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Porcine mesenchymal stem cells - Current technological status and future perspective

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1. ABSTRACT

Similarities of porcine mesenchymal stem/progenitor cells (MSCs) with human counterpart allow them to be considered as a valuable model system for *in vitro* studies and preclinical assessments. Effective isolation and expansion of porcine MSCs from different origins, namely bone marrow, umbilical cord Wharton's jelly, amniotic fluid, umbilical cord blood and peripheral blood has been reported. The differentiation of porcine MSCs into mesenchymal lineages under *in vitro* conditions is consistent and growing evidence has also suggested their transdifferentiation abilities. Results of preclinical studies unveil a time dependent retention, engraftment, migration, *ex vivo* and *in vivo* differentiation characteristics and possibility for genetic modification of MSCs. Findings on immunogenicity and the immunomodulatory capacity of porcine MSCs are encouraging and valuable to understand the host compatibility following transplantation. Furthermore, suitability of porcine MSCs as donors in nuclear transfer offers a greater potential to medicine and biopharming. Here, we highlight recent findings in the areas of porcine MSC sources, differentiation ability, transplantation applications and their potential as nuclear donors for somatic cell nuclear transfer.

2. INTRODUCTION

Stem cells are unique cells that exist in a mitotically quiescent form, have the capacity for self-renewal, and are capable of forming at least one, and sometimes many, specialized cell types (1). Because of their biological properties and potential medical importance, stem cells have gained the main stream attention. Stem cells have long been used to study the differentiation of different cell types during embryogenesis and organogenesis. Recently there has been growing interest in their potential for clinical applications in the repair or replacement of wide range of failing or damaged organs. Stem cells can be broadly grouped into two categories based on their origin from either the embryonic or the adult.

Embryonic stem cells (ESCs) are derived from explanted culture of the inner cell mass of blastocyst stage embryos, have the ability to differentiate into all somatic cell types, and provide scientists with powerful *in vitro* models for the study of normal mammalian ontogeny and complex genetic diseases. However, properties of ESCs have been so far only demonstrated in the mouse and, to a lesser extent, in human. Moreover, the derivation of proven

ESC lines from commercially important species, though 'desirable' have been elusive to date, perhaps, their biotechnology applications could potentially revolutionize agriculture, biomedicine, biopharming and xenotransplantation (2). On the other hand, the adult stem cells (ASCs) possess specialized, tissue or organ specific stem cell types, which can give rise to the differentiated cell types of that specific organ and have in some instances been shown to reprogram or transdifferentiate by expressing the markers and exhibiting the characteristics of cells of different lineage (3). Recent findings have suggested an unexpectedly wide developmental potential and greater plasticity of tissue specific ASCs than previously realized. In addition, few investigators have succeeded in isolating ESC-like cells from several tissues (4), which seem to exhibit various properties of ESCs including unlimited self-renewal without undergoing senescence (5). Eventually, it may lead to the possibility of harvesting these cells from adults to make their own stem cell derivatives available for use. Thus, ASCs might become an alternate choice to ESCs for cell based therapies.

Recently, there is growing interest in the pig as an animal model system to study and evaluate the choice of cells in the development of stem cell based therapy and transplantation (6). Since pigs possess organ physiology, size, and genetic characteristics similar to humans, stem cells of porcine origin would allow some results from other models to be confirmed and extended pre-clinically before realizing the goal of therapeutic applications in humans. Further, development of specific cell lines would be advantageous and is likely to increase its use as a large animal model in a variety of experimental research, including stem cell transplantation. In view of the foregoing, the establishment of stem cells from this source will have a pivotal role in the development of cell replacement strategies.

The current review highlights recent findings in the areas of porcine mesenchymal stem cells (pMSC) sources, *in vitro* differentiation ability, preclinical therapeutic applications and the potential of using these cells as nuclear donors for somatic cell nuclear transfer (SCNT).

3. PORCINE MESENCHYMAL STEM CELLS (pMSCs)

The mesenchymal compartment was first identified as source of progenitor cells when isolated as colony-forming unit-fibroblasts (CFU-Fs) from guinea pig bone marrow (7). These cells were subsequently identified as mesenchymal stem/progenitor cells (MSCs) due to their ability to self-renew and differentiate into multiple mesenchymal lineages (8). The identity of MSC still remains uncertain as there is no single universally accepted immunological definition and little standardization in the procedures used to isolate and culture such cells. In addition, there are no quantitative assays to assess the presence of MSCs in any given population. Recently, however, the minimal defining characteristics of MSCs were ascribed to: first, MSCs are plastic-adherent in standard culture conditions, second, they express the cell surface markers CD105, CD73, CD90, but lack expression of cell markers CD45, CD34, CD14 or CD11b, CD79-alpha or CD19, HLA-DR, and third, they differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* (9). These criteria apply only to human MSC, whereas adherence and tri-lineage differentiation are characteristics of cells from other species, such as porcine as their surface antigen expression is not yet universally well characterized (10). Therefore, MSCs are currently defined by a combination of physical, morphologic, phenotypic, and functional properties, many of which are clearly non-physiologic (11).

3.1. Sources

MSCs reside in a diverse host of tissues not only in fetal, but also through out the adult organism and possess the ability to differentiate into multiple lineages. Effective generation of porcine MSCs from bone marrow (BM) has already been reported by several investigators (6, 10, 12-17). Interestingly, cells displaying properties of MSCs have been isolated from umbilical cord Wharton's jelly (18, 19), peripheral blood (15), amniotic fluid (20) and umbilical cord blood (UCB) (21) in porcine (See Table 1). Putting together the present data, basically all post-natal organs and tissues contain MSCs. However, molecular and functional analyses demonstrate specific and clear differences among these pluripotent or multipotent subpopulations of cells.

3.2. Isolation and primary culture

The isolation and expansion of pMSCs has been feasible, using selected culture medium and protocols, as described for human MSC from adult and fetal tissues (8, 22). The bone marrow is still by far the best characterized source of MSC and almost all that is known about their differentiation is based on studies with marrow derived MSCs. As MSCs are relatively difficult to isolate from the bone marrow of live animals (10), many workers have collected after being sacrificed (6, 13). Moreover, one of the major problems in isolating MSC from pigs remains the lack of appropriate species-specific MSC marker molecules. However, a recent study established a novel, rapid and efficient method for direct isolation of adult MSCs from porcine bone marrow by using high specific nucleic acids called 'aptamers' as a probe to fish out the adult MSCs (23). Tissue samples are usually subjected to fractionation on density gradient solution such as Percoll gradient (13), Ficoll gradient (12, 14, 16, 17, 24-27), Lymphoprep (28), HistoPaque gradient (10, 15) and the mononuclear cell fraction obtained from the buffy coat at the gradient interface or isolated by washing the gelatinous bone marrow in phosphate buffered saline (PBS) (6, 29) and then explanted by plating cells at varying densities. MSCs are considered to be non-hematopoietic precursor cells and their identification is made on the basis of plastic adherent property with morphology as single, stretched cells of fibroblastic appearance the leading to the formation of large clusters/colonies (6). At this stage, however, endothelial cells, lymphocytes, and other fractions of hematopoietic cells may adhere to the culture dish and lead to contamination (30). Enrichment of MSCs has been achieved by expansion and passaging in relatively

Table 1. Expression of porcine mesenchymal stem cell surface markers analyzed using flow cytometry and immunocytochemistry

Source of porcine MSCs[1]	Surface antigens (CD markers)		Technique employed	References
	Positive	Negative		
Bone marrow	CD90, CD44, SWC-3A, MHC-Class I	CD45, MHC-Class II, SLA-DR	FCM[2]	12
	CD90, CD29, CD44, SLA-1, CD106, CD46	CD45, CD14, CD31, CD11b	FCM	62
	CD105	CD31, CD45, CD133	FCM	14
	CD90 (Thy1)	CD11b (ITGAM)	FCM/ ICC[3]	10
	ITGB1, CD44, Vimentin	CD14, CD45 (PTPRC)	ICC	15
	CD44, Cd90, SWC-3A, HLA-Class I	CD45, HLA-Class II	FCM	16
	CD29, CD44, CD90	CD14, CD31, CD45	FCM	28
	CD29, CD44, CD105	CD34, CD45	FCM	59
	CD29, CD49, CD44, CD105	CD133, CD45	ICC	120
Wharton's jelly (Umbilical cord matrix)	C-kit (CD117)	-	ICC	18
Amniotic fluid-derived Mesenchymal cells (AFCs)	CD105, CD90, Vimentin, von Willebrand factor, VE-cadherin, SM alpha -actin, SM22, PECAM	CD34, Sca-1, CD117 (c-kit)	ICC	20
Umbilical cord blood (UCB) MPCs	CD29, CD496, CD105	CD45, CD133	ICC	21
Whole blood MSCs (Blood MSCs)	ITGB1, CD44, Vimentin	CD14, CD45 (PTPRC)	ICC	15

Abbreviations: MSCs: Mesenchymal stem/progenitor cells[1]; FCM: Flow cytometry[2]; ICC: Immunocytochemistry[3]

deprivational, only serum containing medium, ultimately eliminating contamination (10, 11, 14). However, a limitation of the isolation technique used in above studies is that the procedure may not result in a sample of pure MSCs and there remain a heterogeneous mixture of cells with varying proliferation and differentiation potentials. The expansion of MSCs has been documented by using different culture media such as Dulbecco's modified Eagle's media (DMEM) (6, 15, 31), TCM199/DMEM (1:1, 14), alpha MEM (7), advanced DMEM (14, 21). The effect of media type on proliferation of pMSCs under low and high oxygen tensions has been investigated (10). The number and diameter of colonies were not different for pMSCs cultured in alpha MEM, DMEM 2.2, DMEM 3.7, but was significantly lower for cells growing in DMEM/F12. Further, an increased proliferation rate of pMSCs in low oxygen (5%) concentration may have more closely resembled *in vivo* conditions. Many investigators have employed culture media supplemented with 2-20% of fetal bovine serum (FBS) for *in vitro* MSC propagation and expansion. A clear, positive dose response relationship was observed between colony numbers and FBS concentrations, and no colonies were present when FBS was omitted (10). It is likely that the high concentration of serum in the culture medium contained some unknown growth factors. Overall, it is apparent that addition of 10-20% of serum to culture medium provides adequate support for pMSC expansion. However, the use of a culture medium with a low serum concentration (FBS, 2%) but added growth factors such as platelet derived growth factor (PDGF) and endothelial growth factor (EGF) resulted in successful pMSC isolation (12, 27). These data indicate that a growth factor enriched, low serum culture medium is effective for the selection of pMSCs. Despite the multitude of methods and procedures used in the isolation and characterization of MSCs, cells in fact remain the same and appear to retain similar potentials for expansion.

3.3. Expression of cell surface antigens

Porcine bone marrow derived MSCs proliferate *ex vivo* to form a phenotypically homogeneous population of cells that express several surface markers as determined by imunocytochemistry and flow cytometric analysis. However, expression of CD markers in pMSCs has not been yet defined and hence, the most characterized and promising markers with the highest specificities are described here (Table 1). Mesenchymal-like cells collected from human umbilical cord matrix (UCM) i.e., Wharton's jelly and MSCs from bone marrow share a relatively consistent set of surface markers, which is apparently convincing with the hypothesis that they are MSC-like (32). A large number of adhesion molecules, extra-cellular matrix proteins, cytokines and growth factor receptors are expressed by MSCs, and all associated with their function and cell interactions within the bone marrow (8, 33). As a result, antibodies that recognize cell surface antigens cannot be used independently to examine the phenotype of MSCs or for direct isolation, but must be used in combination. There is a consensus that pMSCs do not express hematopoietic stem cell marker in granulocytes, monocytes, natural killer cells, subsets of T cells, and subsets of B cells (6). Expression of CD markers in bone marrow derived cells supports the mesenchymal origin and seem to be conserved across species, because human and rat MSCs also expressed similar markers (for a review, 33). However, pMSCs promote the growth of hematopoietic progenitors by secreting numerous cytokines such as granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and leukemia inhibitory factor (LIF) (14). Differences exist among the reported studies in the surface marker characteristics that may be explained by variations in isolation and culture methods, tissue origin, and/or differentiation stage of the cells (11, 30).

3.4. Long term *in vitro* culture

Even though MSCs offer many advantages over other cell populations to be employed for tissue engineering, one major challenge is obtaining large number of cells, as they become senescent after a few population doublings (PDs). Despite improvements in long-term culture expansion, MSCs display finite life spans, uncharacteristic of immortalized 'stem' cells. In order to successfully use MSCs in therapeutics, it is essential that cells are properly identified; available in sufficient numbers and that they retain their characteristics after *ex vivo* expansion. Furthermore, it will be important to monitor the behaviour of cells during the expansion of cultures since it appears that MSCs undergo subtle changes during prolonged culture that can result in a marked decrease in their proliferation and potency for differentiation. After months

Table 2. Published reports on *in vitro* differentiation/transdifferentiation potential of porcine mesenchymal stem cells

Source and cell types	*In vitro* multilineage differentiation	Representative references
Bone marrow MSCs[1]	Osteocytes, adipocytes, chondrocytes	6, 10, 14, 15, 17, 34
	Osteocytes, adipocytes	13, 23, 82
	Osteocytes	31
	Cardiomyocytes	12, 62
	Neuron-like and Hepatocyte-like cells	64
	Osteocytes, chondrocytes	27
Peripheral blood MSCs	Osteocytes, adipocytes, chondrocytes	15
Umbilical cord blood Mesenchymal progenitor cells (MPCs)	Osteocytes, adipocytes, chondrocytes	21
Amniotic fluid derived mesnchymal cells (AFCs)	Endothelial cells, smooth muscle cells	20
Umbilical cord matrix (UCM) Wharton's jelly	Neuronal cells	18

Abbreviation: MSCs: Mesenchymal stem/progenitor cells[1]

of continuous culture and expansion, MSCs gradually cease division, and this has been associated with lack of telomerase activity (30). In support of senescence, pMSCs grew with regular pace until passage 10, and then they started to slow down and finally arrested growth at passage 15 with 40 PDs (13). This issue remains unclear with respect to cultured pMSCs as in another study; they have not been shown to senesce in culture for at least 80 PDs (27). Clearly, the methods used to amplify MSCs in culture may in part account for the variations since the growth and differentiation of MSCs can be profoundly affected by various serum components, growth factors, and culture conditions (14, 27). Due to inconsistency in culture conditions among few laboratories, comparison of data is not possible. However, phenotypic changes in adult pMSCs induced by prolonged passaging in culture have been demonstrated (14). In ADMEM medium, pMSCs were found to be able to go through more than 100 PDs in 40 trypsin passages without losing their ability to proliferate. Consistent with their robust ability to proliferate, both the early and late passage pMSCs expressed the cell-cycle promoting enzyme p34cdc2 kinase. Late pMSCs, however, exhibited certain features reminiscent of cellular aging such as actin accumulation, reduced substrate adherence, and increased activity of lysosomal acid beta-galactosidase (14). The robust proliferation capacity of the pMSCs may be in part due to the use of young donor pigs since the ability of stem cells to proliferate is frequently described as declining with age (34). Also, with an increasing number of passages and longer time in culture, MSCs tend to lose their multipotency. Early MSCs retained the multipotency, capable of chondrogenic, osteogenic, and adipogenic differentiation upon induction *in vitro*. In contrast, late MSCs were only capable of adipogenic differentiation, which was greatly enhanced at the expense of the osteochondrogenic potential (14). Along with these changes in multipotency, late MSCs expressed decreased levels of the bone morphogenic protein (BMP-7) and reduced activity of alkaline phosphatase (ALP). Late MSCs also exhibited attenuated synthesis of the hematopoietic cytokines G-CSF, LIF and SCF. However, a modified culture medium with low serum content that contained selected growth factors facilitated a high efficiency of isolation of bone marrow-derived pMSCs, expanded through 96 PDs with normal cytogenetic characteristics and *in vitro* differentiation capacity was maintained for at least 80 PDs, (27). The difficulties in expansion and assessing the quality of the cultures, in terms of the content of early progenitor cells which replicate most rapidly and have the greatest potential for multilineage differentiation, are compounded by the fact there are no universally accepted markers selective for MSCs. Until such markers are available and standard procedures for enrichment and culture are developed, then clinical grade cell culture will prove difficult to standardize.

3.5. *In vitro* differentiation

In addition to the general characteristics of MSCs described above, another defining feature is multipotency, or the ability to acquire multiple cellular phenotypes upon exposure to appropriate stimuli. Several laboratories have now demonstrated that MSCs recovered from a variety of adult tissues differentiate into various tissue lineages *in vitro* (Table 2). Studies have been carried out with isolated pMSCs in a well defined culture microenvironment to define the sequential steps and intracellular pathways that are involved in their differentiation into specific lineages. Though investigations were largely limited to the mesodermal differentiation capacity, based on recent reports, however, the spectrum of differentiation of pMSCs does not seem to be restricted to this lineage. The ability to modulate biological effectors to maintain a desired differentiation program, or possibly to prevent spurious differentiation of MSCs, is needed for effective clinical application, as in tissue engineering and regeneration.

3.5.1. Mesodermal lineages

Perhaps the most useful approach for presumptive identification of the MSCs remains functional. The capacity for induced *in vitro* differentiation of MSCs to bone, fat and cartilage is perhaps the single critical requirement to identify putative MSC populations (9). The differentiation into osteocytes that produce mineralized matrices, adipocytes that accumulate lipid vacuoles and chondrocytes that produce type II collagen under *in vitro* conditions is consistent and has been demonstrated by morphology, histochemical, cytochemical, immunocytochemical and polymerase chain reaction (PCR) evaluations (6, 10, 13-15, 17, 21). MSCs are widely regarded as the stem cell for osteoblasts, the cells that synthesize bone matrix (35). The typical default pathway for most MSCs, in culture is osteogenesis and bone marrow derived cells have been well documented for osteogenesis (6, 8, 36). When grown in osteocyte-specific induction media, osteogenic differentiation of pMSCs has been demonstrated in long-term cultures with an increase of ALP activity, a deposition of type I collagen, bone nodule formation and bone related marker gene expression. Clusters of round, calcium producing cells formed above the tightly packed monolayer and mineralization was

demonstrated by staining with von Kossa and Alizarin red S, markers for the osteocyte phenotype (17). ALP activity, an early marker of osteocyte differentiation, though present at basal levels in undifferentiated cells, significantly increased in osteocyte induced cells prior to calcium deposition (6, 17). Also, pMSC treated with dexamethasone (Dex) clearly demonstrated osteogenic differentiation in long-term cultures. Neonatal pig bone marrow stromal cells formed mineralized nodules in the presence of Dex and demonstrated ALP-positive cells along with a calcified type I collagen- rich matrix (36). In pMSC cultures, Dex induced the deposition of mineralized bone matrix and up-regulated the bone-related marker genes, such as osteocalcin, type I alpha 1 collagen and osteonectin (6, 37, 38). Uncommitted stem cells recruited by Dex toward the osteogenic lineage presumably lead to bone cell differentiation at the expense of growth and proliferation (39). Hyaluronic acid (HY) accelerated cellular proliferation, increased ALP activity and osteocalcin gene expression, and inhibited pro-collagen type I synthesis and the expression of type 1 alpha 1 collagen when bone marrow pMSCs were cultivated with HY alone or combined with Dex or recombinant human bone morphogenic protein-2 (rhBMP-2) (31). Induction of pMSCs with interferon-alpha-2b slowed the proliferation, but increased ALP production when the cells were presumably differentiated into osteoblasts (40). Thus, interferon-alpha-2b either induces the differentiation of pMSCs to osteoblasts or up-regulates osteoblasts to increase their ALP production. Evidently, extra-cellular matrix molecules are involved in both modifying cell responses to growth factors and cytokines and in regulating cell motility, growth, and adhesive interactions. A temporal sequence of events has been observed in the process of cellular proliferation with an enhanced expression of ALP occurring immediately after the proliferative period, and later an increased expression of osteocalcin, these genes then would activate the subsequent induction of genes associated with intracellular matrix maturation and mineralization when collagen deposition is promoted (31). Role of bone morphogenetic proteins (BMPs), in particular BMP-2 and BMP-6, have strongly been shown to promote osteogenesis in MSCs (41). Further, wingless (Wnt) pathways have an important modulatory function in osteogenesis (33). The exciting new discoveries of transcriptional mechanisms driving the balance of bone formation and its regulation provide a strong model for osteogenesis, and potentially other MSC lineage differentiation programs. These processes are frequently complex and in most cases require the simplicity of an *in vitro* system to begin to elucidate the molecular cues required to induce differentiation by employing animal models.

Differentiation of adipocytes from MSC has been suggested to be a two-step process: first, determination of preadipocytes, which are growth arrested cells while retaining their fibroblast-like morphology, and second, commitment and terminal development, which is characterized by the formation of lipid vacuoles filling the whole adipocyte (6, 42). Adipogenic induction of pMSC in defined medium containing insulin showed the accumulation of droplets filled with neutral lipids and the expression of distinct adipogenic marker genes. Here, 5-azacytidine (5-azaC) or insulin (43) promotes the determination of preadipocytes. Treatment with 5-azaC alone caused an initial rapid increase of growth, formation of multilayered cultures and some cells had visible lipid vacuoles (13). Incubation of monoloayers in a hormonal cocktail, which blocks the conversion of cAMP to 5VAMP resulting in an up-regulation of protein kinase A. The protein kinase A activity through the action of the regulatory molecule, perilipin results in the up-regulation of hormone sensitive lipase (HSL), which converts triacylglycerides to glycerol and free fatty acids. Indomethacin is a known ligand for the peroxisome proliferator activated receptor (PPAR) *alpha/gamma*, a key early transcription factor in adipogenesis (42). One of the early preadipogenic marker genes is the transcription factor *PPAR gamma* (44) and later in adipogenesis, genes directly related to lipid metabolism are expressed, for example *aP2*, which is induced by *PPAR gamma* (45). Suppression of Wnt signaling is required for the cells to undergo adipogenesis and this is achieved by *PPAR gamma* through accelerating the degradation of beta-catenin by the proteosome (46). The necessary inhibition of Wnt signaling for the progression of adipogenesis provides an interesting insight into the regulation of osteogenic versus adipogenic commitment by MSCs, since *PPAR gamma* activation can inhibit osteogenesis (47). One of the regulators between MSC osteogenesis and adipogenesis is the *PPAR gamma*, which upon activation promotes adipogenesis and inhibits osteogenesis (47, 48). Contrastingly, the Wnt signaling cascade can block adipogenesis and initiate osteogenesis of MSCs (49). This suggests that a fine balance between activated *PPAR gamma* and canonical Wnt signaling controls the differentiation potential of MSCs to either bone or adipose. In addition, notch and RhoA signaling pathways have also been implicated in differentially regulating osteogenesis and adipogenesis (50). However, no study has examined the expression of these regulatory molecules in pMSCs, except the demonstration of down regulated expression of BMP-7 in late pMSCs (14). Thus, a connection between BMPs and Wnt signaling in osteogenic differentiation has been shown to be consistent (31). Further, results of qRT-PCR assays demonstrated that the differentiation was accompanied by time-dependent increases in the selected transcripts previously shown to be enhanced with adipogenesis (6, 22, 44, 51). The genes *aP2* and *PPAR gamma2* are known to be involved in lipid metabolism and trigger terminal differentiation of preadipocytes into adipocytes (44) and expectedly, they increased steadily with adipocyte differentiation. There was also up-regulation in the mRNA levels for LPL, a lipid exchange enzyme and a late marker of adipogenesis at the time of completion of induction. These results evidently showed that phenotypic changes that occur in maturing adipocytes are paralleled by expression of the respective genes. Nevertheless, the expression of genes characteristic of multiple lineages and the possibility to direct differentiation of a large part of the cells towards specific lineages confirm that pMSCs can respond efficiently to inductive signals displaying a considerable level of plasticity.

In vitro chondrogenesis by pMSCs is typically carried out in micromass culture to promote cellular condensation in the presence of transforming growth factor- beta (TGF-beta) superfamily, in the presence of ascorbate and Dex (6, 8, 17, 51, 52). It has been reported that, the interactions between cells, as well as the lack of interaction between cells and substratum, are essential for *in vitro* chondrogenesis and maintenance of the chondrocyte phenotype (53). Formation of cellular mass was perhaps influenced by cell to cell interaction, autocrine and paracrine factors that are essential for lineage progression (8, 52). BMPs and cartilage derived morphogenic proteins (CDMP, also known as GDF-5), belonging to the TGF-beta super family, play a pivotal role in the formation of prechondrogenic condensations and the transition of condensed prechondrogenic cells in to chondrocytes (54). TGF-beta appears to induce chondrogenesis via protein kinases, including extra-cellular signal regulated kinase 1, p38, protein kinase A, protein kinase C, and Jun kinase (55). Together with BMP signaling, co-operation between BMPs and members of the Hedgehog family (Hh) has also been reported (56). Additionally, chondrogenesis is regulated by factors of the Wnt family. The TGF-beta mediated kinase activation also induces Wnt expression which I turn, up-regulates the expression of the adhesion molecule N-cadherin (55). High-density cultures of pMSC stimulated with TGF-beta1 and Dex evolved a compact, cartilaginous structure on the basis of histochemical analysis and cartilage-specific type II collagen deposition (6). Following the treatment with TGF-beta1, a monolayer of pMPCs transformed into chondrocytic cell clusters forming extensively condensed colonies with sulfated proteoglycan rich extra cellular matrix (17). Previous studies have shown that MSCs can differentiate into chondrocytic cells in monolayer in the presence of differentiation factors, such as TGF-beta 1 or TGF-beta 3 (51, 52). In addition, certain other chondrogenic bioactive factors including, TGF-beta 1, fibroblast growth factor and insulin-like growth factor are widely accepted as being main stimulants involved in enhancing chondrogenic differentiation (57). Expression of markers associated with chondrogenesis have been positively characterized in pMSC derived chondrocytes, including transcription factor (sox-9) and extra-cellular matrix genes, such as aggrecan, collagen type II alpha 1, collagen type XI alpha 1 and sulfated proteoglycans (58). However, the specific signaling pathways that induce the expression of these benchmark chondrogenic genes remain generally unknown.

The potentiality of MSCs from mini pig bone marrow to differentiate into endothelial cells was tested by culturing under endothelial cell growth medium (59). Following induction with vascular endothelial growth factor (VEGF), the morphology of the pMSCs gradually manifested to a ball-like appearance, adhered with adjoining cells and formed a tube-like structure. This was further ascertained by an increased number of Factor VIII-positive cells, which formed a tube-like structure. It is possible that multiple lineage-restricted stem cells in the marrow can differentiate independently into their analogous mature cells.

Differentiation of pMSCs into cardiomyocytes may be useful to analyze whether implantation of autologous or even heterologous MSCs, after myogenic differentiation *ex vivo*, the sustained engraftment and improved cardiac function in a porcine myocardial infarct model *in vivo.* While skeletal muscle itself contains stem cells known to be active in regeneration, but these cells are distinct from MSCs. pMSCs have been induced into the myogenic lineage both *in vivo* and *in vitro*. Chemical like an analogue of cytidine, 5-azaC has been reported to induce MSCs into myogenic cells (60). Examination of the myogenic differentiation of MSCs is currently being applied to cardiac muscle as well as skeletal muscle. Upon myogenic differentiation by using 5-azaC as an inducer, bone marrow stromal cells formed myotubes and cells were positive for vimentin, α-smooth muscle actin and desmin (61). Exposure of pMSCs to 5-azaC led to expression of a number of muscle specific protein genes, including phospholamban, skeletal α-actin, myosin LC and muscle specific desmin along with expression of troponin T in longitudinal fibers of treated cells (12). In another study, following treatment with 5-azaC, pMSCs formed a ball like shaped cells, with increased size, but without any signal of beating (13). In addition, morphological observations, expression of myosin HC and presence of lipid vacuoles indicated that 5-azaC promoted unspecific differentiation in their experiments. *In vitro* stimulation of pMSCs with 5-azaC resulted in an increased percentage of cells between 30-50% with cardiomyocyte characteristics, namely, positive for alpha-actin, T-troponin, desmin and/or connexin-43 (62). However, stably transfected and immortalized pMSCs were positive for alpha-actin and T-troponin but not for desmin or connexin-43. The above *in vitro* findings suggest that primary or immortalized pMSCs should be valuable tools for the study of the capacity of those cells to differentiate into cardiomyocytes for cellular therapy. Differentiation potential of porcine amniotic fluid cells *in vitro* in both endothelial growth medium and smooth muscle cell medium showed the enhancement of vascular cell markers, PECAM and VE-cadherin, and smooth muscle marker, alpha-actin (20). *In vivo* studies on the potential application of MSCs in the pig model for the treatment of myocardial infarction are discussed in the later section.

3.5.2. Ectodermal lineages

Similar to that of rodents and human MSCs, growing evidence suggests that pMSCs are capable not only of differentiation to mesodermal cell lineages but also of transdifferentiation to cells derived from ectoderm. It has been reported that, cells derived from UCM i.e., Wharton's jelly shown their ability to become cells with morphological and biochemical characteristic of neurons *in vitro* (18). Pig UCM cells appear poised to differentiate into neurons and were rapidly induced along this pathway by expressing neural markers such as TuJ1, neurofilament-M (NF-M) and tau *in vitro* (63). Later, they successfully demonstrated the xenotransplantation of pUCM cells into non-immune suppressed rats, where they engrafted, proliferated in a controlled fashion, and exhibited tyrosine hydroxylase expression in some cells. *In vitro* studies focused on the capacity of pMSCs to self-renew and

differentiate into neural phenotype. Most recently we have reported that, when treated under neuronal specific media *in vitro*, pMSCs derived from postnatal (immediately after birth) bone marrow exhibited over 80% differentiation into typical neuron-like cells with multipolar, round cell bodies arranged into a network like structure (64). Progressive differentiation of pMSCs coincided with expression of neuronal specific markers and genes, including beta III-tubulin, NF-M, nerve growth factor receptor (trkA) and glial fibrillary acidic protein (GFAP), at levels greater than control cells. The potential for exploiting the capacity of pMSCs to differentiate *in vitro* into mature neural cells and by assessing their function and safety *in vivo* further holds much promise for treating a number of devastating neurological diseases.

The ability of minipig MSCs to transdifferentiate into epidermal cells was demonstrated by culturing under epidermal cell differentiation medium (59). When pMSCs were treated by epidermal growth factor (EGF) in epidermal culture condition medium for 3 days, anti-cytokeratin-positive cells were discernible, accounting for about 3% of cultured and induced cells. Seven days after induction, the cytokeratin-positive cells were increased significantly to 13%. The pMSCs cultured in the control medium did not express any cytokeratin in their cytoplasm. These results give evidence that some MSCs could acquire the phenotype of epidermal cells as long as they were in the lineage-specific culture environment. Nevertheless, condition media might contain some undefined factors, such as insulin-like growth factor (IGF) or basic fibroblast growth factor (bFGF), as these factors could also induce MSCs to transdifferentiate into epidermal cells.

3.5.3. Endodermal lineages

Until recently, it was believed that hepatocytes could only be derived from cells of endodermal origin and their progenitors. We demonstrated the remarkable potential of postnatal bone marrow derived MSC to differentiate *in vitro* into an endodermal cell type with hepatocyte phenotype (64). Upon induction in hepatogenic differentiation media, MSCs acquired a cuboidal morphology and immunofluorescence staining exhibited the structural and functional protein markers of hepatocyte-like cells, including alpha-fetoprotein, albumin and hepatic nuclear factor-1 (HNF-1). Further, the expression of genes related to hepatogenesis showed a time dependent up-regulation for alpha-fetoprotein, albumin and HNF-1. Irrespective of the explanations of stem cell plasticity, the fact that bone marrow stem cell can differentiate into hepatocyte *in vitro* holds great promise for the treatment of inherited and degenerative liver diseases.

3.6. Preclinical therapeutic applications

Preclinical studies performed in porcine are highly relevant to humans and add a growing body of evidence on the safety and effectiveness of the transplantation of MSCs. In terms of effectiveness, results unveiling a time dependent retention, engraftment, migration, *ex vivo* and *in vivo* differentiation characteristics, and possibility for genetic modification strengthen the concept that MSCs form an ideal source of stem cells for cellular therapies.

3.6.1. Repair of myocardial tissues

A novel therapeutic option to prevent the progression toward heart failure involves the introduction of healthy stem cells into the infarct in an effort to repopulate the region, commonly referred to as cellular cardiomyoplasty (65). Cell transplantation by intramyocardial injection into diseased hearts (usually ischemic or infarcted) typically delivers cells into or adjacent to a poorly perfused segment. Whole bone marrow and subpopulation preparations have been used in early clinical trials. Transplantation of MSCs in porcine models has demonstrated that they integrate into implanted organs and contribute to the regeneration of many cell lineages (Table 3). In addition, MSC exhibits properties associated with the avoidance of immunologic detection and subsequent rejection (12, 16). Published data suggest that MSCs may be potentially used as an “off-the-shelf” strategy for allogeneic transfer (66, 67).

It has generally been believed that myocytes do not regenerate after birth. However, the recent finding that MSCs with transdifferentiation potential exist in postnatal tissues opens the possibility of using MSCs to treat myocardial infarction (MI) and heart failure secondary to left ventricular (LV) injury (12). Myocardial transplants of cultured heart cells (68), skeletal myoblasts (69), smooth muscle cells (70) and BM cells (61, 71) have been demonstrated in animal models to prevent failure after myocardial injury. Evidence suggests that exogenous agents such as 5-azaC can induce cultured BM cells to differentiate into cardiac-like muscle cells prior to injection into myocardial infarcts (61). Improved heart function with myogenesis and angiogenesis after autologous porcine BM stomal cell transplantation has been reported. Transplantation of 5-azaC treated BM stromal cells into the myocardial infarct region formed islands of cardiac like tissue, new capillaries, prevented thinning and dilatation of the infarct region, and improved regional perfusion and contractile function. The capability of MSCs engraftment in host myocardium and demonstrated expression of specific proteins related to cardiac muscle, vascular endothelium and smooth muscle (12, 65, 66). In addition, the extent of wall thinning after MI was markedly reduced in treated animals (65). Although the precise mechanism by which MSC implantation limits the extent of myocardial thinning after infarction has yet to be determined, it appears that extra cellular matrix alterations are likely involved (25, 65). The fibrin patch based delivery of autologous MSCs homed into the infracted region and differentiated into myocytes as evidenced by cardiac specific troponin T staining and improved the LV contractile performance and prevented LV aneurysm formation (12). Further this was the first evidence at the gene level to demonstrate that X-gal-positive myocytes were derived from transplanted stem cells and were not endogenous cells expressing beta-galactosidase. Though authors could not either exclude or prove that the *lacZ*-positive myocytes were the result of fusion between MSC with myocytes, the fusion could potentially result in cells having the appearance of blue myocytes. MSC cardioplasty may have significant clinical potential in attenuating the pathology associated with left ventricular wall infarction. Contrary to the BM derived

Table 3. Preclinical applications of porcine mesenchymal stem cells in porcine model

Source	Cell type and concentration used	Clinical condition/ approach	Salient observations	References
Porcine Bone marrow stromal cells	5-azaC induction of stromal cells to a myogenic phenotype Brdu labeling / $100x10^6$ 1.5 ml	Myocardial infarction/ autologous transplantation/ Injection	Induced angiogenesis, prevented thinning and dilatation of the infracted region	61
Porcine Bone marrow	MSCs[1]	Myocardial infarction	Over expression of cardiac tension	25
	MSCs labeled with a cross linkable membrane dye, CM-DiI / 20 million cells/ml	Myocardial infarct model / autologous cell transplantation	Capable of engraftment in host myocardium,may attenuate contractile dysfunction and pathologic thinning	65
	MSCs labeled with AdRSV-LacZ patch with fibrin matrix-MSCs	Myocardial repair	Improved the left ventricle (LV) performance, prevent LV aneurysm formation, prevent transition to heart failure, stem cell differentiation and increased vascularisation	12
	MSCs labeled with membrane dye CM-DiI and DAPI; before injection feridex labeled	Peri-myocardial infarction /allogenic transplantation / percutaneous injection/ 20 million cells/ml	long term engraftment, reduction in scar formation and near normalization of cardiac function	66
	MSCs labeled with BrdU	Myocardial infarction / intramyocardial injection / $2x10^8$ cells	Intramyocardial engraftment and differentiation into cardiomyocytes and endothelial cells	26
	MSCs transduced with Myr-Akt	Myocardial infarction	Improved LV ejection fraction, resistant to apoptosis	28
	MSCs labeled with DiI and transfected with angiogenin gene	Chronic ischemia	Improvement in myocardial perfusion and cardiac function	88
	Vascular Endothelial Growth Factor-MSC	Severe left ventricular hypertrophy / autologous transplantation/ Infusion /30 million cells	No heart failure, engraftment and increased myocardial capillary density	15
	MSCs	Endomyocardial transplantation	No evidence of calcification, teratoma or myocardial infarction	77
	MSCs	Myocardial infarction/ peri-infarct zones in nude mice	Preserved LV ejection fraction, engraftment and peri-infarct zone capillarity	26
	MSCs –Fluorescent dye	Myocardial infarction/ autologous or allogenic /intravenous delivery/$1x10^6$/kg B.wt	Improved myocardial viability and microvascular perfusion	67
Mini pig bone marrow	MSCs labeled with BrdU	Thermal skin injury	Enhanced re-epithelialization and wound healing quality	59
Amniotic fluid derived MSCs (AFCs)	AFCs labeled with CMFDA	Acute ischaemic myocardium / autogenic transplantation	No cardiac troponin expression and needs further *ex-vivo* reprogramming	20
Porcine umbilical cord matrix (UCM) cells	UCM cells labeled with PKH26 and expressing eGFP	Transplantation into rat brain	Cell can survive xenotransplantation and respond to local signals to differentiate along a neural lineage	63
UCM cells	UCM cells expressing eGFP	Transplantation into rat brain with a previous catecholaminergic lesion	Engraftment and no host immune response, cells differentiated in to TH positive cells	89

Abbreviation: MSCs: Mesenchymal stem/progenitor cells[1]

pMSCs, porcine amniotic fluid cells (AFC) could not increment vascular structures in the ischemic/post-ischemic environment (20). Survival of AFC did not seem to be long enough in the periphery of the ischemic lesion and to give rise to a target specific, committed progeny. These observations warrant *ex vivo* genetic and epigenetic manipulations to implement the surviving and differentiation potential of AFC.

The mechanism by which cellular therapy limits the extent of damaged myocardium after ischemic insult remains highly controversial and unclear. Four lines of mechanisms have been proposed to implicate in improving the cardiac function in response to the cellular therapy, which include regeneration of cardiomyocytes, increase of neovascularization, trophic effects and LV scar compliance (12). Results are consistent with the presence of both mechanisms, transdifferentiation of transplanted pMSCs into the cardiac myocyte lineage and increased endogenous repair mechanisms (65, 66). It is possible that stem cells that are committed to differentiate primarily along a particular pathway can switch to another lineage under the influence of signals present in the local environment (72). However, there are doubts about whether transdifferentiation really occurs, as ASCs spontaneously fused with ESCs and exhibited their characteristics, an event that might previously have been referred as transdifferentiation (73). Normal MSCs are known to produce multiple growth factors and cytokines and hence, they might have cell differentiation and paracrine effects in cellular regenerative strategies (74). Transplanted pMSCs have been shown to release cytokines such as VEGF that can exert a trophic effect on host cardiac cells, and not only promotes neovascularization but also regulates cell proliferation, migration and survival (16, 66). Cells also support a contribution of paracrine stimulation of endogenous repair mechanisms (75). It has also been argued that a stimulation of endogenous repair by injected cells might cause the regeneration of stem cell niches (76). Previous studies have shown that a specific microenvironment, which has been known as a 'niche', plays a very important role in deciding the phenotype and differentiation decision of stem cells (77). Multifaceted cell–cell interactions could lead to restoration of these

niches to make the cell therapy effective. Another line of evidence showed that cardiac nerve sprouting and sympathetic hyperinnervation improved the myocardial function, as the consequence of MSC transplantation may not be limited to cardiomyocyte generation (25). Cardiac nerve sprouting was induced by pMSCs without exogenous electrical currents or growth factors. MSCs have been shown to differentiate into nerve cells, migrate to remote sites and differentiated cells over express neurotrophic or other growth factors, which may stimulate both angiogenesis and nerve sprouting (78, 79).

Studies have demonstrated the potential therapeutic effects of allogenic pMSC transplantation after MI (26, 66, 76). Direct myocardial injection of allogenic pMSCs resulted in successful myocardial engraftment and differentiation into cardiomyocytes and endothelial cells, and preserved LV fraction after MI in pigs (26). Allogeneic transplantation of pMSCs resulted in long-term engraftment, profound reduction in scar formation, and promoted the regeneration of new cardiac tissue in the region in which the cells were injected (66). These effects produce early recovery of cardiac energy metabolism, followed by near normalization of systolic and diastolic cardiac function and substantial increases in global cardiac performance. Transplantation of a large quantity of allogenic pMSCs directly into the myocardium demonstrated for the first time that repeated administration of these cells is safe and feasible via percutaneous approach (80). When injected into healthy porcine model, pMSCs did not lead to teratoma formation, calcification or uncontrolled hypertrophy of the myocardium. Transplantation of long term cultured pMSCs into immunodeficient mouse hearts at the time of acute MI promoted the functional improvement in the infarcted heart despite minimal differentiation of MSCs into cardiomyocytes or endothelial cells (27). Authors have opined that beneficial effects of xenotransplantation most likely result from the paracrine effects mediated by cytokines and/or other substances released by engrafted MSC on native cardiac and endothelial cells. Transplantation of BM pMSCs via non infarct-relative artery stimulated cardiomyocyte regeneration and angiogenesis and improved cardiac function, but did not stimulate collateral artery growth (81). Further, pMSCs transplantation combined with hepatocyte growth factor (HGF) therapy was not superior to pMSCs alone transplantation. The exact paracrine stimuli and altered patterns of signaling pathway activation in transplanted cells remain to be defined in future studies. Taken together, all these experimental data support the view that MSCs are capable of secreting substances that are beneficial to stressed native cardiomyocytes. The questions that still need to be addressed are related to type of cell to be used, method of cells delivery, and the optimal time for transplantation (28). Nevertheless, these results collectively indicate that allogenic cells are therapeutically useful and therefore, offer substantial practical advances for the application of this therapy.

The ability of techniques to provide insight into stem cell retention, engraftment and homing for cardiovascular therapy has been assessed using pMSCs in porcine model. In animal models, most techniques for the study of stem cell transplantation require histological analysis to determine the fate and migration of cells (26, 61, 65). Moreover, delivery and tracking of endomyocardial stem cells are limited by the inability to image transplanted cells noninvasively in the beating heart. It has been demonstrated that, pMSCs labeled with a iron fluorophore particle (IFP) imparted useful magnetic resonance imaging (MRI) contrast, enabled ready detection in the beating heart on a conventional cardiac magnetic resonance (MR) scanner after transplantation into normal and infarcted myocardium (82). The ability to perform serial assessment of infarct size and regional cardiac function by MRI while tracking magnetically labeled MSCs offered a method to study the optimum protocols for delivery of MSCs for cardiac regeneration (83). A precise technique allowed guiding intramyocardial MSC injection to desirable targets, such as the border between infarcted and normal tissue (84). MR fluoroscope permitted visualization of catheter navigation, myocardial function, infarct borders, and labeled cells after injection. By providing continuous information, MR fluoroscopy allows targeted intramyocardial delivery and tracking of potential regenerative stem cell therapies.

3.6.2. Genetic modification and myocardial repair

Genetically modified cultured MSCs with genes encoding tissue-specific growth factors and cytokines can induce and maintain lineage-specific differentiation, and hence regarded as a particularly attractive cell type for physiological studies, functional genomics and cell based therapies. Expression of therapeutic proteins can give rise to tissues of different lineage expressing gene products essential for tissue regeneration and repair. Assessment of pMSCs for their capability to express a reporter gene enhanced green fluorescent protein (eGFP) under the control of a specific cytomegalovirus promoter (CMV), through plasmid electroporation and viral transduction has been made (13). Electroporation was shown here to be an efficient method for stably expressing a transgene in pMSCs and subsequently, these transfected cells were able to retain their multipotency. In addition they showed efficient viral transduction using a third generation self inactivating lentiviral vector into pMSCs. Stable genetic modification in the host cells by inserting a retroviral DNA allows following the differentiation process and integration in reconstituted damaged organs (85). A study evaluated the optimization of different non-viral methods, and viral methods by employing recombinant adeno-associated virus (rAAV)-mediated and retroviral gene delivery *ex vivo* for the vectors expressing eGFP and human BMP-2 with respect to gene transfer efficiency, maintenance of transgene expression, and safety issues in porcine MSCs (86). The high efficiency of rAAV-mediated gene delivery observed at high titers was explained by the ability of rAAV vector to transduce non-dividing cells and by its tropism towards porcine MSCs. Though less efficient, further research to improve non-viral gene delivery might be advantageous with respect to safety issues and ease of handling. Ability of pMSCs to undergo transient and stable genetic modifications using a combination of green fluorescent protein (GFP) plasmid with a transfection

reagent and viral vector has been demonstrated (10, 87). Efficient transduction of pMSCs with a non integrating human adenovirus and adeno-associated virus (AAV) vectors represented a potential tool to manipulate cells in culture. Continuing their efforts, Bosch and colleagues demonstrated for the first time that the polyamine based trasnfection reagent, GeneJammer, significantly improved Ad5F35 and Ad5 vector mediated gene transduction in cultured pMSCs (87). Transduced cells retaining their viability and potential to differentiate *in vitro* into mesenchymal lineages further envision the use of genetically modified pMSCs as promising vehicles to target various therapeutic molecules. Moreover, genetic stability and application of growth factors and cytokines along with favorable clonal cell propagation properties make pMSCs an attractive source of cells for preclinical testing in large animal model like porcine.

Augmentation of cell transplantation with proteins or genes might enhance the regeneration achieved with this therapy. Few studies have reported a synergistic effect of cell and gene therapy on heart function in a porcine model. To improve the strategy to overcome the poor survival rate of implanted cells, pMSCs were genetically modified by transducing with Akt, a serine-threonine kinase, to enhance cardiac repair in the ischemic porcine heart (25). Akt-MSCs were more resistant to apoptosis and the levels of extra-cellular signal regulated protein kinase (ERK) activation, and VEGF were higher in H_2O_2 stimulated Akt-MSCs. Characterization of these factors may have an important implication in elucidating the repair mechanism of stem cells. Recent study addressed whether MSCs transplantation would be beneficial to pressure overloaded myocardium undergoing hypertrophy in porcine hearts (16). Autologous transplantation of adenovirus infected VEGF over-expressing pMSCs resulted in significant amelioration of the progression of LV hypertrophy. Engraftment of VEGF-MSCs was associated with significant neovascularization and improved myocardial perfusion, and with improvement of myocardial bioenergetic characteristics and contractile performance (16). Similarly, transplantation of pMSCs, transfected with VEGF gene significantly improved myocardial ischemia, collateral formation and resulted in a favorable trend in LV functional improvement compared with transplantation of pMSCs alone (59). This approach might reduce the host inflammatory response that is a potential undesirable effect of direct adenoviral vector administration. A comparison was made on the effects of autologous MSC transplantation therapy and adenovirus containing angiogenin gene therapy, alone and in combination, on the restoration of cardiac function in the ischemic heart in a pig model (88). Over expressed angiogenin in pMSCs produced stronger beneficial effects than pMSCs transplantation alone on ventricular modulation and the greatest increase in myocardial reperfusion, and the combined cell and gene treatment therefore improved cardiac function in a synergistic manner. The beneficial effect could have been due to angiogenesis, enhanced cell engraftment, or an inhibition of the post infarction remodeling. All these studies collectively indicate the role of pMSCs in cellular therapy after MI, and suggest that genetic modification of MSCs would enhance the effectiveness of the therapy.

3.6.3. Skin regeneration

The possibility of using auto grafting of BM MSCs to enhance wound healing quality in full thickness skin injury has been explored in mini pig model (59). *In vivo* grafting experiments showed that pMSCs could convert into the phenotypes of vascular endothelial cells in granulation tissues, sebaceous duct cells, and epidermal cells in regenerated skin, implying that these grafted pMSCs might have transdifferentiated or interpreted as cell fusion between the grafted pMSCs and pre-existing endothelial cells or sebaceous duct cells. Results also indicated that pMSCs enhanced wound healing quality and generated *de novo* intact skin, resulting in perfect skin regeneration following full thickness injury. Thus auto grafting may help in reduction of scar formation and reestablishment of the normal anatomy and function of the skin, which is still posing a great challenge for scientists and clinicians.

3.6.4. Transplantation of pUCM cells

Useful multipotent stem cells isolated from UCM are closely related to MSCs and have shown to possess potential therapeutic features. The transplantation of pig stem cells derived from UCM into rat brain survived at least 6 weeks without undergoing immune suppression therapy (63). The findings after transplantation further demonstrated that pig UCM cells are relatively non immunogenic, they respond to local cues found in the adult rat and modified their morphology and neurochemical phenotype to resemble neural cells, and cells engraft without stimulating significant immune rejection. A subsequent study from the same group further confirmed that pUCM cells engraft and proliferate without requiring immune suppression of host animal (89). Most importantly, a subset of pUCM cells differentiated into tyrosine hydroxylase (TH) -positive cells within 8 weeks after transplantation into the neurotoxin 6-hydroxydopamine (6-OHDA) lesioned rat brain. Additionally, pUCM cells engrafted without eliciting immune rejection response. These findings of xenotransplantation have collectively provided a therapeutic approach for the treatment of neurodegenerative diseases such as Parkinson's disease.

3.6.5. Tissue engineering

The contribution of implanted BM cells to the repair of an articular osteochondral defect is a critical issue. A better understanding of the mechanism of tissue engineered repair of an articular osteochondral defect such as *in vivo* differentiation and distribution of BM cells offers a promising future. An attempt was made by using autologous chondrocytes as the seed cells for repairing articular osteochondral defects in a porcine model and the results showed that the subchondral bone defect was filled with engineered cartilage leading to a satisfactory repair (90). A recent study explored the feasibility of repairing articular osteochondral defects using autologous BM cells and biodegradable polymers (91). The results strongly indicated that implanted BM cells could differentiate into either chondrocytes or osteoblasts and repair articular

osteochondral defects by forming engineered cartilage and engineered bone. Partially induced BM cells into a chondrogenic phenotype may able to differentiate further into osteoblasts through the process of endochondral ossification. It is also possible that endogenous MSCs might play a role in subchondral bone defect repair through local recruitment in addition to a strong spontaneous repair capability of subchondral bone. However, issues that need to be addressed in future studies include the influence of animal age on the repair and the repair of articular osteochondral defects at weight bearing areas. Furthermore, to regenerate a true functional tissue, it requires the use of fully characterized MSCs, scaffolds and selective differentiating factors that are still to be identified.

3.7. Immunogenecity and immunomodulation features

Studies on immunogenicity and the immunomodulatory capacity of MSCs are more valuable for basic and clinical research on cellular therapy. Allogenic MSC infusions and host compatibility has been the subject of much interest in recent years. Preclinical studies performed using porcine as a model have included various cell types, such as a source of autologous and allogenic, unpurified BM stromal cells, MSCs and *in vitro* differentiated cells. Experimental studies have shown variations in the infusion procedure (intramyocardial, intracoronary, and intravenous), the number of injected cells and the clinical condition of the cardiac receptors. Despite interesting observations of persistence of MSCs after infusion into hosts, little is known regarding host immune response to MSCs after transplantation. *In vitro*, pMSCs fail to induce allogenic responses in mixed lymphocyte reaction assays and they avoid lysis by cytotoxic-T cells and natural killer cells. Miniature pig derived MSCs are reported to have a low immunogenic profile *in vitro* and show suppressed proliferation of human peripheral blood lymphocytes (hPBL) to various stimuli in dose dependent, but antigen dependent fashion (12). These findings suggested the role of FasL and TGF-beta 1 mediated pathways in pMSCs suppressive ability. However, the molecular mechanisms involved in the immunosuppressive properties of pMSCs are still not completely understood. Another study investigated the immunogenicity and the immune modulation ability of undifferentiated and osteogenic differentiated MSCs from mini pig BM (16). SLA class I (P1, P14) was detectable on both differentiated and undifferentiated pMSCs. But SLA class II (DRA and DQA) was detectable and expressed slightly higher in osteoblasts. Interestingly, both cells did not elicit proliferation of hPBLs and showed significant suppression of the proliferative responses of hPBLs to mitogenic stimuli. There is indirect support for an immune-suppressive effect of the MSC-like cells derived from umbilical cord matrix. Transplantation of pUCM cells xenogenically in non-immune suppressed hosts was demonstrated without observing acute immune rejection (63, 89). These results support the hypothesis that UCM cells, which are closely related to MSCs, may have immunosuppressive effects.

Despite the evidence from *in vitro* studies that pMSCs do not elicit a robust T-cell proliferative response or even exert some immunosuppressive effect, it has recently been shown for the first time *in vivo* in a mini pig model that without immunosuppression, whether allogenic MSCs are injected subcutaneously or into ischemic myocardium, cells did elicit a complete immune response (92). Proliferative response was not observed in pMSCs either unstimulated or stimulated with interferon-γ *in vitro* supporting the findings of other studies. Whereas in *in vivo*, all animals receiving skin grafts developed cellular and humoral responses (immunoglobulins M and G) with antibody-complement-mediated cytotoxicity. These results indicate the differences in characteristics of allogenic MSCs *in vivo* and stress the importance of *in vitro* and *in vivo* studies in future.

3.8. Limitations to clinical applications

Many preclinical studies have revealed that *ex vivo* expanded MSCs isolated from different origins represent a viable option for cell based therapies. The cells described from many tissues have shown to be responsible for regeneration of damage and maintenance of tissue homeostasis. Some of these cells appear to be capable of differentiating across tissue lineage boundaries. However, studies proposing such 'lineage switching' of cells remain inconclusive due to either failure to reproduce results or lack of accurate interpretation of experimental findings (for a review, 93). Hence, from the perspective of therapeutic promise, perhaps the greatest need is to understand the mechanisms that govern lineage specific differentiation of these cells as well as on their transformation to stable and functional mature cells. Using the porcine model, although the beneficial responses of MSC transplantation have shown to engraft and populate recipient tissue in a time dependent and tissue specific manner, the molecular mechanisms underlying these beneficial effects remain to be elucidated. Further, *in vivo* studies with different clinical conditions, infusion procedures and doses, assessment of varied parameters, and application of a wide range of detection techniques have resulted in the generation of complex data and hence, a direct comparison of these observations cannot be made. Since most of the studies are short-term, the issue of efficacy endpoints has not yet been addressed, and it requires careful attention by employing long-term studies. Despite the impact of these preclinical studies is difficult to assess on qualitative terms, it is assumed that the data obtained on procedural safety is more reliable and relevant to humans. In this regard, evolving studies on the preclinical therapeutic applications must be performed by a suitable methodological analysis that ensures accurate, reproducible, and sustained data. In addition, a better understanding of the effects of culture expansion on epigenetic events and genetic regulation of MSCs is a critical requirement (11). A clear knowledge of candidate molecules and mechanisms involved in the effectiveness of therapy will also contribute for optimization, and open new alternatives for the utilization of MSCs.

3.9. Generation of hybrid cells

The potentiality of ASCs has been believed to be limited by arrest of growth, and is a major barrier to achieving desirable therapeutic effects (11, 30). A recent

study has been reported for the first time on the generation of experimentally induced cell hybrids by fusion of primary pMSC with an immortal murine fibroblast cell line (29). All tested hybrid cell lines retained osteogenic differentiation, and a few of them retained adipogenic potential. Further, an improvement in the proliferation of primary pMSCs through their treatment with hybrid cell derived media was achieved. These findings show that pMSCs have the capacity to reprogram the adult cell genome after cell fusion. Moreover, these results allow hybrid cells to be employed in various studies to broaden our knowledge of regenerative biology.

4. NUCLEAR TRANSFER (NT)

The procedure of introducing a nucleus from an embryonic or adult donor cell into an enucleated oocyte to generate a cloned embryo is termed as nuclear transfer (NT) (94). NT is used for the purposes of reproductive cloning and more recently for therapeutic cloning (95). It provides a powerful tool for studying the fundamentals of developmental biology and has also numerous potential applications in agriculture and regenerative medicine (96). Reproductive cloning has been successfully applied to range of mammalian species for the production of offspring using a wide variety of donor cell types derived from both fetal and adult tissues (97, 98). The technique of NT allows the simple propagation of endangered species and has potential applications in successfully producing transgenic offspring by manipulating the genomes of somatic or stems cell lines (99). Stem cells derived from NT might also provide a means of screening candidate drugs and compounds with the potential to counter disease progression. Nuclear reprogramming by NT has been a unique phenomenon for functionally testing the nuclear potency of various donor cells and NT remains the tool of choice for studying reprogramming at functional, cellular, molecular and biochemical levels (100). Furthermore, NT has been used to study the role of genetic and epigenetic alterations during development and disease. The development and transplantation of autologous cells from NT-ESC lines to treat various diseases represent a strategy of therapeutic cloning. NT is ultimately aimed at generating uncommitted stem and progenitor cells that may be useful for medical research and cell replacement therapies. Continued research is essential to improve the frequency of development of cloned embryos and to provide basic knowledge on the control of cell differentiation and maintenance of the undifferentiated state. Notably, experiments in a mouse model have suggested that NT for therapeutic applications will work (101, 102). Further, cell transplantation studies derived from NT embryo or offspring in animal models may provide useful research into the effects of mitochondrial DNA heteroplasmy and the potential immune response and risks associated in transfer of mitochondrial related diseases. However, many technical and safety issues must still be addressed before these findings can be translated into actual therapy.

4.1. Factors influencing nuclear transfer

Successful somatic cell cloning with a variety of cell types in several species demonstrated the ability of oocyte cytoplasm to reprogram a somatic donor nucleus to a pluripotent state (96). Although NT has proved to be applicable across a range of species, the frequency of development to term remains relatively low with losses because of developmental abnormalities throughout embryo and fetal development and following birth (97, 99). Numerous studies that address the low efficiency of NT at either the cellular or molecular level have also been reported. A number of factors attributed to affect NT efficiency, include the cell cycle stage of donor nucleus and recipient cytoplasm, genetic background, passage number of cultured cell, loss of imprints, the differentiated state, accumulated genetic damage of the donor cell, or the ability of the oocyte to epigenetically reprogram the donor nucleus (103, 104). Moreover, recipient oocyte age, micromanipulation technique, manipulation medium, activation protocol, and embryo culture method also affected the efficiency of NT (99). Available experimental evidence from diverse model of organisms indicates that the abnormalities and fatalities of cloned animals are due, at least in part, to the faulty or inadequate establishment, maintenance and setting of epigenetic reprogramming during and after NT (94). Epigenetic modification has been central to genome reprogramming in NT and the challenges of epigenetic reprogramming after NT with specialized donor cells include DNA methylation, genome imprinting, X-chromosome inactivation, chromatin remodeling, histone modifications, telomere maintenance and epigenetic inheritance (98, 105, 106). Incomplete reactivation of embryonic genes and failure to inactivate somatic genes has also been held to be the prime causes of NT efficiency (98) Following NT, broadly two major reprogramming events take place, first, reversal to pluripotency, and second, establishment of new differentiation programs (107). Understanding the molecular mechanisms and dynamics underlying the reprogramming process will help to improve the NT technology and facilitate possible therapeutical applications (108). At present the means to enhance the efficiency of NT are not clear, but may involve the use of various strategies which broadly include modification of donor cell characteristics and the NT procedure (101) Although some improvements in the success of NT are to be expected from optimization of existing procedures, greater benefits might be anticipated from intervention to assist reprogramming of the transplanted nuclei.

4.2. Current aspects of porcine nuclear transfer and competency of pMSCs as nuclear donors

SCNT in pigs is a fast advancing area of large animal reproductive biology. Over that last few years, intensive studies made in porcine NT have resulted in birth of cloned pig offspring (109-114). Despite of the low overall efficiency (1-2%), SCNT is believed to be the most reliable approach to produce piglets for the support of human medicine by providing organs for transplantation, animal models for diseases and using animals as living bioreactors to produce therapeutic proteins for human medicine (99, 115). Discussion of more technical details related to SCNT in pigs is beyond the scope of this review and hence, the findings that have been relevant to explain the current status of porcine NT by using various donor cells are briefly outlined below. Perhaps, the most recent

Table 4. Summary of data on development of porcine cloned embryos using porcine mesenchymal stem cells with different donor cell types

Donor cell	Cleavage (%)	Blastocyst (%)		References
		Day-6	Day-7	
Adult fibroblasts	86.0	34.9	37.2	13
Bone marrow MSCs[1]	90.6	40.0	44.7	
Early osteocytes	86.6	31.7	32.9	
Osteocytes	86.0	39.5	38.4	
Adult fibroblasts	82.8±3.4	13.3±3.2	17.5±3.9	15
Bone marrow MSCs	83.1±3.4	25.2±3.2	29.5±3.9	
Skin fibroblasts	44.5	1.77	-	10
Bone marrow MSCs	53.1	4.1	-	
Fetal fibroblasts	50.8±5.2	-	9.5±2.1	17
Bone marrow MSCs	52.2±5.4	-	18.4±3.1	
Fetal fibroblasts	64.7±3.6	-	9.9±1.8	120
Bone marrow MSCs	53.8±3.4	-	20.1±3.0	

Abbreviation: MSCs: Mesenchymal stem/progenitor cells[1]

achievements both in the background procedures (*in vitro* maturation, activation, embryo culture) and the technique of SCNT in pigs and its future possible applications have exhaustively been reviewed (116).

SCNT is a multi-step technology with a number of variables influencing the ability to reproduce a specific genotype. These includes cell type of nuclear donor, enucleation of a recipient ova, transfer of a donor nucleus into a recipient cytoplasm, the method of artificial oocyte activation, *in vitro* embryo culture, and finally embryo transfer (113). To increase the chance of success, efforts must be made to minimize the inefficiencies at each step of NT. Among these steps, the selection of cell type used to produce cloned pigs is of paramount importance as it determines the developmental potency of reconstructed embryos by affecting the successful reprogramming of the donor nucleus (96). In recent years, donor cells used in the production of cloned pigs have been limited to fetal fibroblasts (109, 110, 112, 117) and a few other types of cells (111, 113, 118). Because of their rapid growth and potential for multiple cell divisions before senescence in culture, fetal fibroblasts have been the most commonly used nuclear donors in porcine SCNT (for a review, 116). However, it has been hypothesized that the genome of undifferentiated cells, such as stem cells or partially differentiated multipotent progenitor cells may be more easily reprogrammed by the recipient oocyte. To support this, evidences in mice suggest that less differentiated cell types can increase the efficiency of NT compared with terminally differentiated cell types (104, 119). Initial molecular reprogramming events occurring upon transfer of the donor nuclei to the enucleated oocyte are largely unknown. But the efficiency of reprogramming the donor nucleus by the oocyte cytoplasm and the ability to support the development of cloned embryos is related to the differentiation status of donor-cell type (104). Unlike in mice, non-availability of validated protocols for the derivation and maintenance of an undifferentiated ES cell line in pigs has constrained the comparative studies on the cloning efficiency using ES and somatic cells. However, in recent years, pMSCs have been successfully established, and further employed these cells as nuclear donors for porcine NT (10, 13, 15, 17, 120). Birth of cloned piglets has been demonstrated using pre-adipocytes derived from porcine adult tissues (113). More recently, the successful production of live cloned pigs has been reported from multipotent salivary gland derived progenitor cells (114). However, no reports are available on pMSCs as nuclear donors supporting the development to term.

Several research groups have reported that the *in vitro* development of cloned porcine embryos reconstructed with pMSCs was higher when compared to somatic cells (10, 13, 15, 17, 120). The summary of data on the percentage of embryos that cleaved and developed to the blastocyst stage for the bone marrow derived pMSC compared to other donor cells is shown in Table 4. Interestingly, cloned embryos derived from porcine undifferentiated MSCs and their derivatives along the osteogenic lineage resulted in consistently high preimplantation development compared to adult fibroblasts (13). In addition to enhanced developmental potential of NT embryos reconstructed with pMSCs (15), cloned embryos showed high total cell number and ICM ratio, and low apoptotic positive cells when compared to fibroblasts (17). In contrast, MSCs derived from peripheral blood did not increase the percentage of NT embryos that developed to the blastocyst stage compared with those reconstructed with fibroblasts (15). In another study of our group, in spite of the variations in the expression profiles of genes involved in transcription, DNA methylation, histone deacetylation, apoptosis and embryonic growth observed at different developmental stages, some genes in NT embryos derived from pMSCs closely resembled to that of *in vivo* produced embryos (120). These findings indicated that MSCs with a relatively undifferentiated genome might be more efficiently reprogrammed to re-activate the expression of early embryonic genes. The collective data on the developmental rates, total cell number, ICM ratio, apoptosis and gene expression suggest that pMSCs have a greater potential as donor cells and may have the ability in supporting the preimplantation development of cloned pig embryos to term. Furthermore, bone marrow pMSCs were able to undergo transient and stable genetic modifications with non viral and viral vectors and were shown to be an attractive cell type for therapy models and for NT transgenesis (10, 13, 87). Thus, cloning in pigs using MSCs not only provide new approaches towards improving the efficiency by understanding the mechanisms of nuclear reprogramming involved in development, but also allow the donor cells to undergo specific genetic modifications for producing transgenic pigs for organ and tissue transplantation to humans or to serve as the models of human diseases.

5. CRYOPRESERVATION OF pMSCs

Cryopreservation is an easy and effective method of storing a wide variety of mammalian cells for research and therapeutic applications including transfusion medicine and cell transplantation. Cryopreservation represents a convenient alternative for preserving undifferentiated MSCs and differentiated derivatives for long periods without significantly impairing their viability when further used. To support this, much progress has been made with cryopreservation of human MSCs and several studies have shown that the MSC from different origins can be cryopreserved without significantly altering their proliferation and differentiation capacity (121, 122). Different cryoprotective agents such as dimethylsulfoxide (DMSO), ethylene glycol (EG), glecerol and sugars have been employed for long-term storage and preservation of cells. Recently, a study on the effect of two different cryoprotectants such as DMSO and EG, used alone or in combination, on frozen-thawed pMSCs showed no differences in their viability, apoptosis and gene expression patterns (123). Interestingly, a large quantity of cryopreserved allogenic pMSCs transplanted directly into the myocardium of a healthy swine model demonstrated no evidence of rejection, calcification, teratoma or myocardial infarction and showed the method was feasible (80). In spite of these attempts, a systematic study of cryopreservation for pMSCs is widely lacking and underscores the need for the development and application of effective preservation protocol. It is known that, the benefits of cryopreservation can only be appreciated if viability and function of pMSCs are maintained during the cryopreservation process. Thus, changes in pMSC viability following cryopreservation should be measured by evaluating the changes in cell function, such as proliferative rates and multi-lineage differentiation potential. In addition, freezing, storage and thawing are associated with severe damage and therefore, technical advancement in pMSC cryopreservation, such as choice of cryoprotective agents, their composition in cryopreservation media, cooling rate, etc. is more relevant for current research and clinical applications.

6. Future prospects

Recent advances in understanding the properties of MSCs have made them potentially ideal candidates for cell transplantation in regenerative medicine and tissue engineering. To assure the probable short and long term safety and efficacy of MSC therapy, adequate preclinical trials need to be performed before proceeding into human applications. Despite certain similarities in the molecular regulation of cell function between human and mouse, pig has been proposed as a suitable alternative animal model for developing safe preclinical protocols in biomedical research. Further, the similarities between porcine and human MSCs presented by the available literature allow pMSCs to be considered as a valuable model system for cognitive *in vitro* studies and cell based therapy. Before moving onto functional studies, the critical challenge is to answer relatively fundamental questions regarding the existence, phenotype, and *in vivo* function of MSCs. The cellular and molecular characterization for optimal identification and expansion is required to elucidate the transcriptional regulation of signaling pathways that mediate self renewal and the plasticity of exclusive lineage differentiation in MSCs. Availability of no specific markers has constrained the isolation in large number, development of defined media for expansion and the establishment of immortalized cell line. Several reports have shown the successful engraftment, homing and differentiation of pMSCs at site specific injuries, but their beneficial effects due to local production of cytokines and growth factors rather than direct participation is not ruled out. This necessitates evaluating both of the intrinsic network that define and limit the ability of a stem cell to respond, and of the extrinsic signals that recruit and direct the stem cells *in vivo*. In view of this, it highlights the value of porcine model not only in understanding the mechanisms involved in new tissue regeneration therapies but also in serial investigation of tissue changes and their functional consequences using imaging modalities. In addition, it allows elucidating effects of immunomodulation and host immune response to MSCs to cross the immune barriers. MSCs are also considered in areas of gene therapeutics for targeted delivery of gene products. Ability of pMSCs to undergo stable genetic modifications along with their suitability as nuclear donors in cloning offers a greater potential to both medicine and biopharming. Cumulatively, the results of these experimental and preclinical assessments in a large animal model would strongly facilitate the realization of a major therapeutic role for MSCs in tissue engineering and regenerative medicine in human clinical trials.

7. Conclusion

The potential of MSCs to undergo differentiation in vitro into marrow and non-marrow cell types has been under intense scrutiny in recent years for a number of therapeutic applications. Easy purification and amplification in addition to their multipotency have regarded them as ideal candidates for cell therapies. Despite the recent understanding of cellular and molecular signaling pathways and global transcriptional regulation of MSCs, there are number of fundamental aspects still need to be deciphered before employing them for safe and effective clinical trials. Due to anatomical and physiological similarities to humans, porcine animal model appears to be particularly suitable to address certain basic biological properties and multiple questions of clinical relevance of MSCs. Furthermore, successful isolation, characterization and in vitro differentiation of pMSCs into multilineages along with encouraging findings of transplantation studies provide additional support in developing and standardizing therapeutic strategies for human applications.

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Abbreviations: ASCs: adult stem cells; ESCs: Embryonic stem cells; MSCs: Mesenchymal stem/progenitor cells; SCNT: somatic cell nuclear transfer; pMSC: porcine mesenchymal stem cells IGF: insulin-like growth factor; bFGF: basic fibroblast growth factor; HNF-1: hepatic nuclear factor-1; CFU-Fs:colony-forming unit-fibroblasts; BM: bone marrow; UCB: umbilical cord blood; PBS: phosphate buffered saline; EGF: endothelial growth factor; FBS: fetal bovine serum; UCM: umbilical cord matrix; SCF: stem cell factor; LIF: leukemia inhibitory factor; PDs: population doublings; BMPs: bone morphogenetic proteins, GFAP: glial fibrillary acidic protein

Key Words: Porcine Mesenchymal Stem Cells, Adult Stem Cells, Multipotency, Cell Surface Antigens, Therapeutic Applications, Somatic Cell Nuclear Transfer, Myocardial Repair, Porcine Umbilical Matrix, Review

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