiation potential are some of the demerits of MSCs under long term *in vitro* culture. Vacanti and colleagues have shown that early and late passage porcine MSCs expressed similar cell cycle promoting enzyme p34cdc2 kinase but their cellular aging features such as actin accumulation, reduced substrate adherence, increased lysosomal β -galactosidase activity and reduced differentiation potential, were highly expressed in late passage MSCs [43]. It was observed that pMSCs and fibroblasts show varied telomerase activity and morphology, moreover pMSCs were stable and displayed unaltered phenotype compared to fibroblasts following prolonged *in vitro* culture [55]. Further in ESCs, for maintenance of undifferentiated status, cellular aging and senescence, an up-regulated telomerase activity is required [56]. Whereas, MSC's limited *in vitro* proliferation capacity may be due to down regulated telomerase activity [6].

Table 1. CD markers expression and differentiation potential of porcine mesenchymal stem cells.

| Source of MSCs | Positive marker expression | Negative marker expression | Differentiation potential | References |
|----------------------|----------------------------------|---|---------------------------------|------------|
| Bone Marrow | CD44, CD29, CD90, CD105 | CD14, CD45 | Adipo, Osteo, Hepato | [72] |
| Bone Marrow | CD29, CD105, | CD45 | Adipo, Osteo | [55] |
| Bone Marrow | CD29, CD44 | CD45, CD31 | Adipo, Myocytes | [41] |
| Bone Marrow | CD29, CD90, CD44, CD147, CD105 | CD45 | Osteo | [50] |
| Bone Marrow | CD105, CD44 | CD34, CD45 | Adipo, Osteo | [18] |
| Skin | CD29, CD44, CD90, | | Osteo | [31] |
| Skin | CD44, CD29, CD90, Vimentin | SLA-DR | Neuronal | [30] |
| Skin | CD29, CD9, CD44, CD105 | | Neuronal, Adipo | [32] |
| Wharton's Jelly | CD29, CD44, CD90 | CD45, CD49f, MHC CLASS 11, SLA-DR, PANCYTOKERATIN | Neuronal | [21] |
| Umbilical cord blood | CD29, CD105, CD49b | CD133, CD45 | Adipo, Osteo, Chondro | [19] |
| Endometrium | CD29, CD44, CD144, CD105, CD140b | CD34, CD45 | Adipo, Osteo | [26] |
| Endometrium | CD29, CD44, CD90 | CD19, CD34, CD45 | Adipo, Osteo, Neuro | [27] |
| Adipose tissue | CD29, CD44, CD90 | | Adipo, Osteo, Chondro, neuronal | [58] |
| Adipose tissue | CD44, CD29, CD90, CD105 | CD14, CD45 | Adipo, Osteo, Hepato | [72] |
| Adipose tissue | CD90, CD44 | CD45, CD31 | Adipo, Osteo, Chondro | [81] |
| Adipose tissue | CD90, CD44, CD105 | CD34, CD45 | Hepato | [78] |
| Aorta tunica media | CD90, CD73, CD105, CD56 | CD34, CD45 | Adipo, Osteo, Chondro | [36] |

As cell based therapies are relevant today, hence there is a need for large quantities of potent MSCs sources which can only be achieved through *ex-vivo* propagation using specific media, growth factors without altering the potency or the property of isolated MSCs. Therefore, current research is mainly focused on developing safer protocols in terms of stem cell isolation, expansion and stability, proliferative and differentiation potential during long-term culture.

2.5. Multilineage Potency of MSCs

Multipotent differentiation potential is one of the defined criteria proposed by ISCT, makes MSCs a favorable choice in regenerative therapy [11]. MSCs have a unique quality of multilineage differentiation upon induction with specific differentiation media, supplemented with growth factors. Understanding the molecular mechanism, intracellular pathways and factors responsible for various differentiation abilities of MSCs from different sources has been the matter of great interest since last decades. Initial investigations were

mainly focused on mesodermal differentiation capacities of stem cells, however with advancement in knowledge and technology such as gene targeting and protein engineering, MSC research has reached beyond mesodermal differentiation to multilineage specialized cells differentiation revolutionizing the field of regenerative medicine. Multilineage differentiation potential of porcine MSCs has been shown in Table 1.

2.5.1. Ectodermal Differentiation of pMSCs

Epidermal, neural and hair follicle stem cells are not only ectoderm in origin but also constitute indispensable systems of the body. Senescence, apoptosis, accidental issues, malignancies, environmental cues and sometimes malnutrition causes deformities in the physiology and functioning of these parts. Therefore, it is foremost required to generate same ectodermal parts from other available sources. Multilineage differentiation potential of MSCs has become the boon for such problems as they can differentiate into any of the three

germ layers. Recently, minipig bone marrow mesenchymal stem cells (BM-MSCs) have been successfully differentiated into neuron like cells, exhibiting distinctive neuron like morphological features. These differentiated cells were positively stained for nestin, neurofilament-M (NF-M), neuronal nuclei, β -tubulin, galactocerebroside (GAL-C) and glial fibrillary acidic protein (GFAP). Nestin, GFAP and NF-M expression was further demonstrated with the help of RT-PCR and RT-qPCR [17]. It has been observed that umbilical cord matrix (UCM) derived mesenchymal stem cells i.e., Wharton's Jelly mesenchymal stem cells (WJ-MSCs) have the ability of neuronal differentiation with the expression of neural markers such as TuJ1, neurofilament-M (NF-M), and tau *in vitro* [57]. Neuro-differentiated pUCM cells were successfully xenotransplanted into non-immune suppressed rats, where they expressed tyrosine hydroxylase (TH). Similar *in vitro* neuronal differentiations of UCM-MSCs have been shown by Mitchell *et al.* in their study [22]. *In vitro* neural differentiation of pMSCs has been served as a pivotal tool for the treatment of neurodegenerative disorders, in which cell replacement therapy can be done. While working with the Parkinson's mouse model, Kang *et al.*, (2013) compared the differentiation abilities of UCM derived MSCs and BM-MSCs from porcine, showing advanced features of UCM-MSCs over BM-MSCs. Results were supported by the expression of nestin, TH, neuronal growth factor (NGF), vascular endothelial growth factor (VEGF) and interleukin-6 (IL-6) markers at the site of cell transplantation. When neuro-differentiated cells were transferred into diseased mouse model, growth of dopaminergic neurons was recovered suggesting potential ability of UCM-MSCs in development of viable therapeutic strategies for Parkinson disease (PD) [21]. More recently, porcine endometrial stromal stem cells were successfully differentiated in neuron-like cells with the expression of neuron specific markers. Further they also exhibited electrophysiological property which confirmed the successful neuronal differentiation at functional level [27]. Matured *in vitro* differentiated neural cells with self-renewal ability and safer *in vivo* functions can be proved promising for the treatment of number of devastating neurodegenerative diseases. Easy accessibility, high potency, self-renewability and non-invasive extraction methods have been remained as main focus of stem cell study. Therefore, instead of bone marrow due to its highly invasive and painful extraction procedures, other convenient MSC sources are used from time to time. Porcine neural progenitor derived stem cells have successfully shown spontaneous neuronal differentiation into TuJ1-positive neurons and GFAP-positive astrocytes when cultured for varied long duration of 10 and 130 days respectively. Interestingly, these cells were initially grown in the presence of EGF and bFGF to attain the growth of spherical masses with self-renewal capacity [28]. Huang and colleagues have demonstrated the use of adipose tissue derived stem cells (ADSCs) from infant piglets for *in vitro* neuronal differentiation. It was observed that with the introduction of neuronal media, cells changed their morphology with shrank cytoplasm which further resulted into the formation of axons and dendrite like cytoplasmic projections. Cells displayed retracted cytoplasm towards nucleus which was followed by formation of contracted cell bodies with extended cytoplasmic extensions. Differentiated ADSCs expressed neuronal markers including microtubule associated

protein-2 (MAP-2), neuronal nuclear antigen, and β tubulin-III. Furthermore, immunofluorescence and western blot showed low expression of glial cell marker GFAP by ADSCs [58]. These results clearly evidenced the use of different cell sources for neuronal differentiation *in vitro*.

Skin is the largest organ of body and therefore it has been considered as jack of all trades. Sometime accidental cases, deep and external thermal injuries, hypertrophic scars and burning instances cause highly stressful and ugly effects to the skin which can be treated with early excision, skin grafting, skin substitutes, and engineered growth factors. Such skin related problems can also be effectively treated by administration of potent mesenchymal stem cells at the suffered site. BM-MSCs have shown promising results in quality wound healing at the site of skin injury in porcine minipigs. With the induction of specific Ham's F-12 media containing FBS, VEGF, bFGF and EGF growth factors, pBMSCs showed phenotypes with characteristics of vascular endothelial cells or epidermal cells. Confirmation of epidermal cell phenotype was done with anticytokeratin antibody immunostaining. Factor VIII and cytokeratin cell type markers used for vascular endothelial and epidermal cells respectively showed positive results. All such results indicated the ability of MSCs to get differentiated into desired epidermal lineages to be efficiently used in skin regeneration and wound healing [59]. Recently, Wang in his study on porcine skin regeneration has demonstrated that allogeneic BM-MSCs can be used to increase the skin regeneration efficiency and for the treatment of skin and soft tissue expansion. Different transplantation methods were employed in the study whereas local transplantation of allogeneic BM-MSCs to skin fascia layer showed better results in terms of increased expansion of skin area, thickened epidermis morphology and thinned dermis followed by higher VEGF, bFGF, EGF, and SDF expression was observed as compared to other groups [60].

2.5.2. Mesodermal Differentiation of pMSCs

Multilineage differentiation potential is the key factor of any cell to be called as stem cell in real sense. Basic *in vitro* tri-lineage differentiation capacity that is adipocytes, osteocytes and chondrocytes is one of the defined criteria for putative stem cells [11]. After treatment with specific media and growth factors, under maintenance of required environmental conditions for specified set of time, accumulation of lipid vacuoles, mineralized calcium matrices and type II collagen in differentiated adipocytes, osteocytes and chondrocytes respectively, is the confirmatory criteria for any stem cell to be considered as successfully differentiated. Morphological, histochemical, cytochemical, immuno cytochemical studies and polymerase chain reaction (PCR) analysis is used to check the extent of differentiation of stem cells.

Adipogenic differentiation is based on the time and concentration dependent treatment of stem cells derived from various sources, with three basic chemicals namely insulin, dexamethasone and 3-isobutyl-1-methylxanthine (IBMX). Rho et al, has already discussed the basic mechanism of tri lineage differentiation (Adipo, Osteo and Chondro) by pMSCs under the influence of different chemicals [61]. Porcine MSCs are more efficient for adipogenic differentiation, moreover pMSCs have shown comparable results to that of

human MSCs in the form of lipid accumulation, oil red O staining and gene expression [62]. Noort *et al.*, in their comparative study of porcine and human derived BMSCs have demonstrated that irrespective of the species specific MSCs, differentiation potential also depend on the donor as well as protocol used. Three different protocols used for both pBMSCs and hBMSCs showed similar results, indicating suitability of porcine derived mesenchymal stem cells for human clinical studies. Recently, endometrial stromal mesenchymal stem cells have been shown to express adipogenic markers with positively stained adipocyte induced cells [27]. Recently, in case of pBM-MSCs, use of oleate and octanoate has been shown along with most commonly used adipocyte induction supplements insulin and dexamethasone just for the time period of 10 days instead of 21 days [41]. Further, Oil Red O staining confirmed the successful adipocyte differentiation. RT-PCR and western blot analysis revealed the expression of adipogenic marker genes (PPAR- γ , C/EBP- α , perilipin, aP2) mRNA or proteins (PPAR- γ , perilipin, aP2) respectively. Interestingly, new combination of adipogenic supplements showed better results as compared to other treatment groups including use of insulin and dexamethasone, insulin, dexamethasone and indomethacin and insulin, dexamethasone, indomethacin and 3-isobutyl-1-methylxanthine respectively. One other study has also documented the use of oleate and octanoate for promotion of *in vitro* differentiation of porcine intramuscular adipocytes [63]. Further, Oleate was only found to induce the expression of PPAR γ mRNA, but not that of C/EBP α , whereas octanoate significantly induced the expression of both PPAR γ and C/EBP α mRNA. It was found that oleate and octanoate were responsible for accelerating the induced effect of insulin and dexamethasone on the expression of aP2 mRNA. These findings promote the use of oleate and octanoate in combination with insulin and dexamethasone to obtain better results at gene and protein level within shorter duration of time of 8 to 10 days. *In vitro* chondrogenesis requires induction of chondrocyte specific media in mesenchymal stem cells derived from various stem cell sources. Cartilage development during embryogenesis is initiated by a phase of condensation of mesenchymal precursor cells and for onset of chondrogenesis where cell to cell contacts aroused from condensation appears to be crucial [64]. In case of MSCs, micro mass culture is used to promote cellular condensation supported by the presence of transforming growth factor-beta (TGF- β), ascorbate and dexamethasone [9]. Pellet culturing is commonly used in chondrogenic differentiation of MSCs. Concentration of FBS also plays an important role in deciding the differentiation ability and cellular fate. Dose dependent study of porcine synovial derived MSCs showed that largest pellet size with enhanced Alcian blue staining intensity was observed under serum free conditions, moreover a gradual decrease was found with addition of serum in dose dependent manner [35]. A gradual decrease of cartilage specific genes like aggrecan and collagen II was also observed with increased concentration of FBS but there was consistent expression of the fibrous tissue marker gene collagen I, regardless of concentration of serum present in media. Concentration of TGF- β 1 has been found to be very important for chondrogenesis [65]. Other factors which influence the cartilage formation include biophysical stimuli like tension, compression, fluid flow and hydrostatic pressure [66, 67]. It has been found that

cyclic hydrostatic pressures (HP) can enhance BM-MSCs chondrogenesis at the gene or protein level independent of the presence or absence of TGF [65]. While experimenting with varied concentrations of TGF- β 3 along with HP on chondrogenic efficiency of porcine synovial membrane derived stem cells (SCSCs) and infrapatellar fat pad derived stem cells (FPSCs), Vinardell and colleagues have experienced that there was threefold increase in Sox9 expression along with significant increased accumulation of glycosaminoglycan when HP action was administered under low concentration of TGF- β 3 (1ng/mL) [65]. It clearly shows that HP also acts as important factor for maintenance of chondrogenic phenotype. Mesenchymal stem cells have been proved to be best source for cell therapy based treatments as well as regeneration of any defected tissue or organ of the body. Articular cartilage lesions do not heal adequately, therefore use of mesenchymal stem cells for cell based therapies in cartilage repair is very promising and porcine derived stem cells can be proved best for this kind of task.

MSCs have unique feature of differentiation into any cell lineage under the influence of specific induction media and controlled conditions [11]. Bone marrow, the firstly discovered source for MSCs, has been repeatedly shown to repair bone defects by osteogenic differentiation. Osteogenic differentiation includes long term (three weeks) culturing of MSCs in osteocyte specific induction media with the formation of nodules and calcium deposition which are visualized with Alizarin Red S and Von Kossa staining. Basically osteogenic media contains ascorbic acid, glycerol-2 phosphate and dexamethasone. Demonstration of osteo specific genes like osteopontin, osteonectin, osteocalcin and increased alkaline phosphatase (ALP) activity has been observed with the help of assays like immunohistochemistry, western blot and detection by fluorescence respectively [9, 31, 44, 50]. Wnt/ β -catenin signaling pathway plays an important role in the osteogenesis and bone formation [31]. β -catenin accumulated cytoplasm is translocated to the nucleus to begin osteogenesis related gene expression which is supported by the osteogenic master transcription factor Runx2. Suppression of C/EBP α and PPAR γ by Wnt signaling can also promote osteoblastogenesis of mesenchymal precursors [31]. Recently, it has been shown that N-cadherin is also helpful in the regulation of osteogenesis and bone marrow derived cell migration but prolonged N-cadherin overexpression also leads to inhibition of osteogenic differentiation of MSCs by negative regulation of β -catenin and ERK1/2 signaling pathways [68]. By silencing N-cadherin with shRNA, effects can be reversed. Moreover, blocking of N-cadherin with neutralizing antibody results into inhibition of osteoblast differentiation as N-cadherin mediated adherens junctions play important role in signal transduction during bone development [69]. It has been demonstrated that hyaluronic acid (HA) can increase proliferation and osteogenic differentiation of BM-MSCs. HA stimulates the proliferation of pBM-MSCs alone or in combination with dexamethasone [16]. pBM-MSCs when treated with interferon-alpha-2b not only slowed down the proliferation rate with constant speed but exhibited enhanced ALP production during osteoblast differentiation, indicating that interferon-alpha-2b either induces osteoblast differentiation or helps in osteoblast up regulation to increase their ALP production [70]. Heparin, a type of proteoglycan, has been found to inhibit BMP-2 os-

proteoglycan, has been found to inhibit BMP-2 osteogenic bioactivity by binding to both BMP-2 and BMP receptors. Along with these cellular activities and promotional or inhibitory effects of cytokines and growth factors, many new and exciting discoveries of transcriptional mechanisms are still required to construct a strong model for osteogenesis, and also to explore new programs for potential MSC differentiation. Various studies have revealed the multilineage differentiation potential of porcine mesenchymal stem cells. Apart from osteoblast, adipoblast and chondrogenic differentiation ability, pMSCs have been proved as good source for cardiomyocytes differentiation. Differentiated cardiomyocytes obtained from autologous or heterologous sources can be implanted without any rejection and can be judged for their potency to elicit improved cardiac function in myocardial infarct models *in vivo*. BM-MSCs can be chemically induced to functional myocytes by using demethylation agent 5-azaC [14, 17, 40, 41, 71]. pBM-MSCs treated with 5-azaC also showed 30-50% increased population of cells with cardiomyocytes characteristics positively expressed for α -actin, troponin T, desmin and connexin-43 [14]. Min-qing and coworkers while inducing myocyte differentiation from pBM-MSCs using 5-azaC have also shown positive expression of MyoD, Myf5, myogenin, desmin and S-MyHC by western blotting and immunocytochemistry [41]. Following the treatment with 5-azaC for different durations of 24 h, 3 days and 7 days followed by maintenance of cell culture up to 21 days, pBM-MSCs showed elongated stick like morphology with the formation of extended cytoplasmic processes towards neighboring cells [17]. Moreover, after the completion of induction period, strong positive expression of cardiomyocyte markers including α -smooth muscle actin, cardiac troponin T, α -cardiac actin and desmin was shown by differentiated BM-MSCs. Furthermore, prolonged exposure to 5-azaC did not influence the number of cells that expressed cardiac specific markers. In another study, integration of BM-MSCs into chronologically injured porcine heart resulted into cardiac function restoration and tissue perfusion [71]. These findings confirm the ability of pBM-MSCs to engraft and to differentiate into cardiac cellular elements. All these studies indicate safe, effective and admirable outputs of porcine derived mesenchymal stem cells (pMSCs) in the cardiac myogenic differentiation as well as their immense use in cardiac related disorders.

2.5.3. Endodermal Differentiation of pMSCs

It is very well known that mesenchymal stem cells can be obtained from different cell sources and can be differentiated into various cell lineages. But earlier it was thought that only endoderm based progenitor cells can be used in the generation of hepatocytes, while undergoing differentiation. Recently, studies have shown successful *in vitro* hepatocyte differentiation from various cell sources like bone marrow and adipose tissue [47, 72]. Bruckner *et al.*, recently described comparison between bone marrow and adipose tissue derived differentiated hepatocytes with the promoted hepatocyte-specific functions such as urea and glycogen synthesis, cytochrome P450 activity. It was found that all such results are comparable to cultured primary porcine hepatocytes. Although there was significant decreased CD105 expression after hepatocyte differentiation but the cells attained the

typical polygonal/cuboidal hepatocyte morphology [72]. All the above studies have advocated the use of 5-azacC, fetal calf serum, HGF and EGF in the differentiation medium. Bruckner *et al.*, 2013 have efficiently shown a detailed account of isolation and hepatocyte differentiation of porcine bone marrow mesenchymal stem cells (pBM-MSCs) [47]. Evidenced by different studies, it is clear that *in vitro* differentiated hepatocytes derived from various sources can be proved best for the treatment of inherited and degenerated liver diseases.

3. GENERATION OF INDUCED PLURIPOTENT STEM CELLS (IPSCS) FROM PMSCS

Embryonic stem cells (ESCs) have the potential to differentiate into all the three germ layers but due to limited resource, teratoma formation and ethical concerns, their use has been restricted in clinical applications. On the other hand iPSCs share similar properties to ESCs which can be successfully derived from reprogrammed somatic cells by inducing ectopic expression of transcription factors like Oct4, Sox2, c-Myc and Klf4 [73]. To ensure complete reprogramming other than *in vitro* differentiation potential, chimera formation is a prerequisite. Porcine derived iPSCs have been shown to form chimeras which contribute to all three germ layers and produce live offspring with high efficiency [74], interestingly gross and histological study from porcine chimeras at 2, 7 and 9 months demonstrated normal development without tumor formation. Early studies of mouse iPSCs displayed pluripotent properties but could not produce adult chimeras, showing partial reprogramming [73, 75]. In the similar way, porcine adipose derived mesenchymal stem cells (pADSCs) have been reprogrammed with the use of drug induced expression of human pluripotent factors to produce iPSCs. Reprogramming from pADSCs was found to be much more efficient than fibroblasts and confirmed by short cell cycle intervals, normal karyotypes, Oct4, Sox2, Nanog, SSEA3, SSEA4 expressions and alkaline phosphatase (AP) staining. Addition of Lif-2i medium containing mouse Lif, CHIR99021 and Lif-2i ignored the use of feeder layer and serum conditions [76]. Naïve ESC like cell differentiation was further confirmed by packed dome morphology, Lif-dependency, low expression of TRA-1-60, TRA-1-81 and MHC1, upregulated expression of Stella and Eras, and activation of both X chromosomes. Furthermore, significant upregulated expression of Lin28, Esrrb, Utl1, Dppa5 and differentiation into all germ layer cell types *in vitro* and *in vivo* indicated full reprogramming of naïve like piPSCs [76]. In total, with the elimination of tumorigenicity, iPSC technology can be efficiently used as a valuable tool for the development of complex biomedical models so that it could be easy to introduce or to manipulate genes useful for attainment of desirable traits.

4. STEM CELL THERAPEUTICS - PRECLINICAL ASSESSMENT

Nowadays, porcine has been taken as an excellent model for preclinical and therapeutic studies. Human and porcine have similar immune system. Genetically defined and fixed major histocompatibility complex of inbred minipigs have made reproducible studies of immunologic mechanisms. Therefore, from last few decades porcine has attracted atten-

tion as a valuable preclinical model for medical research. Porcine derived MSCs are highly renewable, proliferative, have high differentiation ability into any specialized cell type and produces non-inflammatory and immunomodulatory responses when used in clinical, therapeutical and transplantation studies. These all qualities attributes to the porcine MSCs as an excellent source of stem cells for cellular therapies. Brief description of pMSCs therapeutic potential in various animal models has been shown in Table 2.

4.1. Cell Transplantation in Acute Liver Failure

Acute liver failure (ALF) is a serious disorder in which rapid deterioration of hepatic function takes place, which ultimately results in jaundice, coagulopathy, and encephalopathy etc. Viral hepatitis, alcohol intake, intoxication above threshold value and disturbances in basic metabolisms are some of the factors responsible for acute liver injury with widespread hepatocellular necrosis. Under these circumstances liver transplantation is the only therapeutic method, but it has many limitations like shortage of donors, high operative risks, high cost and post-transplant rejection. Mesenchymal stem cell based therapy has proven best alternative for such deformities as they have multidirectional differentiation potential, low immunogenicity, anti-inflammatory and anti-apoptotic properties. Recently, administration of IL-1RA loaded chitosan particles with allogeneic MSCs transplantation into ALF swine models showed better synergistic effects including improved inflammation environment, liver function and hepatocyte proliferation as compared to control groups [77]. Moreover, increased levels of hepatocyte growth factor (HGF) and VEGF were observed in the same group. Although clinical application of IL-1RA is restricted due to its high cost and short biological half-life but high biocompatibility, biodegradability and low toxicity makes it a viable choice in clinical applications. Transplantation of porcine adipose derived stem cells (pADSCs) into rat model of acute-on-chronic liver failure (ACLF) has also shown improved survival rate and liver function [78]. Transplanted ADSCs were identified in periportal region of the liver. Time dependent analysis of liver tissues from ADSC/ACLF group displayed normal hepatic trabecular architecture with moderate mononuclear infiltration without any tumor development and hepatic abnormality. These findings revealed the successful therapeutic potential of transplanted pMSCs in the animal model. Due to multi-differentiation potential of MSCs, hepatocyte differentiation from different source derived pMSCs have been documented in various studies [47, 72, 77, 78]. When porcine derived ADSCs and BM-MSCs were characterized for their ability towards hepatogenic differentiation, significant decreased expression of CD 105 was observed from differentiated hepatocytes with the development of typical polygonal morphology of hepatocytes. Both the sources revealed comparable glycogen storage whereas cytochrome P450 activity was twice as high as in bone marrow derived hepatocytes as compared to adipose tissue [47]. Furthermore, more suitable stem cell resources are needed to check all the aspects of hepatogenesis which can give more promising results in the *in vivo* studies of large animal models suffering from acute liver failure.

4.2. Neurodegenerative Disorders

Neurodegenerative disorders occur due to selective loss of specific neurons in specified part of brain, hampering concerned functioning with mental and physical impairment. Alzheimer's disease is the most common neurodegenerative disorder which is followed by the Parkinson's disease in which progressive loss of midbrain substantia nigra dopaminergic (DA) neurons takes place. Degeneration of cell types in peripheral and central nervous system also takes place. According to Hawkes *et al.* (2009) [79], neuronal degeneration may first start in olfactory and enteric nervous system which is followed by brainstem alternations including vagus nerve, pedunclopontine nucleus, locus coeruleus and dorsal raphe nucleus. Finally, DA neuron alterations occur and later on, forebrain neurons such as cortical neurons also get affected. Intracellular inclusions called Lewy bodies and Lewy neurites also found distributed in nervous system under such neurodegeneration. To overcome such a drastic condition of brain, stem cell derived DA neuron generation and their transplantation into substantia nigra of midbrain proves to be the only therapeutic choice. Due to multilineage differentiation potential of stem cells, successful neuronal differentiation has been carried out from various stem cell sources [17, 21, 22, 57, 58]. While comparing *in vitro* differentiation ability of porcine BM-MSCs and UCM-MSCs, Kang [21] has evaluated that UCM-MSCs possess an increased potential to transform into immature and mature neuron like cells as compared to BM-MSCs which shows higher efficiency for adipocytes and osteocytes. When UCM-MSCs were transplanted into right substantia nigra of mouse PD model, improved basic motor behavior was observed with the expression of markers like nestin, tyrosine hydroxylase (TH), VEGF, neuronal growth factor (NGF), and interleukin-6 (IL-6) at the site of cell transplantation. These findings support the potential utility of UCM-MSCs in treatment of PD with no rejection of xenotransplanted MSCs. Furthermore, more attention is needed to understand the mechanism of basic pathways and role of factors involved in neurogenesis so that transplanted MSCs can display more consistent results for longer duration without any complication.

4.3. Wound Healing and Skin Repair

Skin is the most exterior part of animals including human beings. Certain accidental cases, traumatic injuries, exposure to radiation doses in radiotherapy, radio oncology and burning cases cause the deterioration of skin to critical levels. Stem cell based therapies including administration of differentiated mesenchymal stem cells from potent sources and transplantation are the alternative criteria's for treatment of skin related issues. Due to immune privileged properties and physiological as well as anatomical resemblance with humans, pig derived mesenchymal stem cells have been emerged as an excellent source. Auto grafting of BM-MSCs has been demonstrated in many full thickness skin injury mini pig models to ensure their wound healing quality [60, 80]. To explore the utility of auto grafted pBMSCs in the treatment of skin wounds, Fu and coworkers used different combination of MSCs with growth factors like bFGF, EGF along with saline as control group and growth factors treatment devoid of MSCs. It was demonstrated that there was outcome of desirable results in MSCs with bFGF treatment

Table 2. Therapeutic potential of porcine mesenchymal stem cells in various experimental disease models. SMCs: Synovial Mesenchymal cells, UCM: Umbilical Cord Matrix.

| Source | Cell type and concentration used | Clinical condition / Experimental animal model used | Observations | References |
|------------------|---|--|---|------------|
| Porcine BM-MSCs | Implantation of simvastatin treated BM-MSCs | Acute Myocardial infarction (Porcine) | Improved cardiac perfusion, reduced oxidative stress and proinflammatory cytokine expression, inhibition of apoptosis in periinfarct myocardium. | [91] |
| Porcine BM-MSCs | Allogenic BM-MSCs/ 150 x 10 ⁶ cells/ml | Myocardial infarction (Porcine) | Higher ventricular GAP43-positive and sympathetic nerve density, higher tenascin expression and increased cardiac nerve sprouting magnitude. | [93] |
| Porcine BM-MSCs | Allogenic BrdU ^{pos} and Y ^{pos} labelled male MSCs / 2.0 x 10 ⁸ cells | Chronic ischemic cardiomyopathy (Porcine) | Restoration of cardiac function and perfusion along with the formation of gap junctions followed by infarct scar reduction and neovascularization. | [71] |
| Porcine BM-MSCs | Autologous Di-I-labeled BM-MSCs implantation by direct injection / 6 x 10 ⁷ cells | Myocardial infarction (Porcine) | Robust MSCs engraftment, stable hemodynamic variables, attenuation of contractile dysfunction, maintenance of wall thickness | [88] |
| Porcine BM-MSCs | Xenogeneic BM-MSCs injected directly into periinfarct heart zone / 1 x 10 ⁶ 80 PD cells | Acute Myocardial infarction (Mice) | Engrafted MSCs survival for at least 4 weeks, better capillarity in periinfarct zone, Functional improvement in heart, | [45] |
| Porcine ASCs | Autologous ASCs / 50 x 10 ⁶ cells | Cutaneous radiation syndrome (Porcine) | Ultimate wound healing in 80% grafted animals with complete epidermis recovery with favored lymphocyte infiltration in irradiated dermis. | [81] |
| Porcine BM-MSCs | Allogenic BM-MSCs injected intradermally/ CM-Dil labelled/ 2.5/1.5/0.5 x 10 ⁷ cells/ml | Skin regeneration (Porcine) | Increase in expanded skin area, thickened epidermis and thinned dermis with higher VEGF, bFGF, EGF and SDF gene expression. | [60] |
| Porcine GFP-MSCs | Transplantation of IL-1Ra chitosan particles loaded MSCs/ 8 x 10 ⁷ cells | Acute liver failure (Porcine) | Significant improvement in inflammation environment, liver function and hepatocyte proliferation, elevation in levels of HGF and VEGF. | [77] |
| Porcine ASCs | Transplantation of BrdU labelled xenogeneic ADSCs/ 1x 10 ⁸ cells | Acute-on-chronic liver failure (Rabbit) | Improved survival rate and liver function, normal hepatic trabecular architecture with moderate mononuclear infiltration, no tumor development. | [78] |
| Porcine BM-MSCs | Implantation of autogenous tissue engineered undifferentiated BM-MSCs | Osteochondral defect (Porcine) | Smooth fully / partially repaired surfaces with better restoration of subchondral bone and lower pineda score. | [85] |
| Porcine SMCs | SMSCs derived tissue engineered constructs (TEC)/ 4 x 10 ⁵ cells/ml | Partial thickness chondral defect (Porcine) | Chondrogenic tissue like repair, exhibition natural cartilage like mechanical properties, absence of abnormal inflammation and necrosis. | [33] |
| Porcine SMCs | Allogenic SMSCs derived tissue engineered constructs (TEC) | Meniscal lesions (Porcine) | Defects filled with fibrous repair tissue showing good integration with adjacent host meniscal body, absence of any infection or abnormal inflammation. | [35] |
| Porcine BM-MSCs | Autologous BM-MSCs / labelled with PKH-26 / 10 x 10 ⁷ cells/ml | Endotoxin induced pulmonary hypertension and hypoxemia (Ex-vivo) (Porcine) | Reduction in endotoxin-induced pulmonary hypertension and hypoxemia, reduced lung edema, decrease in levels of IL-1 β , TNF- α and IL-6. | [48] |
| Porcine UCM-MSCs | Transplantation of eGFP engineered UCM-MSCs into rat brain / 150 cells | 6-OHDA lesioned rat brain (Rat) | No evidence of host immune response, differentiation into TH-positive cells with gradual increase in percentage | [98] |
| Porcine UCM-MSCs | Xenogeneic PKH26-GL labelled UCM-MSCs transplanted into right substantia nigra / 10 ⁴ cells/ul | Parkinson's disease (Mouse) | Engraftment and no host immune response, differentiation of cells into TH positive cells, improved basic motor behavior | [21] |

group which clearly shows that specific growth factor induced BM-MSCs can elicit better results for clinical skin repair in future [59]. While experimenting with porcine derived BM-MSCs, Wang and colleagues found that local transplanted BM-MSCs can increase the efficiency of skin regeneration and soft tissue expansion by differentiation into vascular endothelial cells and dermal fibroblasts with the use of growth factors like VEGF, bFGF, EGF and SDF. Even after removal of silicon expanders implanted on the back of pigs, there was reduction in skin shrinkage [60]. Adult stem cell sources rather than BM-MSCs have also good response towards skin regeneration by revascularization and immunocompetent cell recruitment of vascular endothelial differentiated cells. Use of porcine derived autologous adipose stem cells (pASCs) transplantation in irradiated mini pigs model lead to improved wound healing without any ill effects [81, 82]. Moreover, *in vivo* grafting experiments using porcine MSCs have shown successful transdifferentiation into vascular endothelial cell phenotypes on administration through local or transplantational route for skin regeneration.

4.4. Tissue Engineering

The role of MSCs containing tissue engineering constructs in repair of osteochondral defects is a critical issue of present era. As cartilage has a limited healing ability, tissue engineering is the best promising criteria for generation of functional tissue substrates to be used in cartilage repair processes [83]. An attempt was made to compare allogeneous tissue engineering, autogenous osteochondral transplantation and spontaneous repair for osteochondral articular defects in pigs [84]. Allogeneous chondrocytes and gelatin-chondroitin-hyaluronan tri-copolymer scaffold were used for tissue engineering based cartilage repair. Research findings revealed that *in vivo* cartilage tissue engineered tri-copolymer can be used for the treatment of full thickness articular defects. A subsequent study from the same group further confirmed that for the *in vivo* engineered treatment of osteochondral defects, it is better to use undifferentiated MSCs than TGF- β induced differentiated MSCs. The desirable results of undifferentiated MSCs group were supported by better cartilage restoration, fibrocartilage mixed with hyaline like cartilage filled defect areas and lower cell scores [85]. It might be due to more homing and differentiation ability of exogenous undifferentiated MSCs as compared to differentiated MSCs. Recently, few other studies have potentially advocated the feasibility of scaffold free tissue engineered constructs (TEC) derived from porcine synovial mesenchymal stem cells to repair incurable meniscal lesions [33, 35]. It was found that within 6 months, allogeneic TEC were able to develop fibrocartilaginous tissue at affected sites and subsequent meniscal body degeneration was prevented. Moreover, there was increased expression of cartilage marker genes like aggrecan and collagen II under serum free conditions and vice versa. However, understanding the fate of implanted cells and elucidation of mechanisms responsible for tissue repair is very much important for future studies.

4.5. Myocardial Tissue Repair

Cardiovascular diseases (CVD) have become the major cause of deaths worldwide. According to World Health Organization (WHO), it is estimated that heart diseases may

account for at least 23.6 million deaths by 2030. It is mainly due to the limited ability of heart for self-regeneration and inappropriate heart failure managements to inhibit myocardial scar formation and replacement of lost cardiomyocyte mass with functional contractile cells [86]. After acute myocardial infarction (MI) self-renewal capacity of heart becomes limited and it undergoes remodeling which results in depressed left ventricular (LV) function [87]. Rapid growing alternative for such problem is the implementation of stem cell based therapy called cardiomyoplasty in which damaged, diseased and attenuated myocardium is regenerated with the attenuation of ischemic heart disease [88]. Transplanted MSCs should have the ability to avoid immunologic detection and rejection by immunomodulation of defense barriers of host body. Myocytes that are not able to regenerate after birth creates major problems when there is any defect in heart regions or in their functioning. Organ transplantation remains the only choice of treatment under such circumstances. However, recent findings have proved that MSCs have the ability of trans-differentiation into cardiomyocytes [14, 17, 40, 71, 89]. Trans-differentiation of MSCs into cardiomyocytes has been successfully achieved with the induction of 5-azaC in the culture media. Similarly, porcine MSCs can be induced to skeletal myogenic differentiation [14]. It has been shown that intramyocardial injection of allogeneic bone marrow derived MSCs results in the improvement of cardiac function in myocardial infarcted porcine model [89]. More interestingly, there was no need of immunosuppression. Similar results have been shown by Kimura and colleagues, who successfully treated porcine model of chronic myocardial ischemia with allogeneic amniotic membrane derived MSCs without using any immunosuppressant [90]. Surgical injection of autologous porcine cross linkable membrane (CM) Di-I-labeled MSCs directly into post-MI swine myocardium showed successful engraftment when engrafted MSCs were observed into scarred myocardium and were positively expressed for cardiomyocyte markers like α -actin, tropomyosin, troponin-T, myosin heavy chain, and phospholamban within 2-weeks post-injection [88]. Another group has reported the potential of allogeneic BM-MSCs to engraft and differentiate into cardiomyocytes, endothelium and smooth muscle cells in a swine model of chronic ischemic cardiomyopathy [71]. In this study, injection of male BM-MSCs was given to female swine and co-localization was identified using Y-chromosome FISH. Further, the presence of approximately 14% of Y-positive MSCs was confirmed to be differentiated cardiomyocytes which were co-stained for the cardio structural proteins such as tropomyosin and α -sarcomeric actinin as well as transcription factors such as GATA-4 and Nkx2.5. Moreover, differentiated myocytes also exhibited coupling capacity with host myocytes via connexin-43. Approximately 10% of the Y-positive cells were shown to be participated in coronary angiogenesis (actinin, smooth muscle protein 22- α expression, calponin) and remaining 76% cells were identified as immature cell clusters in the interstitial compartment. Makkar and colleagues also showed the ability of engrafted allogeneic MSCs to differentiate into vascular cells and cardiomyocytes with the formation of gap junctions with adjacent host cardiomyocytes via connexin-43 [89]. In accordance with these findings to improve the cardiomyocytes differentiation up to 4-fold, Yang *et al.*, 2009 have

advocated the use of statin with intramyocardial injection of BM-MSCs [91]. These findings reveal the potential of MSCs for successful engraftment and differentiation towards cardiomyocytes and their role in the cell based therapies in heart related disorders. But the rate of successful engraftment and their detection after prolonged periods is always not the same. Results may vary according to type and number of MSCs engrafted as well as model selected for engraftment. Instead of source, potency and prolonged stability of transplanted MSCs in MI models, delivery methods of MSCs for myocardial repair is equally important. With the advancement in science and research methodologies, variety of approaches such as intramyocardial, transcatheter, venous, transendocardial, intravenous, intracoronary artery and retrograde venous administrations and bioengineered tissue transplantation have been devised to engraft the differentiated MSCs into patient's damaged myocardium [92]. The basic and initiatory obstacle in the selection, treatment and survivability of MSCs is their extremely low isolation rate as primary cells. Moreover, when these cells are propagated with growth enhancing culture conditions and implanted in diseased host body for proper recovery, they showed poor survival rates. To overcome these problems, pMSCs have been genetically modified by transduction with Akt, a serine-threonine kinase, to improve their ability for cardiac repair in porcine ischemic heart [93]. These genetically modified MSCs were found to be more apoptosis resistant and showed higher levels of VEGF and extracellular signal regulated protein kinase (ERK) activation. In total, all these studies collectively promote the use of pMSCs in treatment of cardiac diseases with possible genetic modifications and other parameters which are mandatory for effectiveness of MSCs based therapies.

5. IMMUNOGENICITY AND IMMUNOMODULATORY FEATURES OF MSCS

Despite immense proliferation and differentiation potential of porcine mesenchymal stem cells derived from various sources, their low immunogenicity, immunosuppressive and immune-modulatory properties makes them an important candidate for cellular therapy in allogeneic settings. Efficient use of allogeneic MSCs for repair of large defects may be an alternative to autologous and allogeneic tissue grafting procedures. Successive isolation, desired differentiation, safer cryopreservation and immuno-modulatory properties have raised the possibility of establishing allogeneic pMSCs banking for future tissue regeneration when needed. Allogeneic MSC infusions and host compatibility with non-production of immunogenic responses has been the matter of great interest in recent years, although a very little is known about host immune response to implanted MSCs. Experimental studies have revealed the successful generation of transgenic animals like pigs that express high levels of human CD46 in a cell and tissue type specific manner, which resembles similar patterns of endogenous CD46 expression as observed in human tissues. Transplantation of human transgenic porcine hearts into baboons did not show any hyper acute rejection with the survivality up to 23 days. These findings suggest the ability of CD46 expressing transgenic pigs to regulate complement activation and overcome hyper acute rejection upon transplantation into non-human primates [94]. Recently, in-

roduction of CD46 (human complement regulatory protein transgene) transgene into Gal-knockout (GTKO) pigs have shown the reduced incidence of rejection, supporting evidence that genetically modified porcine adipose derived MSCs function across the xenogeneic barrier and may have a role in cellular xenotransplantation [17]. Furthermore, GTKO/hCD46 pAD-MSCs expressed lower levels of swine leucocyte antigen I (SLA I), swine leucocyte antigen II DR (SLAIIDR) and CD80 before and after pig interferon- γ stimulation. Several studies have reported successful MSCs engraftment with some functional improvement after direct implantation across both allogeneic and xenogeneic transplant barriers without any immunosuppression and evidence of cellular infiltrate that would indicate an immune response [57, 95]. It has been reported that MSCs are involved in the immunosuppression as evidenced by mixed lymphocyte reaction (MLR) assays in various studies which revealed the failed proliferation of lymphocytes, secretion of cytokines and pro-inflammatory factors [96]. *In vitro* studies related with xenogeneic pig BM-MSCs have also shown to restrict human lymphocyte proliferation [97]. Upon stimulation, pBM-MSCs were found to inhibit the lymphocyte proliferation on dose dependence. Furthermore, addition of anti-FasL or anti TGF- β 1 antibodies caused attenuation of proliferation suppression, while antibody against IL-10 showed no ill effects on proliferative suppression. These findings suggest efficient transplantable abilities of xenogeneic pBM-MSCs due to their low immunogenicity. Porcine umbilical cord tissue (pUTCs) derived stem cells were evaluated for assessment of immunogenicity across a full MHC barrier in miniature swine model [96]. Absence of any detectable adaptive immune response during injection of unactivated UTCs showed low immunogenicity. These findings account for lack of MHCII expression or reduced MHCI expression on unactivated UTCs. Another possibility indicates a direct role of UTCs in modulation of immune response. However, up-regulation of MHCII can be done by exposure of MSCs to inflammatory cytokines such as interferon-gamma (IFN- γ) [95, 96]. Transplantation of porcine umbilical cord matrix stem cells (pUCMSCs) has also been hypothesized to show immunosuppressive effects in non-immunocompromised hosts [57, 98]. These reports strongly support the use of pMSCs in therapeutic applications and xenotransplantation studies due to their low immunogenic profiles and immuno-modulatory features.

6. DELIVERY METHODS, MIGRATION, HOMING AND FINAL FATE OF TRANSPLANTED PMSCS

Despite of MSC's differentional efficiency towards specific lineages, suitable delivery methods are equally important for the transplanted MSCs to function appropriately in cell based therapy. Stem cell based therapy can be made successful by employing efficient delivery methods and long term retention of transplanted cells within the damaged tissue followed by homing of the cells and secretion of factors indispensable for recovery. For safer percutaneous endovascular adipose derived mesenchymal stem cell delivery, a balloon occlusion catheter has been used in the porcine model of liver fibrosis, exhibiting no evidence of vascular complications and portal hypertension [99]. Interestingly, uniform distribution of MSCs in the sinusoidal spaces was

observed immediately after application. Furthermore, successful identification of transplanted MSCs was observed in porcine liver parenchyma. This study shows successful engraftment of cells, as balloon occlusion catheter is responsible for increasing the contact time between MSCs and sinusoidal epithelium. However, detection of transplanted GFP labeled MSCs could not be traced even after 4 weeks of application, showing lack of cellular engraftment for longer duration even after delivery of large number of cells. Using Porcine as an animal model, Barczewska *et al.* has demonstrated an efficient method for the minimally invasive delivery of pBMSCs into the porcine intervertebral disc (IVD). Super paramagnetic iron oxide (SPIO) nanoparticle labeling was used for pBMSCs, and for the efficient delivery transcatheter cannula and an epidural anesthesia catheter was used. Further, labeled MSCs were successfully monitored by MR *in vivo* [100]. This method has been found to be important for diminishing the risk of leakage which can lead to the formation of osteophytes. Finally transplanted MSCs resulted into functional benefit in terms of increased hydration and height of the treated intervertebral disc. Similar minimally invasive study has been conducted by other group of researchers in which allogeneic synovial MSCs has been shown to treat cartilage defects in pigs. Arthroscopic, histological and MRI studies showed that only within 10 minutes, synovial MSCs start migration and adherence to defect site which further cover the defect with membrane followed by the development of cartilage matrix [34]. Interestingly, no specific delivery methods including use of specific equipments such as catheters and scaffolds were used in this study. Although the Dil-labeled cells could be traced at defect sites only for 7 days but arthroscopic and macroscopic observations revealed formation of thin membrane covering over cartilage in one month followed by thick white membrane development at defect site and finally generation of cartilage tissue in MSC- treated knees during second and third month respectively. These findings indicate the secretion of specific tropic factors by the transplanted MSCs which enhanced the cartilage repair. Similar results have been observed by other study in which synovial MSCs have been efficiently shown to repair cartilage defect in a pig model [33]. It is therefore observed that delivery methods, migration, homing and final fate of transplanted MSCs hold promisable and unavoidable factors for any successful cell based therapy. Although non tracing of transplanted MSCs after certain time duration is also unignorable therefore much advanced efforts are needed to resolve this problem so that more success can be achieved in this field.

7. CRYOPRESERVATION OF PMSCS

Mesenchymal stem cells are self-renewable and have multi lineage differentiation potential. Cryopreservation is the only way for their long term storage. Therefore, selection and development of appropriate cryoprotectants (CPA) with less toxicity, higher cell viability, improved differentiation potential and unaltered morphological features is a prerequisite. Term cryoprotectant (CPA) has been coined to describe - any chemical or additive which can be treated with the cells before freezing and ultimately yields a high post-thaw survival of the cells comparable to the results without its use in normal conditions [101]. Different types of mammalian cells

can be easily and effectively stored by well-known cryopreservation method so that they can be further used for research and therapeutic applications including transfusion medicine and cell transplantation, when needed. Cryopreservation is the most reliable method by which all forms of MSCs whether differentiated or undifferentiated, can be preserved for long periods without altering their viability and differentiation potential. Many studies have reported the ill effects of cryopreserved cells in the form of altered doubling time, decreased viability, reduced gene expression and low differentiation potential [102]. All of these ill effects were found to be developed by the toxicity of CPAs, intracellular ice formation and rapid temperature change dehydration [101]. Generally cryoprotectants play their role by two ways: first, the type of protectant used like surface protectants which are impermeable and cannot penetrate inside the cell and the permeable protectants which can enter inside the cells, and the second is freezing rate that is slow freezers, moderate freezers or ultra-fast freezers. Use of different solutes such as dimethyl sulfoxide (DMSO), 1, 2 propane diol, ethanediol, ethylene glycol (EG), glycerol and sugars with high CPA activity have been reported in various studies for long term storage and preservation of cells [101, 102]. But most of the current time research deals with the use of 10% DMSO. As DMSO elicits its toxic effects on the cryopreserved cells, recently many researchers have tried cocktail combinations of DMSO and EG, use of trehalose and sucrose in combination with reduced and variable concentrations of DMSO, dimethyl acetamide (DMA) for the cryopreservation of chicken sperm with rapid freezing (50°C/min) [149, 150]. Park and colleagues in their study of long term dental tissue cryopreservation have also introduced cocktail of glucose, sucrose and ethylene glycol as modified cryoprotectants with a slow ramp freezing rate, showing no detrimental results [103]. Therefore, due to functional similarities between human porcine derived mesenchymal stem cells same cocktail cryoprotectants can be used for long term preservation of pMSCs. Moreover, it has been observed that FBS, used to prepare DMSO has animal origin and proved to be problematic during cell transplantation processes. In spite of these attempts, there is immense need for the development of new and effective preservation protocols. It is well understood that the need of preservation of MSCs can never be ignored, so much more research efforts are needed for the development of an easy and much efficient CPAs along with the special attention to their composition in cryopreservation media, cooling rate for harmless and repetitive good results.

8. LIMITATIONS

It is clearly evidenced by preclinical studies that porcine derived mesenchymal stem cells have great proliferative, differentiation and therapeutic potential. Ex-vivo expanded MSCs isolated from different sources have been efficiently used in cell based therapies with desirable outcomes without any deformities. It has been shown by many studies that not only bone marrow is the putative sources for regeneration of the damaged parts or other clinical studies but other sources such as dental, Wharton's jelly, amniotic fluid, placenta, skin etc. can give similar or better results without much complication. Many of them can be isolated without any invasive or painful procedures. Although MSCs are good enough to be

used in many clinical studies but there are certain limitations which need programmed elimination so that cell based therapies can be used as solution to near about all the disease related problems. One of the major problems is the high cost and short biological half-life of certain materials which are needed to enhance the efficiency of injected MSCs [77]. Other major limitation include non-tracing of transplanted MSCs after shorter durations [34, 99, 100] which sometimes leads to partially recovered disease status. It means they have short effect on the damaged parts and are needed to be injected again for prolonged relief. All the porcine related studies need much more safe and effective clinical trials before their actual use in human studies. It is quite interesting that MSCs can be isolated from almost all the post-natal tissues but their extremely low number and adverse effect of certain chemicals like trypsin during long term culturing to enhance their population also causes their deterioration and limited beneficial effects during *in vivo* studies. Use of fetal bovine serum as growth promotor in the media and DMSO for their preservation also causes problems like xenogeneic contamination and reduced post thaw efficiency. Invasive procedures and non-availability of clinically important MSC sources like bone marrow, pancreas uterine etc. from live animals is one of the big issue from last few decades. Therefore, much more intensive research and development of issue less techniques for isolation, expansion and administration of MSCs for clinical studies are needed.

9. FUTURE PROSPECTS

Selection of highly efficient MSC sources with ability to be successfully used as therapeutic agents in xenotransplantation and regenerative medicine for humans and other economically important species, has always been the main theme of research. Many species like rat, mouse, cat, rabbit, pig etc. have been reported for their potent stem cells. Comparatively larger size, easy assessability and unethical isolation of valuable mesenchymal stem cells from porcine have made it as an ideal candidate for cell transplantation and tissue engineering. Understandings about properties of different origin derived porcine MSCs and their *in vitro* and *in vivo* administration under specific set of conditions have resulted into the treatment of various diseases like Parkinson's disease, hepatic liver failure, osteochondral defects, skin wounds healing and its regeneration and many more. But before proceedings into human application, adequate pre-clinical trials are needed to perform so that clinical limitations could be reduced with the outcome of satisfactory results. Immunomodulatory properties and low immunogenic profile of pMSCs allows the brief understanding of basic mechanisms underlying homing as well as interactive behavior of MSCs with the immune barriers of the body. Moreover, it is very much essential to evaluate the conditions responsible for long term survivability of MSCs inside the host body so that again and again reinsertion of target specific MSCs and their *in vitro* formulation can be ignored. Lack of species specific markers for isolation and low number of isolated MSCs has always been the main problem. In addition loss of telomerase activity and differentiation potential upon prolonged culturing creates hidden but drastic effects on the efficiency of mesenchymal stem cells. Therefore, much more advancements are needed to improve the techni-

cal status of stem cells. Like iPSCs, generation of Tert, Oct and Ras transfected MSCs can be the better choice to overcome potency and senescence related many problems. Different genome of even similar species like human and pigs is the main cause of xenotransplantation failure. To overcome this problem many companies and renowned researchers are progressively collaborating. According to American biologist Dr. Craig Venter, by determining specific aspects of pig genome and their alteration in such a way so as to make porcine lungs compatible with humans, it could be possible to avoid xenotransplantation rejection [104].

CONCLUSION

Mesenchymal stem cells are the specialized cells which can be isolated from embryo as well as post-natal tissues, *in vitro* propagated, genetically modified and can be employed for treatment of many drastic diseases without painful operative procedures or tissue or organ removal. Their differentiation abilities, high proliferation rates, low immunogenic profiles and survivability under *in vivo* conditions without much complication makes them best alternative therapeutic agents in cell based therapies and transplantational studies. Both allogeneic and autologous MSCs have been appropriately used in stem cell based treatments. Moreover, various reports have shown satisfactory and pleasing results in term terms of homing capacity and curative measures but before their actual use in human application, it is very much important to check their various aspects of short and long term efficacy by means of adequate preclinical trials. An anatomical and functional similarity of porcine with humans, porcine animal model has attained much attraction and also proved as best candidate for understanding biological activities, basic mechanisms and their clinical implementations. Furthermore, easy isolation methods, multilineage differentiation potential and high survivability with remarkable achievements in transplantational studies and unethical issues strengthen the use of porcine MSCs in cell based therapies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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