Mini Review

BIO Integration

Isolation, Characteristics, Differentiation and Exploitation of Human Amnion Mesenchymal Stem Cells

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Abstract

Human amnion is a favorable potential source of mesenchymal stem cells (MSC) for future cell-therapy-based clinical applications, because of the painless collection procedure and easy accessibility of the placenta. Human amnion mesenchymal stem cells (hAMCs) have multilineage differentiation ability and high proliferation ability, are non-tumorigenic and have a relatively low risk of rejection after transplantation. Given the beneficial properties of hAMCs, herein, we review the isolation methods and characteristics of hAMCs. Furthermore, we summarize current hAMC applications and preservation methods.

Keywords

Cell culture, cell preservation, human amnion mesenchymal stem cell, stem cells.

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Introduction

Mesenchymal stem cells (MSCs) are non-hematopoietic stromal cells that are well known for their self-renewal ability and mesenchymal cell lineage differentiation. These abundant pluripotent cells can be found in various organs and tissues, such as the brain, spleen, liver, kidney, lung, thymus and pancreas. MSCs from multiple sources exhibit similar fibroblastoid morphology and immunophenotypic profiles, but their growth kinetics and differentiation potential vary depending on cellular origin [1]. Because of the diverse applications of MSCs in many fields, such as regeneration of skin tissue, organ transplantation and brain injury, continued research aims to fully explore the potential of stem cells [2, 3]. However, the use of MSCs derived from the bone marrow can be limited by low cell numbers and aging factors leading to a loss of differentiation ability [4, 5].

Increasing evidence indicates that human amnion MSCs (hAMCs) are a promising alternative source for applications in cell-based therapies, owing to their unique qualities. hAMCs possess several remarkable features of MSCs, including pluripotency, stemness markers, multilineage differentiation potential, anti-inflammatory properties and non-tumorigenicity. In addition, hAMCs can be easily obtained from the placenta, which is often considered medical waste in hospitals. The placental collection procedure is less invasive and more ethically acceptable than those for other sources of MSCs [1, 6, 7]. This review describes hAMC isolation methods, unique characteristics, differentiation potential and preservation methods to facilitate successful cultivation of hAMCs.

Isolation of hAMCs

The human amniotic membrane consists of two cell types of different embryological origin: human amniotic epithelial cells (hAECs) derived from the ectoderm, which are in contact with amniotic fluids, and hAMCs derived from the embryonic mesoderm, which are distributed in the stroma underlying the amniotic epithelium (**Figure 1**). Compared with hAECs, hAMCs exhibit better mesodermal differentiation ability and yield cell types better suited for applications in cell-based therapy [8]. Hence, this review focuses solely on the isolation of hAMCs.

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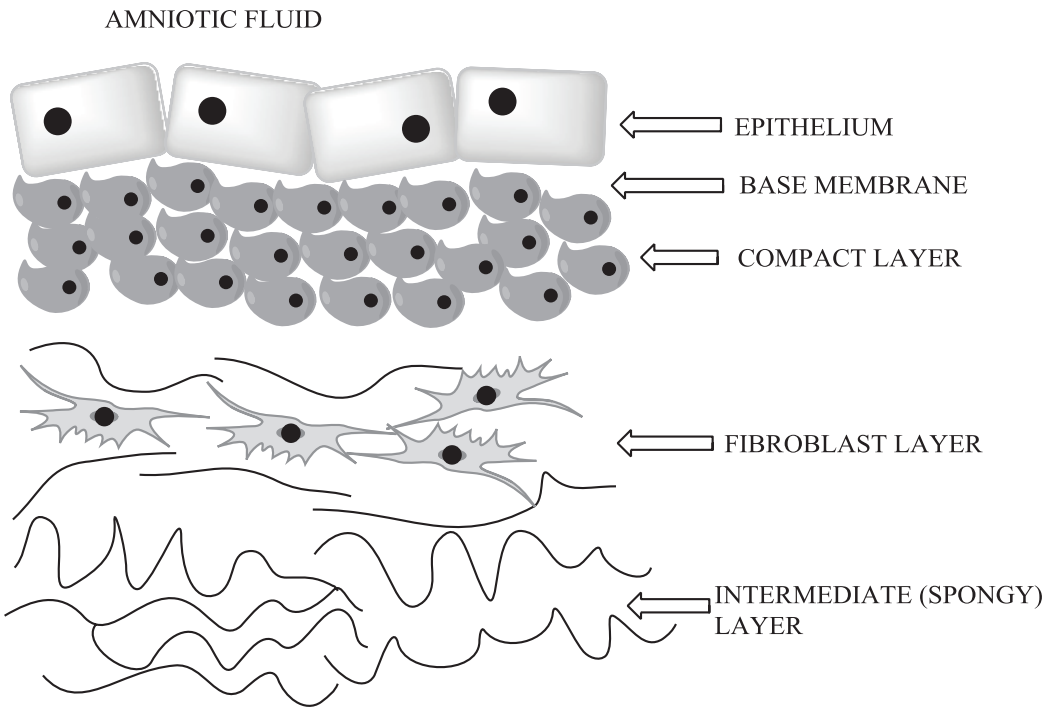

Figure 1 Diagrammatic representation of the human amnion layer.

hAMCs can be acquired through several isolation methods (**Table 1**). The procedure includes mechanical peeling of the amniotic membrane from the underlying chorion, and subsequent digestion of amniotic tissues with trypsin and other digestive enzymes (**Figure 2**) [9]. The method of isolation with trypsin and collagenase has successfully yielded hAMCs with highly pluripotent cells [1]. These cells express stemness markers and can differentiate into multiple cell lineages. Another method using dispase followed by collagenase successfully yields hAMCs with high clonogenic activity and differential potential [10]. Interestingly, a comparison of both methods has demonstrated that the method involving separate culture described by Soncini and colleagues [10] yields more hAMCs than the method involving mixed culture described by Alviano and colleagues [1]. When hAMCs and hAECs are cultured separately, more cells are acquired after isolation and expansion. Furthermore, the method of Soncini and colleagues yields more hematopoietic progenitor cells, which express high levels of embryonic-stem-cell markers, such as stage-specific embryo antigen-4 (SSEA-4) and STRO-1, thus suggesting that both methods result in varying differentiation

Table 1 Methods of Isolation of hAMCs

Reference	Method
[1]	Step 1: 0.25% of trypsin/EDTA for 15 min Step 2: 0.25% of trypsin-EDTA, 10 U/mL DNase I and 0.1% collagenase IV in DMEM
[10]	Step 1: 2.4 U/mL of dispase in PBS for 7 min at 37°C Step 2: 0.75 mg/mL collagenase and 20 g/mL DNase for 3 hours at 37°C
[6]	Step 1: i) 0.03% hyaluronidase i) 0.025% DNase I ii) 0.2% trypsin in DMEM at 37°C, 100 rpm for 30 min iii) 0.2% trypsin in DMEM at 37°C, 400–600 rpm for 30 min Step 2: 0.75 mg/mL collagenase and 0.075 mg/mL DNase I in DMEM at 37°C, 600 rpm for 1 hour
[13]	Step 1: 0.25% trypsin at 37°C for 5 min Step 2: 0.25% trypsin + 0.1% collagenase IV for 5 min at 37°C followed by inactivation with fetal serum
[14]	Step 1: 20 mL of 0.05% trypsin/EDTA at 37°C for 1 hour Step 2: 1 mg/mL collagenase type IV and 25 µg/mL DNase I in EMEM (with 25 mM HEPES and Earle's BSS, without L-glutamine) incubated in a shaker for 1 hour at 37°C
[15]	Step 1: Serial digestion in 0.05% trypsin-EDTA at 37°C for 10 min, 30 min and 60 min Step 2: 0.3% collagenase type 1 at 37°C for 4 hours with constant agitation
[16]	Step 1: i) 0.25% trypsin/EDTA (0.05%) for 10 min at 37°C, 5% CO_2 ii) 0.25% trypsin/EDTA (0.05%) during 40 min with gentle shaking every 10 min Step 2: 0.75 mg/mL collagenase II at 37°C for 90 min, 5% CO_2 with gentle shaking every 10 min

Figure 2 Methods of hAMC isolation, from sample collection to cells culture in flasks.

potential [8]. Both markers play fundamental roles in identifying and isolating MSCs [11, 12].

In general, the isolation of hAMCs through a two-step trypsin/EDTA digestion followed by collagenase digestion can successfully generate hAMCs with high yield [1, 13–16]. The hAMCs isolated with this method display fibroblast morphology, express stemness markers and embryonic-stem-cell markers, and have differential potential. Another method of isolation based on hyaluronidase-trypsin combined with collagenase yields high levels of vimentin-positive cells, thus indicating the presence of epithelial and mesenchymal cells, and the low purity of isolated hAMCs [6]. Using optimized isolation methods is essential to generating highly pure cultures of hAMCs. To exclude unwanted hAECs, trypsin and collagenase digestion have been performed during the isolation process [1, 9], or the culture medium has been changed after 1–2 hours of cultivation, because of the slow adhesion of hAECs to culture flasks [14]. Another method of purifying hAMCs from hAECs is density separation through Percoll gradients [14], which may also be useful in preventing contamination.

Characteristics of hAMCs

The human amnion is considered an alternative source for MSCs, given that hAMCs have attributes including pluripotency, stemness markers, multilineage differentiation potential, anti-inflammatory properties and non-tumorigenicity. Accordingly, hAMCs meet the minimum standard criteria for stem-cell characteristics, according to the International Society for Cellular Therapy: plastic adherent cells with a fibroblast-like or spindle-like appearance, which form clonal colonies, express common MSC markers and are capable of multilineage differentiation when cultured *in vitro* [17].

Amnion-derived stem cells have pluripotent properties, on the basis of their expression of the stem-cell marker octamer-binding transcription factor (OCT)-4 [6]. OCT-4, which functions in renewal-ability maintenance [18], is expressed at higher levels in hAMCs than other types of MSCs, such as bone-marrow-derived MSCs [1]. Other molecular markers of pluripotency, such as Nanog, SOX-2, Lefty-A, fibroblast growth factor (FGF)-4, rex-1 and TDGF-1, are also expressed in cultured hAMCs and are useful biomarkers [19]. Cultured hAMCs secrete growth factors and cytokines including VEGF, IGF-1, HGF, adrenomedullin and PGE2, which accelerate regeneration, proliferation and wound healing [20–22].

Another useful attribute of hAMCs is their expression of stemness markers—molecular signatures that distinguish hAMCs from other differentiated cell types (**Table 2**). Moreover, hAMCs can differentiate into ectoderm (neural cells), mesoderm (cardiomyocyte) and endoderm (liver or pancreas) lineages, the three embryonic germ layers, *in vitro* [6–7, 19, 23]. The differentiation potential of these cells can be induced by growth factors or chemicals [19].

hAMCs additionally have anti-inflammatory properties. These cells express PGE2, which is involved in the

Table 2 Stemness Markers Expressed in hAMCs

	hAMCs	References
Positive markers	CD9, CD29, CD44, CD49d, CD49e, CD 56, CD59, CD73, CD90, CD105, CD166, OCT-3/4, SOX-2, c-Myc, NANOG, KLF-4, FGF-4, HLA-ABC	[22, 44, 58–60]
Negative markers	CD31, CD45, CD34, CD14, CD31, CD133, HLA-DR, CD80, CD117, HLA-DR, DP, DQ	[15, 59, 60]

anti-inflammatory response. hAMCs have been reported to have higher PGE2 production than other MSCs, such as chorion-derived cells, thereby suggesting that hAMCs are a better source of cells from an immunosuppressive viewpoint. The anti-inflammatory functions of hAMCs have been further verified by their expression of the anti-inflammatory proteins interleukin (IL)-1 receptor antagonist; tissue inhibitor of metalloproteinase (TIMP)-1, -2, -3 and -4; and IL-10 [6]. In addition, *in vivo* transplantation of hAMCs has been successfully performed, with only low immunogenicity and no transplant rejection [24, 25].

Notably, hAMCs have no tumorigenicity and thus do not form tumors when transplanted *in vivo*, in contrast to other types of stem cells, such as embryonic stem cells, which pose a substantial risk of tumor formation [26]. Owing to the drawbacks associated with other types of stem cells, efforts have been aimed at finding alternative sources of cells for use in regenerative medicine. hAMCs may provide a safer alternative for cell-based therapy. Furthermore, transduction human telomerase reverse transcriptase (hTERT) in hAMCs does not show tumorigenic potential after long-term culture *in vitro* [27]. hTERT-induced hAMCs maintain fibroblastic morphology, typical MSC markers and differentiation potential.

The amniotic membrane is the only source of MSCs reported to show no contamination from non-fibroblast cells, unlike other sources of MSCs, such as the umbilical cord, cord blood and placenta. Hematopoietic and endothelial cells are common contaminants present in the MSCs isolated from cord blood or placenta. Because the amniotic membrane has no vasculature, amniotic membrane hMSCs are not contaminated by hematopoietic and endothelial cells. In addition, the enzymatic digestion in the isolation protocol of amniotic membrane removes epithelial cells [1].

Beyond the described attributes, the ease of accessibility of source tissues through procedures less invasive than obtaining bone marrow and, more importantly, the absence of ethical concerns, given that placenta is considered a waste product, make hAMCs a favorable novel source of stem cells. hAMCs are easily isolated and expanded through enzymatic digestion while maintaining undifferentiated states [7, 15]. These properties have led to substantial attention being paid to hAMCs as an alternative source of stem cells for applications in cell-based therapy (**Table 3**).

Table 3 The Various Characteristics of hAMCs

Cell Type	Source	Characteristics
Mesenchymal stem cell	Human amnion	Pluripotent properties [4] hAMC markers [**Table 1**] Differentiation potential [6] Anti-inflammatory properties [6] No tumorigenicity [26] No contamination from non-fibroblastoid cells [1] Limited ethical concerns [7]

Preservation of hAMCs

The capability to preserve stem cells is crucial for their utilization in clinical and research applications in stem-cell-based therapies. Because the availability of fresh stem cells from sources such as the bone marrow, placenta and umbilical cord can be difficult to forecast or control, the preservation of these cells for later use in research or clinical settings is greatly needed [28]. Comprehensive quality and safety assessment of stem cells before use is imperative. Furthermore, a chain of custody, i.e., the flow of the cells among various collection and processing centers with clinical-administration oversight, can be maintained through preserved stem cells. Nevertheless, different preservation techniques can greatly affect the physical properties and biochemical composition of hAMCs [29]. Cryopreservation, the most common technique used by most countries for preservation of stem cells, is a mode of preservation wherein cells or any other substances vulnerable to damage due to chemical activity or over time, are frozen to sub-zero temperatures. Before freezing, cells are commonly coated with cryoprotectants, also known as cryoprotective agents (CPA), that act as antifreeze agents; examples include glycerol, trehalose or dimethylsulfoxide (DMSO). However, despite protecting the cells, these CPAs still pose a risk of cryo-injury during cryopreservation [30].

The most conventional methods of cryopreserving cells involve either slow freezing or rapid freezing. In slow freezing, the temperature is gradually decreased at a certain freezing rate. This method is commonly performed with DMSO as the cryoprotectant and is widely used for preserving MSCs originating from the bone marrow and cord blood [31]. However, cellular damage which leads to cell mortality has been linked to this method, notably in the range of vital temperatures from −15°C to −60°C where, the cells ought to pass through this range of temperatures twice (during cooling and warming) [32]. Osmotic damage can occur during the conversion of preserved cells and neighboring materials from the liquid to solid phase or vice versa, and eventually lead to cell mortality [33]. In contrast, a study by Liu *et al.* [34] has demonstrated no significant decrease in the viability and differentiation potential of MSCs derived from adipose tissue when 10% DMSO is used in slow freezing.

In contrast, rapid freezing uses an ultra-rapid freezing rate to cryopreserve cells. Rapid freezing is also known as vitrification, which refers to the "glass formation" resulting from a conversion of the liquid state to the solid state in the absence of crystallization [35]. After direct contact between cells dissolved in cryoprotectant medium and liquid nitrogen,

intracellular and extracellular ice crystal formation are minimized because of the instantaneous cooling rate and high viscosity. Compared with slow freezing, vitrification uses a higher concentration of cryoprotectant. Rapid freezing is a simple, efficient and non-damaging technique for preserving cells [36, 37]. Vitrification has been demonstrated to successfully preserve human embryos, oocytes, hematopoietic progenitor cells derived from human cord blood and human embryonic stem cells. Further studies have indicated that hAMCs retain their viability, proliferation and differentiation potential after a two-step exposure to equilibration and vitrification solutions, which were originally used for preserving human blastocysts [37, 38]. hAMCs have been successfully vitrified with 20% ethylene glycol as an equilibration solution, and a vitrification solution consisting of 40% ethylene glycol, 18% Ficoll 70 and 0.3 M sucrose [37].

A newly proposed cryopreservation method is based on double-bagging and storage in a freezer without liquid or gas-stage nitrogen in a cryogenic chamber [39]. This method has a low likelihood of bag rupture and bio-hazard release, and eventually increases stem-cell viability and improves neutrophil engraftment. Neutrophil engraftment is an important determinant of successful transplantation, in which the absolute neutrophil count should be at least 500 cells/mm^3 during the first three consecutive days after transplantation. With engrafting immediately after transplantation, MSCs may prevent early bacterial and fungal infections [40]. Furthermore, advances in micro/nano technologies, such as freezing cells in nanoliter droplets on highly hydrophobic nano-rough surfaces, and the progression of nontoxic nanoscale bio-inspired CPAs, are encouraging cryopreservation research [30]. However, cells are vulnerable to several stresses during cryopreservation, such as mechanical, thermal and chemical stresses [41]. Therefore, the major hurdles during cryopreservation are the alteration of cell function, and the cell damage and mortality associated with the choice of cryoprotectants, and the cooling and thawing process. Thus, many clinical applications are restricted because of the loss of cells during storage.

Currently, most cryopreservation research is focused on adult stem cells derived from sources including the bone marrow, umbilical cord blood and adipose tissue. Therefore, a large gap remains in research on the cryopreservation of hAMCs to establish protocols for long-term preservation. Unfortunately, the currently published methods are not specific to stem-cell type. Thus, fundamental studies are greatly needed for the development of suitable techniques to maximize the ability of preserving hAMCs to further exploit their potential in clinical applications.

Applications of hAMCs

The self-renewal and immunomodulatory properties of MSCs have generated substantial interest in regenerative medicine applications, specifically in the fields gene therapy and cell biology. In addition, the technology to preserve stem cells has been exploited in clinical and research applications of stem-cell-based therapies. The large numbers of MSCs obtained from expanded culture enables tissue engineering and cell engraftment [42]. Recent years have seen increasing documentation of hAMC applications in various experimental disease models, such as those for cardiovascular, neurological, diabetic, gastrointestinal and respiratory diseases [43, 44–49].

Past studies have shown that hAMCs secrete physiologic concentrations of cytokines that are important in wound restoration, such as angiogenin, platelet-derived growth factor, transforming growth factor-β2 (TGF-β2), vascular endothelial growth factor, TIMP-1 and TIMP-2. These cytokines enhance proliferation, diminish inflammation and control various processes that are essential for injury repair and wound healing [20]. The immunosuppressive properties and the ability of hAMCs to secrete angiogenic factors make them suitable for treating cardiovascular diseases, such as critical limb ischemia and myocardial infarction. hAMCs have been found to partially rescue experimental critical limb ischemia and to improve cardiac function and the revascularization of ischemic myocardium after embedding of a fibrin graft consisting of hAMCs within failing rat hearts, in an experimental model of cardiac grafting [43, 44]. According to preliminary laboratory experiments, hAMCs can develop into heart muscle cells and assist in repairing heart damage [50].

The ability of stem cells to differentiate into functional neuronal-progenitor-like cells enables hAMCs to be used in treating neurological diseases, such as traumatic brain injury and spinal cord injury. hAMCs significantly decrease the viability and migratory ability of microglia [45]. The transplantation of neuronal progenitors derived from hAMCs has been found to improve neurological function and brain histology after traumatic brain injury [51]. hAMCs release neurotrophic factors at high levels, which have been suggested to ameliorate experimental traumatic brain injury [52]. In addition, hAMCs have been studied in experimental spinal cord injury, and their transplantation into the spinal cord has been found to decrease activated macrophages/microglia and apoptotic cells. The levels of brain-derived neurotropic factors and VEGF in the injured spinal cord also increase significantly after hAMC transplantation, thus contributing to angiogenesis and axonal regeneration [45].

hAMCs are also commonly used in disease models of diabetes. In mice with streptozotocin-induced type I diabetes, transplantation of hAMCs-generated pancreatic islet cells has been found to increase body weight and normalize hyperglycemia [53]. Moreover, hAMCs transplanted into the kidney have also been found to restore body weight and normalize blood glucose levels, whereas hyperglycemia recurs after the removal of the graft-bearing kidneys. Another report has shown that hAMCs encapsulated in insulin-producing islet cells in polyurethane-polyvinyl pyrrolidone macrocapsules protect transplanted cells against immune rejection and normalize hyperglycemia [46, 47]. In a model of respiratory disease, reports have suggested that hAMCs may be used to treat clinical pulmonary fibrosis. hAMCs used in bleomycin-induced lung fibrosis have been found to decrease neutrophil infiltration and fibrosis associated with bleomycin instillation [48].

hAMCs have been demonstrated to be favorable candidates for cell-based therapy for rheumatoid arthritis [39]. The availability of hAMCs may enable the development of

skin appendages, such as hair follicles and sweat glands, without the possibility of immune rejection [54]. The lack of expression of HLA-II antigens in hAMCs results in an absence of induction of allogeneic or xenogeneic lymphocyte proliferation reactions; consequently, lymphocyte sensitivity and the proliferation of peripheral blood mononuclear cells have been successfully restrained [55]. A clinical study has been conducted to exploit the potential of hAMCs in the treatment of acute graft-versus-host disease, which is commonly treated invasively with bone-marrow-derived MSCs [49]. Only one clinical study has documented MSCs derived from amniotic membranes, whereas 125 and 43 clinical studies have documented MSCs from the bone marrow and umbilical cord [56]. Numerous encouraging preclinical studies on hAMCs to date support that the distinct properties of these cells may provide future clinical opportunities for treating countless diseases [46, 48–50, 57]. Until then, challenges such as cell preservation, the translation of research into good manufacturing practices, marketing of unproven stem cell-based interventions and clinical use must be addressed.

Conclusion

In this review, we presented an overview of hAMC isolation methods, characteristics and preservation techniques. Increasing interest in hAMCs has led to more studies aimed at determining the optimal protocol for hAMC isolation and preservation. In addition, recent advances in hAMC differentiation incorporating external factors, including small molecules, growth factors, ECM components and co-culturing methods, has led to promising developments in cell-based therapies using hAMCs in the new era of clinical applications, without restrictions due to ethical concerns regarding cell harvesting, given that the cells are isolated from human biological waste. Therefore, the possibilities of novel discoveries and applications of hAMCs in bio-medicine and regenerative technology are bright, and these cells may be key to future developments in human health.

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Competing interests

The authors declare that they have no competing interests.

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