Amniotic Stem Cells: Potential in Regenerative Medicine

Guest Editors: Mahmud Bani-Yaghoub, Patricia Wilson, Markus Hengstschläger, Toshio Nikaido, and Duanqing Pei
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This special issue of *Stem Cells International* focuses on the potential applications of amniotic cells in regenerative therapies to repair tissues and organs that have been damaged by trauma, disease, or aging. Amniotic cell populations have historically included cells in amniotic fluid that have been sloughed from external and internal surfaces of the developing fetus and potentially from the amnion, the innermost extraembryonic membrane of the amniotic sac. Amniotic fluid is recovered by amniocentesis, a technique that was initially used to assess fetal health in cases of Rh blood group incompatibility [1] and to determine the sex of the fetus by the presence or absence of the Barr body in amniotic cells [2, 3]. These pioneering studies [4] provided accessible methods for *ex vivo* culture of amniotic cells as a source of normal, rather than transformed cancer cells for biomedical research. Fauza and colleagues [5] were among the first to explore use of amniotic cells for tissue engineering and repair of congenital defects *in utero*. Ovine amniotic fluid and later human amniotic fluid proved to contain populations of rapidly proliferating mesenchymal, also known as stromal, cells that have since dominated research efforts. Similar to multipotential stromal cells (MSCs) derived from bone marrow [6, 7] and adipose tissue [8, 9], amniotic stromal cells differentiate into connective tissue lineages and show beneficial trophic support and immunomodulatory activities that promote self-repair [10–13]. Trophic support and immunomodulatory activities are not unique to stromal cells; several active clinical trials are testing for the safety and efficacy of amniotic membrane derived epithelial cells that show similar properties (http://www.ClinicalTrials.gov/).

Unique features of amniotic cells present opportunities for regenerative medicine and biomedical research that are not shared by other cell sources, including pluripotent stem cells as defined by the International Society for Stem Cell Research (http://www.isscr.org/). A unifying feature of amniotic and placental cell sources is *ex vivo* culture from some of the earliest stages of fetal development, ranging from 15 weeks to 17 weeks of gestation with amniocentesis sampling to near birth at 9 months with delivery of full term placenta. Another unique feature reflects signaling cascades during gestation; amniotic and placental cells are exposed to paracrine signaling among the mother, the fetus, and the placenta. These transient conditions may confer distinctive properties to amniotic cells that are not shared...
by other cell sources. The consensus of the diverse papers offered here is that continued research and development may prove amniotic cells to be superior cell sources for selected applications.

Further progress would be facilitated with identification of useful biomarkers for amniotic cells. In a review article by M. G. Roubelakis et al. in Greece “Amniotic fluid and amniotic membrane stem cells: marker discovery,” readers are provided with the most recent approaches used to investigate stem cells in amniotic membrane and fluid. The authors have assembled a comprehensive list of markers employed to analyze these cells with the techniques focused on transcriptomics, proteomics, and metabolomics. The authors further emphasize the importance of developing efficient methods to isolate homogeneous stem cell populations prior to their use for cell-based therapies.

Biomarker identification is furthered by a research article by M. P. Dobrèva et al. in Belgium and the Netherlands “Periostin as a biomarker of the amniotic membrane” examining the target genes of bone morphogenetic proteins (BMP) as amniotic membrane markers. Comparative gene expression analyses combined with in situ hybridization assays presented in this paper has led to the identification of periostin as a reliable marker expressed in the amnion throughout gestation. Furthermore, the authors have established a transcriptional signature for extraembryonic tissues, using a carefully selected panel of genes, encouraging the development of other biomarkers in this area.

While the first two papers in this special issue have focused on biomarkers, a review article by R. J. Hodges et al. in Australia presents “Amnion epithelial cells as a candidate therapy for acute and chronic lung injury”. The authors discuss the plasticity and immunomodulation properties of amnion epithelial cells in repair lung injury. In addition, they address the therapeutic potential of these cells with references to purity of cell population and the transcription factors they express. Key features of amnion epithelial cells and their role as exogenous stem cells compared to resident endogenous lung stem cells have been presented. The authors provide a list of factors that are potentially involved in the repair mechanisms elicited by amnion.

In a paper focused on mesenchymal stem cells, C. M. Raynaud et al. in Qatar and USA. present a “Comprehensive characterization of these cells from human placenta and fetal membrane and their response to osteoactivin stimulation.” The authors have employed transcriptomics, immunocytochemistry, cytokine array analysis, and other complementary methods to evaluate the similarities and differences between placenta- and membrane-derived mesenchymal stem cells before and after differentiation. Using established protocols to isolate these cells from both sources, the authors show that placenta- and membrane-derived mesenchymal stem cells respond differently to osteoactivin during osteogenic differentiation.

A concise review of “Amniotic fluid stem cells: future perspectives.” By M. Rosner et al. in Austria discusses the progress made by this group as well as others, since their first report on connection between amniotic fluid cells and stem cell research. The comparison among AFS cells, ES cells, adult stem cells, and iPS cells has been followed by an emphasis on the importance of monoclonal genomically stable human AFS cell lines and their capacity to form embryoid bodies. As a novel approach, this paper has regarded AFS cells as a source for cell-based therapies for kidney disorders such as acute and chronic renal failures and acute tubular necrosis. While promising, the authors have emphasized that standardized protocols are required to isolate monoclonal populations of AFS cells.

The challenge inherent to population complexity in amniocentesis samples as well as the potential effects of paracrine signaling prompted P. G. Wilson et al. in the USA, “Clonal populations of amniotic cells by dilution and direct plating: evidence of hidden diversity.” to first establish an efficient method to generate amniotic cell cultures, avoiding the need for refrigeration and centrifugation of samples. Application of this method allowed isolation of a large number of clonal lines from independent amniocentesis samples. As proof of concept for clonal identity, clonal populations of stromal and epithelial cells were compared with each other and with control cell populations. The results of flow cytometry, immunocytochemistry, differentiation tests, and transcript analysis revealed clear differences not only among stromal cell clones and epithelial cell clones from the same donor, but also between mixed cell populations from different donors.

Functional communication between the grafted and host cells plays a pivotal role in delivering therapeutic agents to the damaged tissues. In a research paper by A. Jezierski and colleagues in Canada “Human amniotic fluid cells form functional gap junctions with cortical cells,” the authors show that amniotic fluid cells express high levels of connexin 43, a gap junction protein known for its role in brain development and function. Furthermore, AF cells establish intercellular communication with astrocytes, suggesting a novel role for AF cells to deliver therapeutic factors, including specific microRNAs and small molecules, to the damaged tissues. In particular, this approach may facilitate neuroprotection in the early stages of graft-host interaction during which intercellular coupling via gap junctions precedes the integration of transplanted cells into the neuronal circuit.

A key feature of this special issue of Stem Cells International is to provide an overview of the recent progress in the field of amniotic stem cells by independent research groups from different continents. In an article by K. Rennie et al., authors from Canada, Austria, China, and Japan discuss applications of amniotic membrane and fluid in stem cell biology and regenerative medicine. While amniotic membrane is widely used as a natural scaffold for clinical applications, using standardized protocols to establish homogeneous clones from amniotic membrane or fluid cells has been described as a critical step towards development of desired phenotypes for cell transplantation. The paper has also recapitulated the use of amniotic fluid cells in several disease models, hoping that more progress will be made towards effective regenerative therapies in the near future.
The editors hope that the original and review articles integrated in this special issue provide more insights into the advancement and challenges associated with the use of amniotic cells in regenerative medicine.

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Patricia Wilson
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Toshio Nikaido
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Applications of Amniotic Membrane and Fluid in Stem Cell Biology and Regenerative Medicine

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The amniotic membrane (AM) and amniotic fluid (AF) have a long history of use in surgical and prenatal diagnostic applications, respectively. In addition, the discovery of cell populations in AM and AF which are widely accessible, nontumorigenic and capable of differentiating into a variety of cell types has stimulated a flurry of research aimed at characterizing the cells and evaluating their potential utility in regenerative medicine. While a major focus of research has been the use of amniotic membrane and fluid in tissue engineering and cell replacement, AM- and AF-derived cells may also have capabilities in protecting and stimulating the repair of injured tissues via paracrine actions, and acting as vectors for biodelivery of exogenous factors to treat injury and diseases. Much progress has been made since the discovery of AM and AF cells with stem cell characteristics nearly a decade ago, but there remain a number of problematic issues stemming from the inherent heterogeneity of these cells as well as inconsistencies in isolation and culturing methods which must be addressed to advance the field towards the development of cell-based therapies. Here, we provide an overview of the recent progress and future perspectives in the use of AM- and AF-derived cells for therapeutic applications.

1. Introduction

Regenerative medicine involves the use of living cells to repair, replace, or restore normal function to damaged or defective tissues and organs [1, 2]. Stem cells are viewed as promising candidates for use in cell-based therapies, owing to their capacity for self-renewal and differentiation into diverse mature progeny. However, the source of stem cells, in order to maximize the safety and efficacy of regenerative therapies, is clearly of great importance. Both adult and embryonic stem cells are commonly used to develop therapies for various preclinical models of disease and injury. Recently, induced pluripotent stem (iPS) cells, which are obtained by genetically reprogramming adult somatic cells to a pluripotent state, have also been proposed as an alternative cell source for use in regenerative medicine [3, 4]. However, a number of limitations hamper the clinical applicability of stem cells derived from either adults or developing embryos. While embryonic stem cells (ES cells) are highly proliferative and capable of differentiating into cells of all adult tissues, they pose a significant risk of tumour formation [5]. Furthermore, since ES cells are obtained by the destruction of embryos, they face serious ethical objections that have yet to be resolved. In contrast, although adult stem cells carry a reduced risk of tumorigenicity and fewer ethical restrictions, they are limited in number, have diminished differentiation
Table 1: Comparison of ES, AM and AF stem cells.

<table>
<thead>
<tr>
<th>Source</th>
<th>Embryonic stem cells</th>
<th>Amniotic epithelial cells</th>
<th>Amniotic mesenchymal stromal cells</th>
<th>Amniotic fluid cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>Inner cell mass of preimplantation embryo</td>
<td>Amniotic membrane</td>
<td>Amniotic membrane</td>
<td>Amniotic fluid</td>
</tr>
<tr>
<td>Ethical issues</td>
<td>Yes [55]</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Clinical trials</td>
<td>Yes [55]</td>
<td>Yes [56]</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Figure 1: The isolation of human fetal membranes from the placenta. (a) Note the texture and elasticity of the membranes. (b) Human amniotic (left) and chorionic (right) membranes can be readily separated from each other for further purification procedures.

capacity, and reduced proliferative potential [6, 7] which render the production of a sufficient number of cells for use in cell-based therapy difficult. Finally, despite major advances in iPS technology in recent years, reprogrammed cells often have an imperfectly cleared epigenetic memory of the source cells [8]. In addition, iPS cells are vulnerable to genomic instability [9, 10]. Due to the drawbacks associated with ES cells, adult stem cells and iPS cells, much effort has been directed at finding an alternative source of cells for use in regenerative medicine.

Subpopulations of multipotent cells exist in both the amniotic membrane (AM) and amniotic fluid (AF). Amniotic fluid cells are obtained during amniocentesis, an important diagnostic procedure performed worldwide to evaluate the health status of the fetus during pregnancy. Amniotic epithelial (AE) and amniotic mesenchymal stromal (AMS) cells are isolated from amnion that is normally discarded following birth. These cells are therefore readily available, easily procured, and avoid the ethical issues that are associated with the use of ES cells. Subpopulations of AF- and AM-derived cells with stem cell characteristics can be maintained in the undifferentiated state in culture, but are capable of differentiating into cells representing all three germ layers under appropriate conditions [11, 12]. Unlike ES cells, AF and AE cells have not been found to form teratomas when transplanted *in vivo* [11, 13–16], and may be a safer alternative to ES cells. A comparison of AF, AE and AMS stem cells with ES cells is provided in Table 1. The use of amniotic fluid- and membrane-derived cells as cell-based therapy for a variety of indications has been extensively explored in the past decade. Here, we briefly review the findings regarding the use of AM and AF in tissue engineering and cell replacement strategies in a number of injury and disease models.

2. Amniotic Membrane

2.1. Amniotic Membrane Is a Natural Scaffold with Multiple Clinical Applications. Human amniotic membrane (Figure 1) is the innermost fetal layer, lining the amniotic cavity and protecting the fetus during pregnancy. The outer layer, termed chorionic membrane, further separates the fetus from maternal tissues. Reports focusing on the physiological functions of fetal layers have shown that amniotic membrane not only provides a physical support for the fetus, but also serves as a metabolically active filter through a direct interaction with amniotic fluid. In particular, the transport of water and soluble materials as well as the production of
growth factors, cytokines, and other bioactive molecules are regulated by amniotic membrane [17]. In addition to its role during pregnancy, amniotic membrane allows the initiation and maintenance of uterus contraction at birth [18].

The translucent, avascular, low immunogenic, anti-inflammatory, anti-scarring, and wound healing properties of amniotic membrane allow this material function beyond its role in vivo and assume a wide range of applications in regenerative medicine [19, 20]. In fact, the clinical use of amniotic membrane has a long history, with the first reports on its application in treatment of skin burns and wounds more than a century ago [21–23]. These groundbreaking studies played a significant role in advancing the use of amniotic membrane in surgery, especially in areas such as reconstruction of the corneal and conjunctival surfaces, treatment of open ulcers and traumatic wounds, and skin transplantation [17, 20, 24, 25]. In parallel, the shelf life of amniotic membrane has been extended by irradiation, air-drying, lyophilization, cryo-preservation, and glycerol preservation techniques. These methods are expected to further expand the use of amniotic membrane in ophthalmology to treat corneal, conjunctival and limbal lesions, burns, scars and defects as well as general surgery to reconstruct skin, genitourinary tract and other surfaces [25–31]. However, the efficacy of amniotic membrane in clinical applications can only be enhanced by retaining its biological properties in the long term. This issue is especially important because the presence of key growth factors such as EGF, FGF, TGF, HGF in amniotic membranes may account for their clinical effects and mechanisms of action. Currently, a series of standardized guidelines are being developed in a number of countries to optimize the production of surgically suitable amniotic membrane from donor placenta.

2.2. Stem Cells in Amniotic Membrane. In addition to these strategies, various histological, biochemical, and cellular biology techniques have been used to isolate and determine the suitability of the cells in amniotic membrane for other clinical applications. Epithelial cells can be readily identified as a single layer adjacent to the amniotic fluid on one side and the basement membrane on the other side [17, 32, 33]. While epithelial cells reside on the inner layer of the amniotic membrane, mesenchymal stromal cells form the outer layer [17, 32, 33]. Both cell types have been extensively investigated for their biological properties, using a number of in vitro and in vivo models. In particular, the expression of several cellular and molecular markers has confirmed the presence of stem cells in epithelial and mesenchymal stromal cultures. Subpopulations of both AE cells and AMS cells express pluripotency markers, including OCT4, SOX2, and NANOG [13, 15, 34, 35]. AE cells express embryonic stem cell markers such as SSEA4, TRA-1-60, and TRA-1-81 [13, 36], while reports on the expression of ES cell markers by AMS cells have been inconsistent [20]. Technical issues have prevented researchers from determining whether a single human AE or AMS (hAE or hAMS) cell can differentiate into cells representative of all three germ layers after clonal expansion [37]; therefore, it remains unclear whether the human amnion harbours true pluripotent stem cells, or a mixed population of multipotent progenitor cells. Nevertheless, it is widely accepted that multiple cell types can be derived by culturing either AE or AMS cells under appropriate conditions. Several laboratories have reported neural [13, 15, 35, 38, 39], hepatic [13, 15, 40–43], cardiac [15, 34, 44], osteogenic [15, 45, 46], chondrogenic [39, 47] and adipogenic [15, 46] differentiation of both AE and AMS cells.

2.3. Amniotic Membrane-Derived Cells in Tissue Engineering and Cell Replacement. The development of biological substitutes to replace damaged or dysfunctional tissue may involve the construction of “replacement parts” in vitro for later transplantation, or the direct administration of cells to the damaged tissue [57]. AE and AMS cells have been employed for both purposes. For instance, after inducing osteogenic differentiation of human AMS cells seeded onto microcarriers, the resulting bone-like structures could be used as building blocks to form a large (2 × 1 cm) bone construct in vitro [58]. AE cells have been used to form tendon-like structures [59], and a double-layered skin graft (using both AE and AMS cells) capable of repairing skin defects in mice [60]. Human AE and AMS cells have also been shown to reduce liver damage in a chemically-induced model of cirrhosis [61, 62] and improve cardiac function after experimental cardiac infarction [34, 44, 63]. Furthermore, both AE and AMS cells were able to replace insulin-producing pancreatic beta cells in diabetic mice to restore normal glucose levels [64–66]. Comprehensive reviews of the differentiation potential and therapeutic use of AE and AMS cells in experimental models are available in the literature [18, 20, 37, 67–69].

3. Amniotic Fluid

3.1. Amniotic Fluid Is a Dynamic Environment Containing Diverse Cell Types. Human amniotic fluid is a dynamic environment, which undergoes multiple developmental changes in order to sustain fetal growth and well being (Figure 2). The amniotic cavity first appears at 7-8 days after fertilization and in early gestation the amniotic fluid originates mostly from maternal plasma that crosses the fetal membranes [70]. Fetal urine first enters the amniotic space at 8–11 weeks gestation [70], and in the second half of pregnancy, fetal urine becomes the major contributor to amniotic fluid [71]. At this time, fetal skin keratinisation is complete, leading to reduced water transport across the skin and a decrease in AF osmolality. For the remainder of gestation, fluid volume is determined by different mechanisms including fetal urine production, oral, nasal, tracheal and pulmonary fluid secretion, fetal swallowing, and the contributions of the intramembranous pathway [72].

Amniotic fluid contains electrolytes, growth factors, carbohydrates, lipids, proteins, amino acids, lactate, pyruvate, enzymes, and hormones [73–76]. In addition, fluid secretions from the fetus into the AF carry a variety of fetal cells, resulting in a heterogeneous population of cells derived from fetal skin, gastrointestinal, respiratory and urinary tracts, and the amniotic membrane [77]. As the fetus develops,
the volume and composition of the amniotic fluid change drastically, and the complement of cells detected in amniotic fluid samples taken at different gestational ages varies considerably [78, 79].

Despite this heterogeneity, cultures of amniotic fluid cells obtained by amniocentesis have been used for decades for diagnostic purposes, including standard karyotyping as well as other genetic and molecular tests. AF samples are routinely used in the evaluation of fetal lung maturity, metabolic diseases, fetal infections, and intrauterine infections. These tests have recently been complemented by applying chromosomal microarray (CMA) as a more efficient prenatal genetic screening tool to detect fetal abnormalities [80]. In this multicenter study, nearly 4400 AF samples were used to assess the performance of CMA compared with karyotyping for prenatal cytogenetic diagnosis. Interestingly, CMA analysis allowed the detection of additional genetic abnormalities in about 1 out of every 70 samples that reported a normal karyotype during routine prenatal diagnosis. These results further emphasize the importance of AF cells in providing clinically important information about the fetus. In addition, this technology can be used to routinely follow the status of different subpopulations of amniotic fluid cells in culture and identify the most suitable clones for cell-based therapies.

Generation and banking of monoclonal human AF stem cell lines with specific chromosomal aberrations or monogenic disease mutations may also help study the functional consequences of disease-causing mutations [81, 82]. As a promising approach, the use of prolonged siRNA-mediated gene silencing in AF stem cells [83] may advance our understanding of the functions of specific genes and shed light on the pathogenesis of certain naturally occurring diseases [84].

3.2. Stem Cells in Amniotic Fluid. The fact that amniotic fluid is commonly collected for routine diagnostic testing and is a widely accessible source of fetal cells, prompted an interest in examining the possibility that AF might contain multipotent fetal-derived cells [85]. In 2003, Prusa et al. discovered the existence of a small population of actively dividing cells in human amniotic fluid which express OCT4, a marker of pluripotent stem cells, as well as stem cell factor, vimentin and alkaline phosphatase [86]. In the same year, In’t Anker et al. reported the isolation of mesenchymal stem cells with multilineage differentiation capacity from amniotic fluid [87]. A subsequent study used immunoselection for c-kit (CD117, receptor for stem cell factor) to isolate a population of cells with high self-renewal capacity that expressed some common ES cell markers (OCT4 and SSEA4) as well as markers of somatic stem cells (CD29, CD44,
CD73, CD90, and CD105) that are not typically detected in ES cells [14]. Several AF-derived clonal cell lines were established that exhibited the capability to differentiate into cell types from all three germ layers (including adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic cells) [14]. A number of other studies have also investigated the differentiation capacity of clonal AF-derived cells [88–93]. However, evaluation of the differentiation potential of AF-derived cells has relied heavily on expression of selected markers. Thus, further research is required to demonstrate that differentiated cells are capable of acquiring functional characteristics of the desired cell type, especially in vivo.

3.3. AF Cells in Tissue Engineering and Cell Replacement. Because they are readily accessible, pose little to no ethical concerns, and do not form teratomas in vivo, amniotic fluid-derived cells have been investigated as a promising alternative source of cells for use in tissue engineering and cell-based therapies. Kaviani et al. first demonstrated that mesenchymal cells from ovine or human AF could be seeded on synthetic scaffolds, as a prelude to using these cells for tissue engineering [94, 95]. Since that time, amniotic fluid-derived cells have been used in experimental settings to repair different tissues, including cartilage grafts for fetal tracheal reconstruction [96], tendons for diaphragm repair [97, 98], bone grafts [99–101], and heart valve leaflet [102–104]. In vivo administration of amniotic fluid-derived cells as a strategy for cell replacement has had beneficial effects in various injury models, including acute bladder injury [105], acute tubular necrosis of the kidney [106], hyperoxic lung injury [107] and ischemic heart [108]. The use of AF cells in tissue engineering and cell replacement has been extensively reviewed elsewhere [11, 12, 20] and is summarized in Table 2.

4. Complementary Applications of AE, AMS, and AF Cells

4.1. Paracrine Action of AF- and AM-Derived Cells in Tissue Repair. A common theme among several studies attempting to use AF, AE, or AMS cells for tissue repair in injury models is that, despite improving organ function, these cells often do not differentiate into the desired cell type or integrate fully into the target tissues [105, 129]. This issue may be particularly pertinent in neural applications, since the ability of AF-derived stem cells to differentiate into neurons has been a matter of debate [130, 131] and definitive evidence that AF, AE, or AMS stem cells can be induced to become mature functional neurons in vivo is still lacking. Nevertheless, the use of amniotic membrane- and fluid-derived cells for nervous system repair has met with some success. c-kit+ AF cells injected into injured chick embryo spinal cord increased embryo survival and reduced injury-induced haemorrhaging, although the cells failed to undergo terminal differentiation into neurons [132]. Pan et al. [113, 114] reported that AF-derived mesenchymal stromal cells improved motor function and electrophysiological indicators of nerve function in a sciatic nerve crush model, in the absence of stem cell penetration into the nerve. AF cells have also been shown to improve memory and sensory/motor functions following focal ischemia induced by middle cerebral artery occlusion (MCAO) in mice as soon as 4 days after cell injection [109]. Although the fate of the injected cells was not examined in that study, it is doubtful that AF cells could have differentiated into mature neurons capable of effectively integrating into the host circuitry to restore function on such a short time scale. Therefore, it is unlikely that cell replacement could directly account for the beneficial effects of AF cells in this study. In a rat model of Parkinson’s disease, implantation of AE cells into rat striatum prevented the degeneration of nigrostriatal dopaminergic neurons, when administered prior to the neurotoxin 6-OHDA [133], and attenuated motor disturbances in rats that had previously been subjected to 6-OHDA-induced degeneration [134]. Subsequent work showed that administration of AE cells into the lateral ventricle had a similar effect, which was maintained over 10 weeks despite the fact that the majority of the transplanted cells either did not survive, or did not exhibit a dopaminergic phenotype at the end of the experiment [135]. These results further suggest that the positive effect of the transplanted AE cells was not due to their ability to replace lost nigrostriatal neurons.

In a number of cases, the favourable outcomes observed after AF or AM cell transplants have been attributed not to the direct replacement of lost cells, but rather to factors secreted by the cells which may serve a protective or reparative function. Such paracrine mechanisms have also been postulated to explain some of the positive effects of other stem cell types in animal models of organ/tissue injury [136–138]. Studies in which conditioned media (CM), rather than AF or AM cells themselves, have been used in injured tissues support the notion that secreted factors mediate, at least in part, the beneficial effects of the transplanted cells. For instance, AF-CM [139] and AMS-CM [140] both reinstated blood flow in a murine hindlimb ischemia model, and AF-CM increased perfusion to an ischemic skin flap [141] likely owing to the presence of proangiogenic growth factors and cytokines, including VEGF, SDF-1, and TGF-β present within the media. AF-CM was also shown to stimulate other endogenous repair mechanisms, such as proliferation of dermal fibroblasts near the injury site in a mouse excision wound model [142] and recruitment of endothelial progenitor cells to ischemic skin flap [141]. Other paracrine mechanisms, such as the production of trophic factors [114, 143], immunomodulation [144, 145], and creation of a supportive milieu for regeneration [146] might also contribute to the ability of AF- or AM-derived cells to limit damage and/or stimulate repair of injured tissue.

4.2. AF- and AM-Derived Cells for Delivery of Beneficial Factors. Although AF- and AM-derived cells appear to have natural protective and reparative functions, they may also be used for efficient biodelivery of specific factors to enhance the protection or repair of damaged tissue through genetic modification. Accordingly, it was recently reported that AF mesenchymal stromal cells engineered to express elevated levels of GDNF ameliorated motor deficits in rats subjected to sciatic nerve crush, beyond the improvement observed
Table 2: Applications of AF stem cells.

<table>
<thead>
<tr>
<th>AF cell source</th>
<th>Target tissue</th>
<th>Animal/disease model</th>
<th>Delivery route</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Brain</td>
<td>Normal and twitcher neonatal mice</td>
<td>Intracerebroventricular injection</td>
<td>[14]</td>
</tr>
<tr>
<td>Human</td>
<td>Brain</td>
<td>Mouse cerebral ischemia</td>
<td>Intracerebroventricular injection</td>
<td>[109]</td>
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<tr>
<td>Human</td>
<td>Brain</td>
<td>Rat cerebral ischemia</td>
<td>Intrastriatal injection</td>
<td>[110]</td>
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<tr>
<td>Rat</td>
<td>Brain</td>
<td>Rat cerebral ischemia</td>
<td>Intravenous injection into the jugular vein</td>
<td>[111]</td>
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<tr>
<td>Human</td>
<td>Brain</td>
<td>Mouse motor cortex injury</td>
<td>Injection or implantation of cells seeded on biocompatible scaffolds into the motor cortex</td>
<td>[112]</td>
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<tr>
<td>Human</td>
<td>Nerve</td>
<td>Rat sciatic nerve crush injury</td>
<td>Injection or implantation of cells and fibrin glue into the injury site</td>
<td>[113–117]</td>
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<td>Human</td>
<td>Nerve, Muscle</td>
<td>Rat sciatic nerve crush injury</td>
<td>Intraepidermal injection</td>
<td>[118]</td>
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<tr>
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<td>Rat cardiac infarction</td>
<td>Intracardiac injection of cells or cell sheet fragments</td>
<td>[119, 120]</td>
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<td>Rat</td>
<td>Heart</td>
<td>Rat cardiac infarction</td>
<td>Intracardiac injection</td>
<td>[108, 121, 122]</td>
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<td>Rat pulmonary hypertension and heart failure</td>
<td>Intravenous injection into the tail vein</td>
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<td>Heart valve</td>
<td>Fetal sheep</td>
<td>Closed-heart implantation of cells seeded on biodegradable scaffolds in utero</td>
<td>[104]</td>
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<tr>
<td>Mouse</td>
<td>Skeletal muscle</td>
<td>Mouse spinal muscular atrophy</td>
<td>Intravenous injection into the tail vein</td>
<td>[124]</td>
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<tr>
<td>Human</td>
<td>Bone</td>
<td>Mouse subcutaneous implantation</td>
<td>Subcutaneous implantation of cells printed on biocompatible polymers</td>
<td>[14]</td>
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<tr>
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<td>Bone</td>
<td>Rabbit chest wall/sternal defects</td>
<td>Bone graft implantation of cells seeded on biocompatible scaffolds into the injury site</td>
<td>[99]</td>
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<tr>
<td>Human</td>
<td>Bone</td>
<td>Rat subcutaneous implantation</td>
<td>Subcutaneous implantation of cells seeded on biocompatible polymers</td>
<td>[101]</td>
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<tr>
<td>Sheep</td>
<td>Cartilage</td>
<td>Fetal lamb tracheal reconstruction</td>
<td>Tracheal implantation of cells seeded on biocompatible scaffolds in utero</td>
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<td>[97]</td>
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<td>Injection into the renal cortex</td>
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<td>Intravascular injection</td>
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<td>Abdomen</td>
<td>Rat</td>
<td>Intraperitoneal injection</td>
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<td>Rabbit</td>
<td>Fetal membranes</td>
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<td>Injection into the plug followed by fixation to the fetal membranes</td>
<td>[126]</td>
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<tr>
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<td>Nonspecific</td>
<td>Fetal lamb organs</td>
<td>Injection into the fetal peritoneal cavity in utero</td>
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<tr>
<td>Mouse, Human</td>
<td>Hematopoietic</td>
<td>Mouse</td>
<td>Intravenous injection into the retro-orbital vein</td>
<td>[128]</td>
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</tbody>
</table>

with green fluorescent protein (GFP)-transduced cells [147]. To extend this research to CNS applications, we are currently assessing the neuroprotective capacity of GDNF-expressing AF cells in a mouse motor cortex injury model (unpublished data). Both AE [148] and AMS [149] cells have also been used to deliver neurotrophic factors (GDNF and BDNF, resp.) to ischemic rat brain, and in both cases, enhanced recovery using GDNF- or BDNF-expressing cells was observed, relative to GFP-expressing cells.

AF- and AM-derived cells might be suitable for delivery of diverse compounds for a variety of diseases. For instance, a handful of recent studies have made use of AF cells for biodelivery of anticancer therapeutics. Yin et al. engineered AF mesenchymal stromal cells to express the antiangiogenic factor endostatin and the prodrug-activating enzyme secretable carboxylesterase 2 (sCE2) to treat glioma. sCE2 converts the antitumour drug CPT11 into its active form.

By injecting the engineered cells along with glioma-forming cells prior to treatment with CPT11, the AF cells boosted the conversion of the prodrug to its active form selectively at the tumour site, inhibiting proliferation, increasing apoptosis, and decreasing the population of glioma stem cells [150]. Similarly, expression in AF cells of cytosine deaminase and thymidine kinase, which act as suicide genes by converting two cancer prodrugs to their active toxic forms, inhibited the growth of breast tumours in a xenograft mouse model and prevented both the damage to the surrounding tissue and the physical side effects that were observed when the active drugs were directly administered [151]. These studies highlight a potential role for AF cells in biodelivery of a wide range of compounds.

Presumably, all of the above-mentioned studies have relied on bulk release of secreted factors into the extracellular space to mediate the beneficial effects of AF or AM cells.
However, we are also investigating the possibility that AF cells could be used for direct cellular delivery of certain types of molecules via gap junctional communication, as has been suggested by Brink et al. [152] for bone marrow mesenchymal stromal cells. AF cells express connexins, the proteins that make up gap junction hemichannels, and are capable of establishing gap junctional communication with cultured cortical cells, as evidenced by dye transfer [112]. Given the induction of connexin expression surrounding a surgical lesion to motor cortex, [112] as well as in other models of brain injury [153–155], it is hoped that AF cells might be capable of delivering small molecules through gap junctions to host cells, in an effort to protect the surrounding tissue or promote repair mechanisms.

5. Current Limitations in the Use of AM and AF Cells

Recent evidence suggests that diverse subpopulations of multipotent cells in amniotic fluid differ in marker expression, morphology, and/or growth kinetics [16, 156]. Furthermore, amniotic membrane-derived cells are not as homogeneous as previously thought. Different culture conditions and methods for isolating and expanding cells with stem cell characteristics might introduce a bias towards producing particular subpopulations of cells [11]. In addition, the gestational stage at which AF is collected [79] and the passage number of the cultured cells [157] will likely influence the resulting cell phenotypes and behaviour. At present, it is not clear exactly what effects these methodological differences have on the outcome of studies, but there is an agreement that cells used by different research groups may not represent identical biological properties. While this renders the comparison of different studies very difficult, it also prompts the question of whether different subpopulations of multipotent cells in AF and AM have distinct differentiation capacities. There is, in fact, some evidence that this is the case [156, 158, 159]. Further exploration of this issue is required, and hopefully it will be possible to exploit these differences to isolate cells with greater potential to differentiate into desired functional cell types. This should be done in conjunction with an examination of the role of culture conditions in directing AF, AE, and AMS cell differentiation towards particular cell fates.

Furthermore, it is possible that predifferentiation of AF- or AM-derived cells toward a desired phenotype prior to transplantation might promote engraftment in some tissues [160, 161]. This issue warrants further investigation, especially considering the low rate of differentiation of transplanted AM- or AF-derived cells observed in many studies.

Finally, although AM and AF-derived cells reportedly possess low immunogenicity and can survive transplantation into xenogeneic or allogeneic hosts [14, 20, 61, 62, 146, 162], one study found that AF cells were rejected upon transplantation into immunocompetent animals due to the recruitment of host T and B lymphocytes, natural killer cells and macrophages [163]. In another case, poor survival of amniotic epithelium grafts was observed in mice that received repeated transplants, because of immune rejection [164]. Other studies have also reported a low rate of survival of transplanted AF cells [114, 165, 166], which may be a result of immune rejection. Thus, as for ES cells, whose status as immune-privileged has been questioned [167], further research is required in order to understand the immunological properties of AM- and AF-derived cells, and to enhance graft survival.

Future Perspectives. There is a need for the establishment of national and international registries of cell lines derived from amniotic membrane and fluid in order to make these lines available to researchers worldwide. This strategy will facilitate the development of guidelines for the derivation and characterization of new cell lines and provide detailed protocols for culturing and differentiating existing lines. It is expected that the proposed approach would reduce methodological variabilities, which are compounded by the inherent heterogeneity of amniotic cells. In addition, the creation of a library of information pertaining to the research and (pre)clinical use of AF, AE, and AMS cells would allow researchers to choose the most appropriate cell line for a particular application, hopefully leading to more rapid development of effective regenerative therapies.

Acknowledgment

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References

8 Stem Cells International


Research Article

Clonal Populations of Amniotic Cells by Dilution and Direct Plating: Evidence for Hidden Diversity

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Fetal cells are widely considered a superior cell source for regenerative medicine; fetal cells show higher proliferative capacity and have undergone fewer replicative cycles that could generate spontaneous mutations. Fetal cells in amniotic fluid were among the first normal primary cells to be cultured ex vivo, but the undefined composition of amniotic fluid has hindered advance for regenerative applications. We first developed a highly efficient method to generate clonal populations by dilution of amniocentesis samples in media and direct plating without intervening refrigeration, centrifugation, or exposure of cells to the paracrine effects in mixed cell cultures. More than 40 clonal populations were recovered from 4 amniocentesis samples and representative clones were characterized by flow cytometry, conventional assays for differentiation potential, immunofluorescence imaging, and transcript analysis. The results revealed previously unreported diversity among stromal and epithelial cell types and identified unique cell types that could be lost or undetected in mixed cell populations. The differentiation potential of amniotic cells proved to be uncoupled from expression of definitive cell surface or cytoplasmic markers for stromal and epithelial cells. Evidence for diversity among stromal and epithelial cells in amniotic fluid bears on interpretations applied to molecular and functional tests of amniotic cell populations.

1. Introduction

The mission of regenerative medicine is to repair or replace tissues and organs that have been damaged by trauma, disease, or aging with living bioengineered tissue that restores function [1, 2]. Among cell sources for regenerative applications, stromal cells have gained increasing interest. Stromal cells, also known as multipotent stromal cells or MSCs, have been isolated from virtually all adult and postnatal tissues and organs [3]. Recent studies focus on use of stromal cells as a cell-based delivery system for trophic factors to repair damage and immunomodulatory activities to suppress damaging effects of inflammation, autoimmune, and graft-versus-host disease (GVHD) that can cause rejection of transplanted organs and tissues [4–7]. Fetal MSCs may be superior to other sources of MSCs with respect to proliferation capacity [8, 9]. Fetal cells can be obtained with minimally invasive methods during routine amniocentesis [10] and easily transitioned to ex vivo culture [11]. However, amniocentesis samples are complex mixtures of cells that are sloughed from fetal and placental surfaces that are exposed to amniotic fluid [12, 13]. Standards for cell-based therapies require cell populations that satisfy criteria for safety and efficacy [14–16]. Incorporation of amniotic cells into regenerative applications would be advanced by a better understanding of the complexity within amniotic cell populations and variation among amniocentesis samples from different donors.

Similarities and differences among amniotic cells have been classified to a large extent on cell shape. Clones of amniotic cells were first isolated almost 4 decades ago with cloning rings and classified on the basis of colony morphology, see Hoehn and Salk (1982) for a contemporaneous review [11]. F-type colonies consisted of “spindle-” shaped
fibroblast-like cells forming dense, multilayered colonies that are reminiscent of confluent stromal cell cultures. E-type colonies were formed by "epithelioid" cells with smooth margins and juxtaposed cell boundaries. AF-type colonies were the most common colony type from amniotic fluid, representing ~70% of colonies in one study, and considered to be specific to amniotic fluid. AF colonies consisted of fibroblast-like cells in a radial arrangement surrounding a dense amorphic cell aggregate that was resistant to enzymatic methods to generate single cells. While it is unclear whether AF-colonies reflect a cell type unique to amniotic fluid or the cell culture methods used, these pioneering studies set the stage for ex vivo culture of cells from amniotic fluid and provided the first widely used source of normal, rather than transformed, cells for biomedical research.

Current criteria for evaluating stromal cell identity and function have been based on bone-marrow-derived MSCs. These BMMSCs are the best studied stromal cells and are currently in clinical trials for treatment of several pathologies (http://www.clinicaltrials.gov/). BMMSCs are derived from bone marrow aspirates and adhere to plastic culture wares, in contrast to hematopoietic derivatives of bone marrow that proliferate in suspension [17]. The International Society for Cellular Therapy has established criteria for assigning BMMSC identity [18, 19], including adherence to plastic, differentiation into mesenchymal lineages of fat, bone, and cartilage, and expression of the cell surface markers endoglin or CD105, ecto 5′ nucleotidase or CD73, and Thy-1 or CD90. More than 95% of cells must express these markers, although the acceptable levels of absolute or relative expression have not been established. The relationship between these criteria for stromal cell identity and the potential therapeutic properties of stromal cells is not clear, in part because expression is correlative rather than causative and because the definitive set of cell surface antigens is not unique to stromal cells. Outstanding questions include whether stromal cell populations vary in expression of BMMSC-definitive markers and whether expression profiles are predictive of differentiation potential.

The diversity of cells in amniotic fluid bears on interpretation of molecular analyses and functional tests since the outcomes could reflect additive effects of one or more cell types. The presence of both epithelial and stromal cells in amniotic cell cultures has been reported in most [20–22] but not all studies [11, 23, 24]. Epithelial cells have been noted to quickly disappear during propagation of mixed cell cultures [25]. Amniotic cell cultures acquire a uniform stromal cell appearance [26] that can reflect replicative senescence of epithelial cells [11]. Loss of epithelial cells in culture could also reflect epithelial to mesenchymal transition (EMT), a molecular pathway in which epithelial cells become stromal cells with the immunoreactive profile and differentiation potential that is expected of MSCs [27, 28]. The complexity of amniotic cell populations could be addressed through analysis of clonal populations. Clonal populations of amniotic cells have been established with cloning rings [20], immunosolation of cells expressing the receptor for Steele Factor or CD117 [29], enzymatic treatment of preestablished cultures to generate single cells followed by limiting dilution [24, 29], and variations on these methods [30]. In each case significant effort is required to generate clones and amniotic cells are exposed to paracrine signaling in mixed cell populations during isolation.

The initial goal of this work was to develop an efficient method to establish independent clones from uncultured amniocentesis samples with minimal manipulation and without ex vivo expansion in mixed cell populations. We further asked whether clonal populations of stromal and epithelial cells differed from each other and from BMMSCs. Clones were characterized by phase microscopy, flow cytometry, in vitro differentiation, and high-resolution immunofluorescence imaging. The results revealed phenotypically and functionally distinct stromal cell clones and, for the first time, clonal populations of long-lived epithelial cells. Our findings show that the differentiation potential of amniotic cells need not mirror expression of cell surface markers of other amniotic cell clones or expression profiles of BMMSCs. We show that clones of amniotic stromal cells and epithelial cells can share nearly indistinguishable profiles of cell surface markers and coexpress cytoplasmic markers for epithelial and stromal cells, but differ in adipogenic and osteogenic potential. Analysis of multiple nonclonal mixed cell populations from different donors by high-resolution imaging identified most, but not all, of the same cell types in clonal populations and revealed clear differences between mixed cell populations. Taken together, our results shed new light on the differences among amniotic cells and raise questions of their source.

2. Materials and Methods

2.1. Cell Culture. Amniocentesis samples were donated with informed written consent, approved by the Institutional Review Board of Wake Forest University Health Sciences (2008) and deidentified for research. The age of the mother, period of gestation at which amniocentesis was performed, or the results of genetic testing were not approved for disclosure.

All amniocentesis samples were maintained at room temperature prior to cell culture. Samples used to derive mixed cell populations were diluted 1:1 to 1:2 in serum-containing media and plated in 1 or 2 wells of a 6-well plate. Samples used to derive clonal populations were diluted as described in Table 1 and distributed among one or more 24-well plates. Media and any nonadherent cells were routinely combined with approximately one-half volume of fresh media, transferred into new plates after 48–72 hrs, and discarded after another 72 to 96 hrs, allowing cells a total of 5 days to 7 days to adhere to culture wares. Cells were routinely maintained in Chang’s Media, which contained α-MEM supplemented with 15% FBS, 1% glucose and 1% penicillin/streptomycin, 18% Chang B, and 2% Chang C (Irvine Scientific). All cell cultures were maintained at 37°C with 5% CO2 in humidified incubators. Media components were obtained from Gibco/Invitrogen unless stated otherwise. Tissue culture wares (BD Falcon) were not pretreated with extracellular matrix proteins except for plates used in assays for differentiation potential as detailed below.
Table 1: Independent cell populations from uncultured amniocentesis samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution vol. mL</th>
<th>24 well plate</th>
<th>Clones</th>
<th>Doublets</th>
<th>Senescent clones</th>
<th>Total clones</th>
<th>Total viable clones</th>
</tr>
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<tbody>
<tr>
<td>RC</td>
<td>25</td>
<td>1</td>
<td>13</td>
<td>1</td>
<td>13</td>
<td>12</td>
<td>9</td>
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<td>1</td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>9</td>
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</tbody>
</table>

a Amniocentesis samples were donated with informed consent under approved IRB protocol.
b Diluted samples were plated in 1 to 5 tissue-culture-treated plates. Clones were assigned unique identifier based on position and plate of derivation.
c Populations arising from a single spherical cluster in a single well.
d Population derived from 2-well separated spherical clusters in a single well.
e Populations that did not continue to proliferate and were discarded after 2 weeks in culture.
f Total number of populations obtained from a single amniocentesis sample.
g Total number of viable populations expanded and cryopreserved for long-term storage.

Primary subconfluent cultures were passaged as needed to maintain healthy populations. Cells were passaged with Accutase diluted 1:4 in calcium and magnesium-free Dulbecco’s phosphate buffered saline (DPBS) with standard methods. Clonal populations in a single well of a 24-well plate were maintained as independent lines. Populations were passaged for expansion into the next largest culture volume: from 24 wells to 35 mm wells or to 60 mm plates before expansion into 100 mm plates and/or cryopreservation. Mixed cell populations that were derived from a single sample were harvested with enzymatic treatments, pooled, and cryopreserved in multiple aliquots with standard methods for long-term storage in liquid nitrogen.

BMMSCs were expanded from cryofrozen mononuclear cells that were derived from human bone marrow (Lonza, 2 M-125 C). Cryovials were thawed in a-MEM media supplemented with 10% heat-inactivated FBS, 1% glutamine, and 1% penicillin/streptomycin in 15 cm treated culture dishes. Some cells attached after several days and unattached cells were discarded in the first media change. The attached cells were then expanded in Chang’s media which better supported continued growth and simplified media preparation. BMMSCs at an early passage were frozen in small aliquots and thawed as needed.

2.2. Imaging and Immunocytochemistry. Cells were propagated in multiwell tissue culture plates on cover glass or in multiwell Permanox chamber slides (Nunc) for immunostaining. Samples were washed with Dulbecco’s phosphate-buffered saline (DPBS) and fixed for ~20 min with 2% paraformaldehyde (EM Sciences) in DPBS. Fixative was made fresh by dilution of 16% paraformaldehyde and frozen in aliquots at ~80°C. Aliquots of the fixative were thawed when needed, used once, and then discarded. Following fixation, cells were briefly washed twice in DPBS with or without ~ 0.1% (v/v) Triton-X and 0.1% (v/v) Tween-20 (Sigma) for permeabilization as needed. Cells were routinely blocked for ~30 min in blocking buffer containing 3% bovine serum albumin (BSA fraction IV, Jackson Immunolabs) in DPBS with or without detergents as appropriate. Cells were incubated with primary antibodies in blocking buffer at room temperature (RT) for ~1 hr and then washed for a total 30 min. Secondary antibodies in blocking buffer were applied for at least one hr at RT or overnight at 4°C. Primary antibodies for immunofluorescence were used at the following dilutions: AE1/AE3 (1:100) DAKO; vimentin (1:100) and fibronectin (1:100) Santa Cruz, N-cadherin (1:100) Pharmingen. Alexa Fluor conjugated secondary antibodies (1:1,000) were obtained from Invitrogen/Molecular Probes.

Stained samples were washed as before and mounted in Prolong Gold Plus (Molecular Probes) mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) for viewing. Unless noted otherwise, wide-field images were captured with Image-Pro software using a QImaging CCD camera mounted on a Leica upright microscope using a 20X dry objective (NA 0.40) and imported into Photoshop for presentation. Immunostaining was repeated in at least 2 technical replicates and in more than 3 independent trials for each marker/combination tested. The images shown throughout this paper are representative; conclusions were based on at least 3 fields of view for each replicate and inspection of more than 100 cells.

2.3. Flow Cytometry. Cells for flow cytometry were enzymatically treated with Accutase to generate single-cell suspensions. Cells were collected by centrifugation at 300 x g for 5 min, washed once in DPBS, and collected again by centrifugation. Cell pellets were resuspended in approximately 200 μl of DPBS before immediate dilution into 20 ml of 2% paraformaldehyde in DPBS. Cells were fixed for 20 min with gentle rocking, collected by centrifugation as described above, washed once in DPBS, and either used immediately or...
stored at 4°C. Prior to staining for flow cytometry, cells were collected by centrifugation and then resuspended in blocking buffer for 30 min. Cell suspensions in blocking buffer were filtered with 40 μm mesh baskets to eliminate cell clumps and then dispersed into individual 15 ml conical tubes for immunostaining. Cells were stained with fluorochrome-conjugated mouse monoclonal antibodies or with matched isotype control antibodies in blocking buffer for at least 1 hr at room temp. Cells were then washed twice with DPBS and resuspended in ~300 μl blocking buffer for analysis by flow cytometry.

Fluorochrome-conjugated antibodies were diluted in blocking buffer as recommended by the vendor: CD90 Fluorescein isothiocyanate or FITC (Millipore), CD90 Allophycocyanin or APC (BD Pharmingen), CD105 Alexa Fluor 647 (BD Pharmingen), CD105 FITC, (BD Pharmingen), CD73 APC (BD Pharmingen), SSEA4 Alexa Fluor 647 (BD Pharmingen), CD44 FITC (BD Pharmingen), and CD29 Alexa Fluor 488 (Molecular Probes/Invitrogen). Isotype controls were obtained from BD Pharmingen. Populations were gated to include only live cells and to exclude background staining that could be attributed to nontspecific staining by isotype antibody controls. Data was based on detection of 10,000 events for each marker. Data collection was performed with a FACSCalibur (BD Biosciences) flow cytometer and the results were imported into FlowJo 7.6.4 for analysis and presentation.

2.4. Adipogenic and Osteogenic Differentiation. Healthy cells were cultured to ~90% confluence in Chang’s media on cultureware pretreated with a 1:300 dilution of growth-factor-reduced Matrigel. Media were then switched to α-MEM supplemented with 10% FBS and agents for osteogenic [0.1 μM dexamethasone, 10 mM β-glycerol phosphate, 50 μM ascorbic acid 2-phosphate] or adipogenic [1 μM dexamethasone, 5 μg/ml insulin, 0.5 mM isobutylmethylxanthine (IBMX), 60 μM indomethacin] differentiation [31–33]. Media without differentiation treatments were used as a negative control and all media were exchanged every 3–5 days. Osteogenesis and adipogenesis were assessed with alizarin red and oil red-O staining, respectively, using standard histochemical methods. Each cell population was differentiated in at least 2 trials with more than 3 technical replicates, using undifferentiated cells in the same culture plate for controls and BM-MSCs for negative and positive controls, respectively.

2.5. Transcript Analysis. Total RNA was extracted with RNAeasy kits (Qiagen) with DNase treatment to eliminate genomic DNA according to manufacturer directions. RNA was converted to cDNA with Superscript III First Strand Synthesis kits (Invitrogen). TaqMan gene expression assays were used to detect transcripts of glucuronidase-β (GUSB), HAS939627, E-Cadherin (CDH1) HAS0123894, and N-cadherin (CDH2), HAS983056. Ct values of replicate assays were averaged and averages were normalized to expression of GUSB, an internal control that was included in all experiments.

3. Results
3.1. Dilution and Direct Plating of Uncultured Amniocentesis Samples. Centrifugation is commonly used to concentrate cells in amniotic fluid and minimize dilution of media, but it was unclear whether this manipulation is necessary to recover viable amniotic cell populations. Newly isolated amniotic fluid samples of less than 2.0 ml were diluted 1:1 or 1:2 in serum-containing media and directly plated in individual wells of a 6-well tissue-culture-treated plate. As early as the first 48 to 72 hrs of plating, a few adherent cells were detected in each sample. These populations, designated as ChM mixed cell populations, were expanded into larger culture wares within a few days. Virtually all primary cultures expanded as monolayers. Subsequent passaging with trypsin generated colonies with centrally located aggregates, reminiscent of descriptions of AF-type colonies in seminal works [11]. With rare exceptions, cultures passaged with Accutase expanded as monolayers without aggregate formation, suggesting that aggregate formation may reflect cell culture methods rather than a specific feature of a unique class of cells in amniotic fluid [11]. These findings together indicated that cell concentration is unnecessary for recovery of proliferating cells and that reducing the concentration of serum by ~1:1-2 does not preclude cell attachment and proliferation.

We next tested whether multiple independent populations could be isolated simply by diluting amniocentesis samples into larger volumes of media. The four subsequent samples that we received were diluted in 25 to 125 mL of serum-containing media and plated into one or more 24-well plates treated for tissue culture. Media and nonadherent cells were transferred as replicates to new plates after 48 hr to 72 hr. The 4 amniocentesis samples RC, LB, GW, and PB generated 12, 9, 14, and 9 independent viable cell populations, respectively (Table 1). Each population was assigned a name reflecting its source and its isolation address: the sample (RC, LB, GW, PB), primary or secondary (2) transfer, plate number (1–5), row (A–D), and column (1–6). Similar numbers of cell populations were derived from the 4 samples that we tested, but the sample size (n = 4) precludes meaningful tests of statistical significance. Nonetheless, 44 independent viable cell lines were generated within 2 to 4 days of plating less than 8 mls of uncultured amniocentesis sample using this simple and highly efficient method.

3.2. Clonal Populations Expand from a Discrete Point Source. Cell populations developed as expected of single-cell clones; daily monitoring by phase microscopy showed continual expansion of a small spherical cluster of cells, most often at the edge of the well (Figures 1(a), and 1(b)), but occasionally near the well center. We did not detect dispersion of cells throughout wells during population expansion, suggesting that any cell migration was limited to short distances. The clonal character of cell populations was consistent with detection of 2-well-separated expanding spheres in single wells, as if each cluster was initiated from 2 different founder cells. On the basis of these observations, we designate individual populations arising from a single spherical cluster...
as clonal populations and only such populations were studied further.

Expanded clonal populations could be classified by phase microscopy on the basis of cell morphology, designated here as stromal or epithelial to use terminology that is widely applied in cell biology and consistent with descriptions of cells in mixed cell populations that were established by others [11, 34, 35]. Stromal cell populations were dominated by large, well-separated flat cells with multipolar morphology and irregular cytoplasmic extensions (Figures 1(c), and 1(e)) that resembled those of BMMSCs (Figure 1(f)). Stromal cell populations typically contained very small cells with very little cytoplasm and small masses of cytoplasm without detectable nuclei. Epithelial populations consisted of spherical cells with a centrally located nucleus (Figure 1(d)), appearing as a phase refractive dome surrounded by flattened phase transparent cytoplasm. Primary populations of epithelial cells tended to be juxtaposed to each other in islands of multiple cells. All of the amniocentesis samples produced at least one apparently senescent population that did not expand enough to passage or failed to proliferate after passaging. While we did not attempt to expand and characterize all populations, simple inspection during colony expansion indicated that approximately half of the clones classified \( n = 22 \) were stromal and half were epithelial. Although AF-type clones fitting the description of Hoehn and Salk [11] were not typical, one GW clone consistently developed aggregates during repeated passages for reasons that are not yet clear. These findings show that morphologically distinct clonal populations can be isolated from uncultured amniocentesis samples simply by dilution and direct plating of fresh uncultured samples.

3.3. Analysis of Cell Surface Marker Expression by Flow Cytometry. Expression of cell surface markers was tested by flow cytometry to determine whether the profiles of amniotic stromal cell clones were comparable to those of BMMSCs and to determine whether flow cytometry could be distinguished between clonal populations of stromal and epithelial cells. Cells were immunostained with antibodies against CD73, CD90, and CD105, the minimal set of cell surface markers that are required for assignment of BMMSC identity [18] as well as additional markers that are widely used to characterize MSCs [29], including integrin\( \beta_1 \) that is detected with CD29 antibodies, the CD44 glycoprotein receptor for hyaluronic acid, and the stage-specific embryonic antigen 4 (SSEA4). Profiles were first established for BMMSCs as a positive control for immunodetection procedures. The results showed that nearly all BMMSCs expressed CD73 as well as CD29, CD44, and CD105, the minimal set of cell surface markers that are required for assignment of BMMSC identity [18] as well as additional markers that are widely used to characterize MSCs [29], including integrin\( \beta_1 \) that is detected with CD29 antibodies, the CD44 glycoprotein receptor for hyaluronic acid, and the stage-specific embryonic antigen 4 (SSEA4). Profiles were first established for BMMSCs as a positive control for immunodetection procedures. The results showed that nearly all BMMSCs expressed CD73 as well as CD29, CD44, and SSEA4 (Figure 2(a)). Large subsets of BMMSCs expressed CD105 and CD90, ~86% and 60% respectively, using FITC-conjugated antibodies (Figure 2(b)), but the proportion was less than the 95% frequency expected [36]. The proportion of CD105 and CD90 immunopositive cells increased to 100% and 97%,
Figure 2: Flow cytometry of cell surface markers. Cell populations BMPSCp5, PB4A2p20, PB3B5p14, and PB1C4p15 were stained with antibodies against (a) CD73, CD29, CD44, SSEA4 and (b) against CD90 and CD105. Cell populations are indicated in vertical text on the left of the corresponding row of histograms for each marker. The markers and conjugated fluorochromes are indicated at the bottom of the corresponding column of each histogram. The x-axis of all histograms corresponds to the mean florescence intensity (MFI) in log scale. The y-axis is the percentage (%) of events in linear scale that were detected at each position of MFI along the x-axis. Cell populations were gated to exclude presumptive dead cells and debris. The histogram of isotype controls is depicted by black line and the marker that is assayed is depicted in the filled histogram. 10,000 events were scored for all populations. The percentage of immunostained cells, excluding those stained by isotype controls, is indicated within each histogram.
respectively, with antibodies conjugated to the long wavelength flours Alexa Fluor 647 (AF647) and allophycocyanin (APC) that increase the sensitivity of detection (Figure 2(b)). Both sets of FITC- and APC/AF647-conjugated antibodies were used in the remaining experiments as an added measure of confidence for expression of CD90 and CD105 in amniotic cell populations.

Clones from the PB amniocentesis sample were analyzed: two stromal cell clones, PB4A2 and PB1C4, and an epithelial cell clone PB3B5. Like BM MSCs, nearly all PB4A2 and PB3B5 cells expressed CD73 as well as CD29, CD44, and SSEA4 (Figure 2(a)). PB4A2 stromal cells and PB3B5 epithelial cells showed very similar profiles of CD105 and CD90 expression; less than ∼14% and ∼7% of these cell populations expressed CD105 and CD90, respectively, as assayed with FITC-conjugated antibodies. The proportion of CD105 immunopositive PB4A2 and PB3B5 cells rose to 60% and 75%, respectively, with AF647-conjugated antibodies, but CD90 detection showed little change. In comparison with BM MSCs that were run in parallel, the mean fluorescence intensity (MFI) associated with immunostaining of CD105 and CD90 in PB4A2 stromal cells and PB3B5 epithelial cells was only modest or extremely low, indicating that CD105 and CD90 are not highly expressed. Two conclusions can be drawn from these findings. First, expression of CD29, CD44, CD73, and SSEA4 did not distinguish between BM MSCs and clones of PB4A2 stromal cells and PB3B5 epithelial cells. Second, flow cytometry did not distinguish between populations of PB4A2 stromal cells and PB3B5 epithelial cells. Finally, CD105 and CD90 expression in both PB4A2 stromal cells and PB3B5 epithelial cells was notably lower than in BM MSCs.

PB1C4 stromal cells differed from other cell populations; 67%, 63%, and 57% of cells expressed CD73, CD29, and CD44, respectively, and only ∼17% of cells expressed SSEA4 (Figure 2(a)). Fewer than 8% of PB1C4 stromal cells were immunopositive for either CD105 or CD90, even with AF647/APC-conjugated antibodies. Finally, the antibodies tested generated a single major peak in signal intensity in all other populations, but profiles of both control and test populations of PB1C4 cells showed multiple major peaks and a broad range of signal intensities. Although the molecular basis for the differences between PB1C4 and other cell populations is not clear, these data together with the data above show that dilution and direct plating can generate distinct clonal populations of stromal cells.

3.4. Osteogenic Differentiation. Results from flow cytometry suggested that amniotic cells would not show differentiation given that cell surface marker expression differed significantly from BM MSCs. Clonal cell populations were tested for osteogenic differentiation using standard methods. Subconfluent cultures of PB4A2p19, PB3B5p14, PB1C4p11, and BM MSCp5 cells were seeded in multiwell plates at near-confluent densities. Cells were subsequently maintained in differentiation media for 3 to 4 weeks, exchanging media every 3 to 5 days. Cells in media without differentiation supplements were used as negative controls. Following fixation and staining with alizarin red, robust deposition of calcium was detected in both BM MSCs and PB4A2 populations, but neither PB1C4 cells nor PB3B5 cells showed calcium deposition (Figure 3). Together with the results of flow cytometry, differential osteogenic potential of PB4A2 and PB1C4 populations supports recovery of distinct clones by dilution and direct plating. Further, the absence of robust expression of CD90 and CD105 suggests that expression of these markers is not predictive of osteogenic potential.

3.5. Adipogenic Differentiation. Parallel experiments tested for adipogenic differentiation using standard methods of differentiation followed by staining with oil red-O to detect fat droplets (Figure 4). Phase microscopy showed the appearance of fat droplets accumulating in BM MSCs and PB1C4 cells within 2 weeks after induction (data not shown). Staining with oil red-O showed large bright red droplets in approximately 30% of BM MSCs and approximately 10% of PB1C4 cells. Although PB4A2 populations showed only occasional cells (<1%) with similar clusters of large droplets, approximately 10% to 30% of PB4A2 cells had clusters of small bright oil red reactive droplets. Similar clusters of small oil red-O reactive droplets were also present in BM MSCs and PB1C4 cells. PB3B5 cells did not show oil red-O reactive droplets, indicating that PB3B5 cells do not have adipogenic potential and that small oil red-O reactive droplets are not artifacts of staining. Taken together with the evidence for osteogenic potential, these findings indicate that clonal populations of PB4A2 and PB1C4 cells have distinct differentiation potentials.

3.6. Immunostaining for Intermediate Filaments Typical of Epithelial and Stromal Cells. The results thus far showed differentiation of stromal, but not epithelial, cell populations. We next asked whether differentiation potential could be correlated with expression of stromal rather than epithelial cell markers. Cell populations were immunostained with panantibodies against keratins, a superfamily of intermediate filament family proteins that is expressed in epithelia [37], and vimentin, another member of intermediate filament superfamily that is widely used as a marker for stromal cells. BM MSCs cells showed well-organized immunopositive vimentin filaments as would be expected for stromal cells, but only a low level of diffuse keratin staining was detected in BM MSCs that was attributed to background staining. PB4A2 and PB3B5 cell populations showed bright immunopositive networks of both vimentin and keratin although the intensity of keratin immunostaining showed more variation in comparison to that of vimentin. In contrast to the other populations tested, the clonal population of PB1C4 cells did not show immunostaining of either vimentin or keratin networks. These findings show that expression of keratins or vimentin did not correlate with osteogenic and adipogenic differentiation potential. Further, amniotic stromal cells can be discriminated from one another on the basis of keratin and vimentin networks.

3.7. Immunodetection of Fibronectin and N-Cadherin. The absence of vimentin networks raised the question of whether
PB1C4 stromal progenitors expressed other stromal cell markers. Fibronectin is an extracellular matrix (ECM) protein that is highly expressed in stromal cells, although it is not exclusive to these cells [38]. N-cadherin is a cell adhesion molecule that is expressed in mesenchymal cells [39], in contrast to E-cadherin which is highly expressed in epithelial cells [28]. Double labeling experiments showed coexpression of fibronectin and N-Cadherin in all populations tested (Figure 6). However, differences were detected; BMMSCs showed elaborate networks of fibronectin while PB4A2 and PB3B5 cells showed sparse fibronectin filaments except localized areas of high cell density. PB1C4 cells showed dramatic immunostaining of fibronectin filaments, even in low-density cell cultures. Fibronectin filaments in PB1C4 cells appeared as almost parallel arrangements of short filamentous structures that were reminiscent of porcupine quills in contrast to the cross-hatched networks of long filaments in BMMSCs. These results together with the results of flow cytometry and differentiation assays indicate that PB1C4 cells represent a unique clonal population of stromal cells that was isolated by dilution and direct plating.

3.8. Differential Expression of Vimentin and Keratins in Mixed Cell Populations. Variation among amniotic cell clones predicted that ChM mixed cell populations would contain that same mixture of epithelial and stromal cell types. We tested this prediction using several of the ChM mixed cell populations that we isolated from different donors at the onset of this study. Preliminary inspection by phase microscopy indicated that ChM populations varied in the apparent proportion of epithelial and stromal cells; epithelial cells were highly enriched in the ChM1 population, but few were detected in other ChM populations (data not shown). Immunostaining with antibodies against keratin and vimentin or keratin and fibronectin revealed diversity in the size and shape of cells within and between ChM populations. The ChM1 population contained many large spherical cells with prominent networks of keratin and vimentin, representing 85% of cells ($n > 200$) as well as smaller...
Figure 4: Adipogenic differentiation. Populations of BMMSCp5, PB4A2p19, PB1C4p11, and PB3B5p14 cells were maintained in adipogenic media for 3 to 4 weeks. Representative bright field images show robust adipogenic differentiation in BMMSCs and PB1C4 cell populations as indicated by oil red-O stained spheres. Arrows and inserts indicate very small oil red-O positive spheres in BMMSC, PB4A2, and PB1C4 populations that were not detected in PB3B5 populations. Magnification is identical in all panels.

3.9. Epithelial Cell Populations Vary in Expression of E-Cadherin and N-Cadherin Transcripts. Coexpression of stromal and epithelial cell markers by immunofluorescence (Figure 6) raised the question of whether PB3B5 epithelial cells expressed transcripts of E-cadherin. Although all of the tested clones were immunopositive for N-cadherin, PB3B5 cells were immunonegative for E-cadherin (data not shown) as expected of epithelial cells. Sensitive gene expression assays were used to test for E-cadherin and N-cadherin transcripts in clonal populations as well as in the ChM1 mixed cell population. Primary cultures of BMMSCs and epithelial cells derived from human urothelium were used as controls for stromal and epithelial cells, respectively. N-Cadherin transcripts were detected in all control and amniotic cell populations; however E-cadherin transcripts were only detected in ChM1 cells and in control uroepithelial cells (Table 2). E-Cadherin expression in both control and ChM1 cell populations was confirmed by immunofluorescence analysis (data not shown). These findings show diversity among amniotic epithelial cells that can be defined by the presence or apparent absence of E-cadherin expression.

4. Discussion

4.1. Isolation of Phenotypically Distinct Clonal Populations by Dilution and Direct Plating. This work provides proof of concept that dilution of amniocentesis samples and direct plating, without refrigeration or centrifugation, is a highly efficient method to generate unique clonal populations. Clonal identity of PB4A2, PB3B5, and PB1C4 populations...
Table 2: Transcript analysis of E-cadherin and N-cadherin in clonal and mixed cell populations of amniotic cells.

<table>
<thead>
<tr>
<th></th>
<th>BMP5</th>
<th>UECp6^b</th>
<th>PB1C4p6</th>
<th>PB4A2p16</th>
<th>PB3B5p11</th>
<th>ChM1p4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUSB^a</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
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<tr>
<td>CDH1^c</td>
<td>0.00</td>
<td>30.59</td>
<td>0.00</td>
<td>0.00</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>CDH2^d</td>
<td>13.22</td>
<td>0.27</td>
<td>16.29</td>
<td>12.98</td>
<td>18.08</td>
<td>23.56</td>
</tr>
</tbody>
</table>

^a Ct values from 2 replicate TaqMan assays were averaged and normalized to expression of glucuronidase-β (GUSB).
^b Primary cultures of human uroepithelial cells.
^c Gene expression assay for E-cadherin.
^d Gene expression assay for N-cadherin.

Table 3: Similarities and differences among cell populations.

<table>
<thead>
<tr>
<th>Morphology^a</th>
<th>Differentiation^b</th>
<th>Contact inhibition^c</th>
<th>Coexpressed markers stromal/epithelial^d</th>
<th>Cadherin^e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stromal</td>
<td>Epithelial</td>
<td>Fat</td>
<td>Bone</td>
</tr>
<tr>
<td>PB4A2</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PB1C4</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>PB3B5</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>BM MSC</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ChM1</td>
<td>+</td>
<td>+</td>
<td>ND^f</td>
<td>ND</td>
</tr>
</tbody>
</table>

^a Morphology judged by phase microscopy of cells in newly established cultures. ChM1 mixed cell populations contained both cell types.
^b On the basis of alizarin red and oil red-O staining of differentiated cell populations.
^c Nonoverlapping epithelial cells in confluent cultures with cobblestone appearance.
^d Visible networks of keratin and vimentin in the same cell by high-resolution immunofluorescence microscopy.
^e Detected with TaqMan gene expression assays and/or by immunofluorescence analysis.
^f ND: not determined.

Figure 5: Immunostaining for vimentin and keratin. BM MSCp5, PB4A2p16, PB1C4p12 and PB3B5p11 cell populations were stained for the stromal cell marker vimentin (green), the epithelial cell marker keratin (red) and a fluorescent chromatin dye (blue). Note that virtually all PB4A2 and PB3B5 cells showed keratin staining, although the intensity varied. The clonal population of PB1C4 stromal progenitors did not show immunostaining of prominent networks of either vimentin or keratin. Scale (50 microns) is identical in all panels.
is reflected in the phenotypic differences among these clonal lines (Table 3). One inference of our findings is that cell populations in amniotic fluid have greater diversity than can be appreciated by assignment of amniotic cells as epithelial or stromal cell types on the basis of morphology by phase microscopy.

Dilution and direct plating allow efficient recovery of cell types that might otherwise be lost or undetected in mixed cell populations. This view is supported by 2 observations. First, we isolated clones of long-lived epithelial cells; PB3B5 cells have been in culture for more than 25 passages. This is significant because others have noted that clonal populations of epithelial cells are difficult to maintain beyond 5 or 6 passages [35, 40, 41] and that amniotic cell cultures either show or acquire a uniform stromal or fibroblast-like morphology during culture [26, 42–44]. Long-lived clonal populations like PB3B5 could reflect unique epithelial cell types and/or propagation of epithelial cell clones without the paracrine effects that may be present in mixed cell populations. A second observation supporting recovery of undetected cell types is isolation of atypical PB1C4 stromal cells; these progenitors possess adipogenic differentiation potential, but do not show detectable immunostaining of intermediate filaments vimentin or keratin. Given that polymers of intermediate filaments provide strength to the cytoskeleton and protect cells from shear force [45, 46], we speculate that cell shearing may underlie the complexity of PB1C4 cells that was detected by flow cytometry (Figure 2). Clonal populations of long-lived epithelial cells and atypical stromal cells, like PB3B5 and PB1C4, respectively, have not been previously identified in amniotic cell cultures.

4.2. Cell Surface Marker Expression Can Be Uncoupled from Differentiation Potential. Flow cytometry is widely used to characterize stromal cell populations from a variety of sources and coexpression of CD73, CD90, and CD105 is one criterion for MSC identity [18]. Several of our findings suggest that expression of these cell surface markers is not predictive of the differentiation potential of amniotic cells. First, significant proportions of all populations tested expressed CD29, CD44, CD73, and SSEA4, whether or not the populations showed evidence of differentiation potential. Second, both PB1C4 and PB4A2 populations showed osteogenic and/or adipogenic differentiation potential, but the frequency and intensity of CD90 and CD105 expression was very low or, in the case of PB1C4 cells, almost undetectable. Because analysis of BMMSCs was run in parallel in these experiments, low or undetected levels of CD90 and CD105 were not due to technical differences between experiments. Third, PB3B5 epithelial cells and PB4A2 stromal cells (Figure 1) showed almost indistinguishable profiles of surface marker expression by flow cytometry (Figure 2), but only PB4A2 cells showed differentiation potential (Figures 3 and 4). These findings show that adipogenic and osteogenic
differentiation potential of amniotic cells can be uncoupled from the cell surface markers that are widely used to gage stromal cell identity and potential differentiation capacity.

Several groups have profiled expression of cell surfaces markers in populations of amniotic cells and tested for differentiation potential to generate connective tissue lineages [23, 29, 43, 47, 48]. It is difficult to compare results from different studies, in part because studies vary in technical methods and in the benchmarks for assigning positive and negative results. Results may also vary because the composition and gestational age of the tested amniotic cell populations vary. Given that the tissue of origin and the conditions used to culture cells are known to impact the differentiation potential of stromal cells [3], the relationship between differentiation potential and expression of cell surface markers may vary for similar reasons. In addition to these influences, future work may show whether expression of cell surface markers and differentiation potential are impacted by paracrine effects on cells in mixed cell populations.

4.3. Coexpression of Epithelial and Stromal Cell Characteristics. Keratin is expressed in epithelial cells and known to be prevalent in amniotic cell populations [34]. High-resolution immunofluorescence imaging showed coexpression of keratin and vimentin in elaborate filament networks in subsets of amniotic cells, including the PB3B5 clonal
population of epithelial cells (Figures 5 and 6) and in spherical cells in mixed cell populations (Figure 7) that likely correspond to the epithelial cells detected by phase microscopy. Diversity among epithelial cells is suggested by expression of E-cadherin; this epithelia-specific adhesion molecule was not detected in PB3B5 cells although it was detected in the ChM1 cell population (Table 2). The basis for these apparent differences among epithelial cells is not clear, but differences could reflect variable lifetimes in culture or paracrine signaling in ChM mixed cell cultures that is not present in clonal populations. Differences could also reflect derivation from different fetal sources; fetal skin is a good candidate source of amniotic epithelial cells [34] and it is feasible that amniotic epithelial cells may be derived from placental membranes [49], released naturally or by needle puncture during the amniocentesis procedure. In addition to these sources, epithelia that line the internal surfaces of the fetus are also potential candidates, including epithelial cells from the gastrointestinal tract, lungs, and urinary tracts among others.

The clonal population of PB4A2 cells showed coexisting epithelial and stromal cell characteristics; PB4A2 cells coexpressed keratin with vimentin and showed multipotential differentiation potential to generate bone and fat. Coexisting epithelial and stromal cell character raises the question of whether stromal cells in amniotic fluid can result from EMT [27, 28]. Precedence for stromal cell derivation through EMT comes from studies in which epithelial cells were induced to release from mammary gland epithelium [50]. These epithelial cells transitioned into MSCs that express stromal cell markers and differentiated into fat, bone, and cartilage [51]. Although derivation by EMT is feasible, it is unlikely to be the exclusive source of stromal cells since PB1C4 stromal progenitors did not show expression of epithelial markers and mixed cell populations included many stromal cell types that lacked keratin expression. Further work is needed to show whether EMT contributes to the pool of stromal cells in amniotic fluid and whether EMT impacts the diversity among amniotic epithelial cells.

4.4. Novel Clonal Populations of Stromal Cells as Resources for ECM Proteins. The extracellular matrix (ECM) is of critical importance to tissue engineering and manufacture of bioengineered organs. Decellularization is perceived to leave behind detergent insoluble ECM that provides form and organization for revascularization and function [52]. While there is considerable advance in tissue and organ engineering, reseeding of decellularized and bioengineered organs with viable, proliferation-competent cells remains a challenge [52–56]. Recent work showed that preseeding biodegradable scaffolds with BMMSCs improved performance of grafted constructs [57]. Within this framework, an outstanding feature of the PB1C4 population was the widespread deposition of fibronectin in low-density cultures (Figure 6). Clonal populations of stromal progenitors like PB1C4 from amniotic fluid offer a cell-based source and/or delivery vehicle for fibronectin and other ECM proteins to improve outcomes with bioengineered scaffolds.

Conflict of Interests

The authors have no commercial associations that might create a conflict of interests in connection with this paper.

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References

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Research Article

Periostin as a Biomarker of the Amniotic Membrane

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Tracing the precise developmental origin of amnion and amnion-derived stem cells is still challenging and depends chiefly on analyzing powerful genetic model amniotes like mouse. Profound understanding of the fundamental differences in amnion development in both the disc-shaped primate and human embryo and the cup-shaped mouse embryo is pivotal in particular when sampling amniotic membrane from nonprimate species for isolating candidate amniotic stem cells. The availability of molecular marker genes that are specifically expressed in the amniotic membrane and not in other extraembryonic membranes would be instrumental to validate unequivocally the starting material under investigation. So far such amniotic markers have not been reported. We postulated that bone morphogenetic protein (BMP) target genes are putative amniotic membrane markers mainly because deficiency in one of several components of the BMP signaling cascade in mice has been documented to result in defective development of the early amnion. Comparative gene expression analysis of acknowledged target genes for BMP in different extraembryonic tissues, combined with in situ hybridization, identified Periostin (Postn) mRNA enrichment in amnion throughout gestation. In addition, we identify and propose a combination of markers as transcriptional signature for the different extraembryonic tissues in mouse.

1. Introduction

The amnion is the innermost extraembryonic membrane that surrounds the foetus of amniotes and delineates the fluid-filled amniotic cavity, thereby providing a confined environment within the conceptus and conferring protection and shock resistance. In most amniotes, the amnion is a thin and avascular transparent membrane. In recent years, human term amnion has attracted considerable attention because amniotic-ectoderm- and mesoderm-derived cells can differentiate into cells from the three germ layers in cell culture. Furthermore, resident stem cell-like cells in the amniotic ectoderm have been reported (reviewed in [1, 2], this issue). In addition, the low immunogenicity of the amnion makes this “medical waste” tissue of great interest for (regenerative) medicine. Indeed, the amnion has been used for over a century as a wound dressing.

Recently, studies aiming to explore the presence and origin of amniotic stem cells have been accomplished, using much more powerful genetic model organisms, such as, mouse and rat. Despite the fundamental differences in amnion development in the disc-shaped primate embryo and in the cup-shaped mouse embryos (for review: [2]), amniotic-membrane and amniotic-fluid derived cells with stem-cell-like features have been isolated from mouse and rat [3, 4].

In human, both amnion and chorion surround the embryo and both membranes fuse during the second trimester of pregnancy, while the yolk sac remains rudimentary [2]. In contrast, in mouse, the chorion will never fuse with the amniotic membrane after the physical separation of the amniochorionic fold shortly after gastrulation at embryonic day (E)7.0 [6]. The chorion becomes incorporated in the chorionic disk of the placenta, whilst the amnion...
becomes surrounded by the visceral yolk sac, except in the part of the chorionic disk. Importantly, amnion on the one hand and yolk sac and chorionic disk on the other hand remain spaced by the fluid-filled exocoelomic cavity (Figures 1(a)–1(c)). In mouse embryos, the amnion consists throughout gestation of a simple bilayered membrane of squamous mesoderm and ectoderm, which face the exocoelomic and amniotic cavity, respectively.

Awareness of the fundamental different surrounding tissues of human and mouse amnion is important when collecting amniotic membrane from nonprimate species to isolate the so-called amniotic stem cells [2]. The availability of molecular markers that are specifically present in amnion and not in the other extraembryonic membranes, or in the respective fluids, would therefore be helpful to characterize unequivocally the initial starting material from which stem cells are isolated.

Spatial gene expression studies rarely include information on expression in amnion, in part because this tissue appears prone to be neglected or discarded, but also because it is often hard to distinguish low expression levels from background staining due to the flat cellular architecture of this stretched membrane. To our knowledge, amnion-specific genes have not explicitly been reported, but the physiologic features of the amnion may hint towards putative candidate amnion markers. The amnion is a very elastic tissue that resists to increasing stretch. Hence, progressively, a basal lamina composed of collagen, laminin, nidogen, and fibronectin fibers forms between the amniotic ectoderm and mesoderm [7, 8]. The amniotic epithelium acquires
Figure 2: RNA profiling of putative amnion markers in mouse extraembryonic tissues by RT-qPCR. (a)–(b) Validation of microdissected amniotic membrane tissues collected at different stages of development by relative RNA expression analysis of markers for (a) nonneural ectoderm (Ap-2) and allantois (Tbx4) and (b) primitive red blood cells (ζ-globin) and yolk sac endoderm (Afp). (c)–(e) Expression analysis of acknowledged target genes of SMAD-mediated BMP signaling (Tbx2, Tbx5, Hand1, Hand2, Msx1, Msx2) and (f) Postn, in extraembryonic tissues. Relative RNA levels were obtained by setting the sample with lowest expression for each target to 1. The expression of different targets cannot be directly compared. Abbreviations: Am: amnion; Al: allantois; YS: yolk sac.

an increasing number of microvilli at the surface, which may be associated with enhanced filtering and transport capacity across the membrane. Mouse models with impaired amnion development may also tip-off candidate amnion markers. Remarkably few mouse mutants displaying defects in amnion formation have been described (reviewed in [6]), in contrast to the many mutants with defects in allantois [9] or placenta [10]. Many of the mutants that display defects primarily related to amnion development seem to point at impaired bone morphogenetic protein (BMP) signaling (Bmp2, Smad5) or are found in genes that encode putative modulators of BMP signaling (Amm, Bmp1). Amnionless
Figure 3: *In situ* hybridization on sections of mouse embryos. (a) RNA localization (blue) of *Postn* in E8.5 mouse embryos. Boxed areas are shown at a higher magnification. *Postn* appears localized in the amniotic mesoderm. (b) RNA localization (blue) of *Postn* in E9.5 mouse embryos. Abbreviations: Am: amnion; Al: allantois; AmM: amniotic mesoderm; AmEC: amniotic ectoderm; Ch: chorionic plate; Ht: heart; Hd: head; Pl: placenta; YS: yolk sac.

Figure 4: (a) RNA profiling of *POSTN* in human extraembryonic tissues of three individual embryos by RT-qPCR. Gestational age is represented by weeks (w) and days (d). (b) Relative RNA expression of *Postn/POSTN* in mouse and human amnion samples from different developmental stages. In human amnion *POSTN* is expressed at very high levels during the first trimester, followed by a significant drop in expression, whilst *Postn* expression in mouse amnion is stable during gestation. Mouse and human developmental stages are positioned along an arbitrary interval scale. Mouse and human gestation approximates 19.5 days and 38 weeks respectively. Mouse and human developmental stages do not match pairwise. Abbreviations: Am: amnion; Ch: chorion, E: embryonic day; Pl: placenta, UC: umbilical cord, 8–38 w: weeks of gestation.
(Amn) mutants develop the most specific defects because they lack an amnion, whereas chorion, yolk sac blood islands, and allantois develop normally [11]. Bmp2 null embryos fail to close the amnion [12]. Mice deficient in Smad5, an intracellular mediator of BMP signaling, also show delayed amnion closure, in addition to local amnion thickenings that contain ectopic stem cell-like cells, haematopoietic and endothelial cells [13–15]. Several ligands may elicit BMP signaling in amnion. Bmp4 is expressed abundantly in mouse amnion, but Bmp2 and Bmp7 transcripts have also been reported in amnion and adjacent tissues [16–18].

The crucial role for BMP signaling in amnion development made us hypothesize that target genes for BMP are candidate markers of the amniotic membrane. Given the poor documentation of the expression of such target genes in the developing amnion and its neighboring tissues, we have performed a comparative gene expression analysis of several such target genes in different extraembryonic tissues. Transcripts for periostin (Postn) appeared to be highly enriched in mouse and human amnion at different stages of development. Periostin is a secreted ECM protein that can interact with different ECM proteins and integrins and that is induced by transforming growth factor (TGF)β and BMPs in tissues undergoing remodeling or active stress (reviewed in [19]). In situ hybridization analysis confirmed the amnion-enriched localization of Periostin mRNA in amnion. We propose to use a combination of Periostin and AP-2 as biomarkers for developing mouse amniotic membrane.

2. Materials and Methods

2.1. Collection of Mouse and Human Extraembryonic Tissues. Wild type mouse embryos (CD1) between E8.5 and E18.5 were isolated in ice cold PBS, followed by collection of the amnion, allantois, and visceral yolk sac tissues. The material was washed in ice cold PBS and immediately frozen and stored at −80°C until further processing. BRE:LacZ embryos are transgenic for a gene composed of a BMP-responsive element (BRE) from the Id1 promotor that drives β-galactosidase synthesis that reports BMP-SMAD activity [5]. These embryos were isolated between E7.0 and E8.0 in ice cold PBS, and further processed for β-galactosidase staining. CD1 embryos were collected at E8.5 and E9.5 in ice-cold PBS, fixed in 4% paraformaldehyde in PBS and further processed for in situ hybridization (ISH).

First and second trimester human extraembryonic tissues were isolated in ice cold PBS and immediately frozen in RLT buffer (Qiagen) until further processing (LUMC). These tissues were collected from voluntary abortions without medical, fetal or obstetrical complications. Human term amnion was collected following planned cesarean sections at the Obstetrics and Gynaecology division, UZ Leuven. Collection of mouse and human tissues was approved by the ethical commission from the KU Leuven (097/2008) and by the Medical Ethical Committee of the Leiden University Medical Center (P08.087), respectively.

2.2. β-galactosidase Activity and In Situ Hybridization. After brief glutaraldehyde/formaldehyde fixation, BRE : LacZ heterozygous embryos were washed in PBS and stained for β-galactosidase overnight at 30°C in a staining solution of 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside (X-gal, Fermentas, R0941), as described before [5]. Stained and postfixed embryos were subsequently washed, dehydrated, paraffin embedded and sectioned at 6 μm. Slides were counterstained with Mayer’s Hematoxylin.

For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C, washed with PBS and saline, dehydrated and embedded in paraffin, and sectioned
at 6 μm. In situ hybridization on sections with DIG-labeled antisense riboprobes against Tbx2 [20], Tbx5 [21], Msx1 [22], Msx2 [23], and Postn [24] was performed using an automated platform (Ventana Discovery, Ventana Medical Systems).

2.3. Gene Expression Analysis. RNA was extracted and purified with RNaseasy purification columns (Qiagen, RNaseasy Mini or Micro kit, 74104 and 74004). Reverse transcription was performed using SuperScript III reverse transcriptase (Life Technologies), oligo-dT and random primers (Life Technologies). Real-time qPCR was performed on LightCycler 480 Real-Time PCR System using LightCycler 480 SYBR Green I Master mix (Roche, 4707516001), and all reactions were in technical duplicates. Primers were designed with the online tool of IDT (http://www.idtdna.com/). Gapdh and Ubc (mouse) and GAPDH and β-Actin (human) were used as reference genes for normalization. Mouse primer sequences are (forward primer first) Afp: GATGAAAACCTATGCCCCTCC, CAAAAGGCCCCGAGAATCTG; Ap-2: CATTACCCTCTCATCTAG, TTTGCACACGTACC; CAAAGT; TACCCTCCTACGTCACTAG, TTTCGCACACGTACC; Hand1: CGAAGAACGGAAATAGTGATCG; Hand2: AGGCCCTCA-AGGCGGAGATCAA, CCTGTCCGGCTTTGGTTTTC-ΔΔ; TTTAGCTCCAGCGCCCAGACTT; Tbx5: AGTGGGCAGCGTTAGCT; Ap-2: CCTCC, CAAAAGGCCCGAGAAATCTG; Tbx2: ATCGACAACAACCTGCTTA-TC, TGCACCACCAACTGCTTACTT; RPL19: TCTCTGCGAA TGTCA-AAAAGGGTCATACACGTACTTC

3. Results

Immediately after amnion formation the mouse amnion separates and demarcates the extraembryonic region from the embryonic region of the conceptus. At that stage the amnion does not surround the developing embryo yet (Figure 1(a)). At the early organogenesis stage the embryo undergoes an axial rotation and hence it gets enveloped by its amnion and yolk sac (Figures 1(b)–1(c)). Mouse amniotic membrane is composed of amniotic ectoderm in continuity with the embryonic ectoderm and of amniotic mesoderm sharing its borders with the mesothelium that delineates the visceral yolk sac and the allantois/umbilical cord in the most posterior part of the amnion (Figure 1(a)).

To investigate whether BMP elicits SMAD-mediated BMP signaling around and beyond the stage of amnion closure, we monitored BMP-SMAD activation in amnion of BRE: LacZ reporter embryos. These mouse embryos report the activation of expression of target genes for activated BMP-SMADs [5] (Figures 1(d)–1(h)). Before amnion closure, active BMP-SMAD signaling is most predominant in the amniotic ectoderm component of the amniochorionic fold (Figure 1(d)). After closure high BMP-SMAD signaling levels persist in both layers of the amnion, and the signaling domain starts to expand into the extraembryonic-embryonic junctional region, more specifically in anterior embryonic ectoderm and mesoderm and the most posterior embryonic mesoderm and extraembryonic mesoderm of amnion, allantois, and yolk sac (Figures 1(e)–1(h)).

Amniotic membranes and visceral yolk sacs (called from now on yolk sacs) were microdissected from E8.5, E9.5, E13.5, and E18.5 mouse embryos, covering early organogenesis, organogenesis, and preterm stages of development. In addition, allantoises were isolated from E8.5 and E9.5 embryos. Tissues from at least five embryos were pooled for the E8.5 and E9.5 samples, whilst tissues from older gestational stages were processed individually, and the mRNA was isolated and cDNA synthesized. To assess the quality of these samples, the presence of transcripts encoded in different extraembryonic tissues was evaluated by quantitative reverse transcription-PCR (RT-qPCR). Tbx4 was used as an allantois-specific marker [25], and Afp and ζ-globin markers of the visceral endoderm and primitive haematopoietic cells, respectively, the latter developing in the mesoderm of the visceral yolk sac [26, 27]. An appropriate amnion-specific marker was lacking at this stage but Ap-2 (Tfap2A) was included in the analysis, as we previously reported that this marker for nonneural surface ectoderm and neural crest cells [28] is also abundantly expressed in amniotic ectoderm [13]. This analysis showed that microdissected amniotic membrane samples were not contaminated with allantois (Figure 2(a)) or yolk sac (Figure 2(b)) tissue.

Identically the same amnion, visceral yolk sac, and allantois samples were profiled by RT-qPCR for the expression of 7 selected BMP targets (Tbx2, Tbx5, Hand1, Hand2, Msx1, Msx2 and Postn) as a first selection criterion for identification of a putative amnion marker(s).

The transcript levels of the T-box transcription factor encoding gene Tbx2 are high in allantois and yolk sac
when compared to the amnion (Figure 2(c)). The expression in the allantois seemed most predominant at E8.5 and correlates with the expression domain that has been reported previously by *in situ* hybridization [29]. This differential enrichment in the allantois becomes unlike the *Tbx4* mRNA expression progressively lost from E9.5 onwards. The expression domain of *Tbx5* has been documented by *in situ* hybridization in the allantois of E7.5 embryos [30]. The *Tbx5* profiling does not result in a stable and robust pattern of expression in the different extraembryonic tissues (Figure 2(c)). Hence, we consider neither *Tbx5* nor *Tbx2* as candidate markers for amniotic membrane.

*Hand1* and *Hand2* encode basic helix-loop-helix transcription factors that are essential for heart and extraembryonic development [31], with *Hand1* seemingly as the favorable candidate marker for amnion (Figure 2(d)). However, in addition to robust expression in the amnion [32] high expression of *Hand1* has been demonstrated in the yolk sac of a *Hand1*:LACZ-reporter mouse strain [31]. Moreover, *Hand1*-deficient embryos show defects in yolk sac vasculature, suggesting that *Hand1* is important for yolk sac development [33].

*Msx1* and *Msx2* are Msh homeobox-containing transcription factors involved in neural tube, heart, tooth, limb, and craniofacial development, and are reported to be immediate effectors of BMP signaling (reviewed in [34, 35]). *Msx1* expression has been reported for chick amnion [36], and *Msx2* mRNA was detected in human placenta [37]. This made us investigate the expression of these two genes in mouse extraembryonic tissues. RT-qPCR results showed high expression of both transcripts in amnion early during gestation but also in allantois (Figure 2(e)).

*Postn* encodes a poorly described extracellular matrix (ECM) protein. Its expression has been reported in heart and also in the amnion of developing embryos [14, 38], albeit its expression in the other extraembryonic tissues has not been documented. In independent experiments, *Postn* expression was consistently found to be enriched in amnion throughout mouse development (Figure 2(f)), but it was also detected in the yolk sac and allantois. Indeed, *in situ* hybridization analysis confirmed the high expression levels of *Postn* in mouse amnion at E8.5 and E9.5 and highlighted that expression is predominant in amniotic mesoderm (Figures 3(a)–3(b)). *Postn* expression was not detected in the allantois at E8.5, but expression was clearly detected in allantois/umbilical cord at E9.5. Likewise, *Postn* expression was only detected sporadically in mesothelium cells of the visceral yolk sac at E8.5, but *Postn* expression was observed throughout the mesothelium of amnion, allantois, and yolk sac at E9.5 (Figures 3(a)–3(b)).

In human, RT-qPCR analysis of three individual embryos demonstrated that *POSTN* gene expression is highly enriched in amnion during the first trimester of gestation, in comparison with the other extraembryonic tissues: chorion, placenta and umbilical cord (Figure 4(a)). *POSTN* expression in the umbilical cord was relatively high as well, similarly to the results for E9.5 mouse allantois (Figure 2(f)). Thus, *POSTN* may be considered as amnion marker in humans too, but it needs to be specified that its expression was only validated by RT-qPCR in isolated extraembryonic tissues (Figure 4(a)). Unlike in mouse, *POSTN* levels in human amnion decrease progressively during gestation (Figure 4(b)).

*Postn* expression is enriched in the amniotic membrane, but its expression in extraembryonic tissues is not exclusive to the amnion. To use *Postn* with confidence as an amnion marker we have analyzed a combination of markers for different extraembryonic tissues on extraembryonic samples of E8.5 and E9.5 mouse embryos (Figures 5(a)–5(b)). Based on these results, we conclude that the amniotic membrane is a tissue that expresses relatively high levels of *Postn* and *Ap-2*, and ignorable levels of ζ-globin, Afp and *Tbx4* (Figures 5(a)–5(b)).

## 4. Discussion

Given that *Bmp4* expression is patent in developing mouse amnion [16], that genetic mouse models deficient in several components of the BMP signaling cascade develop early amnion defects [6], and that active BMP-SMAD signaling is ongoing in amnion during early organogenesis development, we hypothesized that target genes for BMP are putative amniotic membrane markers. Based on this information, we followed an educated guess approach and identified *Postn* from a preset selection of 7 acknowledged BMP target genes as an amnion-enriched marker gene throughout gestation.

All selected BMP target genes were clearly expressed in mouse amniotic membrane, whereas expression of another BMP target gene, the allantois-specific marker *Tbx4* [25], was indeed not detectable in amnion. The spatial-temporal expression pattern of the different selected BMP target genes followed different trends in the different extraembryonic tissues during development which suggests that they do not all belong to one synexpression group. For instance, *Postn* transcript levels were constantly high in amnion samples throughout gestation, whereas the *Hand1* and the *Msx2* transcripts appeared enriched in the amnion early during organogenesis, but this became less prominent in function of time. The spatial-temporal regionalization of the expression patterns of the respective BMP target genes suggests that the extraembryonic tissues under investigation are exposed to dynamic levels of signaling by (different) BMP morphogens and/or a different regionalization of transcriptional coactivators and repressors may result in a different transcriptional response.

*Postn* is a secreted ECM protein that has been related to bone and heart development as well as to cancer [19]. The protein is associated with areas of fibrosis; it can directly interact with other ECM proteins, such as fibronectin, tenasin-C, collagen I, collagen V and heparin sulfate proteoglycans. Periostin serves as a ligand for specific integrins, such as αvβ3, αvβ5 and α4β1 but interacts also with focal adhesion kinases and can thus affect the ability of cells to migrate [19, 39]. *Postn* is expressed in fibroblasts or in cells that adopt fibroblast-like characteristics following an injury event [19]. The extracellular and secreted nature of the *Postn* protein makes this a less appropriate amniotic membrane...
marker for FACS-based sorting of cells. Nonetheless, Postn is an interesting BMP target gene in the context of the highly stretched amniotic membrane. The expression of Postn has been reported to be induced by BMPs, but also by TGFβ1, and by mechanical stretch [40]. Secreted growth factors of the TGFβ and BMP families are well known for their involvement in endocardial cushion development within the embryonic heart tube. Postn mRNA has been shown to be expressed in the developing mouse embryonic and fetal heart and localizes to the endocardial cushions suggestive of a role in valvulogenesis and valvular disease [41]. Indeed, loss of Postn results in the inappropriate differentiation of mesenchymal cushion cells and valvular abnormalities via a TGFβ-dependent pathway during establishment of the mature heart [19, 40, 42, 43]. So far no amnion defects have been reported in Postn null mice. It has, however, been demonstrated recently that Postn interacts with BMP-1. This interaction probably results in enhanced deposition of BMP-1 and BMP-1-mediated proteolytic activation of lysyl oxidase on the extracellular matrix, which promotes collagen cross-linking [44]. BMP-1, despite its misleading name, is not a BMP-related growth factor but a Procollagen C-proteinase. Intriguingly, mice deficient in BMP-1 do develop an amnion defect [7].

In the last few years, ECM has been recognized as an important source of regulatory signals in normal tissues and tumors (reviewed in [45]). Recent studies indicate a link between cancer stem cells and their metastatic niches. Together with another ECM protein-tenascin C–Postn plays a key role as metastasis niche component for breast-derived tumour-initiating cells that invade the lungs [46]. By enhancing Wnt and Notch signaling in cancer cells, Postn and tenasin C provide physical and signaling support for metastasis-initiating cells. Postn deficient mice develop mammary tumors, but their ability to metastasize to the lungs is significantly diminished compared to tumors in wild-type mice. Malanchi et al. propose that the role of Postn in progression of lung metastasis is to concentrate Wnt ligands in the metastatic niche for the stimulation of stem-like metastasis-initiating cells [46]. Postn promotes tumor metastasis and facilitates invasion in the tumor microenvironment also in colorectal, pancreatic, oral, prostate, esophageal, and ovarian cancer [47–52]. Perhaps the presence of Postn in amnion may contribute to the reprogramming capacities of amniotic membrane cells in cell culture.

Postn is enriched in mouse amnion, and its expression level remains fairly constant during gestation. Postn mRNA appeared localized in the amniotic mesoderm, while the amniotic ectoderm appeared negative (Figure 3(a)). This was especially clear before embryo turning at E8.5 when amnion is less stretched. POSTN can be considered a suitable amnion marker in humans too, although its transcript levels decrease progressively during gestation. The timing of the observed decrease seems to correlate with the phase when amnion and chorion physically fuse, and it is therefore tempting to speculate that changes in mechanical stretch and pressure/contact with the chorion as compared to the previously surrounding fluid attenuates the POSTN levels in the amniotic membrane. During the first trimester of gestation, POSTN is highly enriched in human amnion comparing to the other extraembryonic tissues (chorion, placenta and umbilical cord), but it is not restricted to amnion only, as demonstrated by its expression in the umbilical cord. The expression of POSTN throughout gestation may be dynamic in different extraembryonic tissues because POSTN mRNA has been detected by in situ hybridization in the stromal cells of human term placenta [53]. In this study the authors do not report POSTN expression levels in other nonplacental extraembryonic tissues. In any case, analyzing sets of markers for human amniotic membrane instead of using a single marker gene is preferable, and more research needs to be performed in this direction.

Our in situ hybridization analysis confirms that mouse Postn is enriched in amnion during early organogenesis and highlights furthermore that it is expressed in amniotic mesoderm. However, Postn expression can be observed in mesothelium of the visceral yolk sac and allantois as well. To bypass making premature conclusions on amnion identity based on analysis of Postn expression levels only, we suggest to categorize amnion, visceral yolk sac and allantois by expression profiling of a set of different marker genes. Mouse amniotic membrane is characterized during early organogenesis by high levels of Postn and Ap-2, low levels of ζ-globin expression, and ignorable levels of Afp and Tbx4 (Figure 5). Visceral yolk sac can be classified as the tissue with high expression levels of Afp and ζ-globin, with moderate expression of Postn and Ap-2, and absence of Tbx4, whereas the allantois is characterized by high Tbx4 expression level and weak expression of Postn, Ap-2 and ζ-globin. Afp expression was undetectable in the allantois (Figures 2(b) and 5). Since ζ-globin is a marker of primitive haematopoietic cells in the yolk sac, it is not advisable to use this marker beyond early organogenesis development.

In summary, we propose using Postn and Ap-2 as a marker set enriched in mouse amniotic membrane and we propose a combination of markers as transcriptional signature for the different mouse extraembryonic tissues. The unbiased identification of additional markers, preferentially of amnion-enriched intracellular proteins and/or membrane proteins, and the use of panels of amnion markers should be further encouraged.

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References


Research Article

Human Amniotic Fluid Cells Form Functional Gap Junctions with Cortical Cells

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The usage of stem cells is a promising strategy for the repair of damaged tissue in the injured brain. Recently, amniotic fluid (AF) cells have received a lot of attention as an alternative source of stem cells for cell-based therapies. However, the success of this approach relies significantly on proper interactions between graft and host tissue. In particular, the reestablishment of functional brain networks requires formation of gap junctions, as a key step to provide sufficient intercellular communication. In this study, we show that AF cells express high levels of CX43 (GJA1) and are able to establish functional gap junctions with cortical cultures. Furthermore, we report an induction of Cx43 expression in astrocytes following injury to the mouse motor cortex and demonstrate for the first time CX43 expression at the interface between implanted AF cells and host brain cells. These findings suggest that CX43-mediated intercellular communication between AF cells and cortical astrocytes may contribute to the reconstruction of damaged tissue by mediating modulatory, homeostatic, and protective factors in the injured brain and hence warrants further investigation.

1. Introduction

Recent advances in regenerative medicine have boosted efforts to explore the therapeutic potentials of stem cells to repair damaged tissue in the injured brain (reviewed in [1–4]). In particular, the transplantation of embryonic stem cells [5], fetal neural stem or progenitor cells [6–8], or bone-marrow-derived stem cells [9, 10] into the injured brain has been explored extensively. However, human embryonic stem (ES) cells and fetal neural stem cells are subject to ethical considerations and the risk of tumor development, whereas adult neural stem cells have limited proliferation capabilities and lineage restriction. Therefore, other stem cell sources, such as human amniotic fluid (AF) [11–13], are being considered for therapeutic applications. There is evidence that AF contains stem cell subpopulation(s) [14] isolated based on c-Kit (CD117—the receptor for stem cell factor [15]) expression, which share some of the characteristics of embryonic and adult stem cells [14]. For instance, several reports have shown that AF cells can differentiate along the adipogenic and osteogenic [16–18], myogenic [19, 20], and endothelial [21] pathways. Furthermore, AF cells have also been shown to harbour the potential for neurogenic differentiation, using different induction protocols [14, 18, 22–25]; however, the proof that these cells can differentiate into functional neurons remains elusive [26, 27]. Nonetheless, the versatility of AF-derived cells for therapeutic applications has been investigated in various animal injury models in the central and peripheral nervous system [14, 28–32]. Although it has been suggested that AF-derived cells exert beneficial effects on the ischemic brain to an extent comparable with the neuroprotective effect of embryonic neural progenitor cells [32], it remains to be determined whether or not these cells are capable of integrating into the brain and developing functional connectivity with the host tissue to support neuroregenerative and protective
capabilities. The success of this strategy depends on the formation of a rapid and efficient intercellular communication between grafted AF cells and the host tissue followed by the reestablishment of functional networks. In fact, a recent report by Jäderstad et al. [33] clearly shows that an essential step in the functional integration of grafted ES cells, even before mature electrochemical synaptic communication, is cell-cell coupling via gap junctions. This integration is, at least in part, dependent on the formation of gap junctional intercellular communication (GJIC), which is considered to be an indispensable mechanism for the propagation of information among cells in the CNS. Gap junctions are composed of two juxtaposed, membrane-bound connexin hemichannels; each composed of six connexin subunits, which are joined to bridge the cytoplasm of two neighbouring cells [34, 35]. This consolidation allows the transfer of small ions and molecules, nutrients, metabolites, second messengers, and more recently miRNAs [34, 36]. Hence, intercellular communication between graft and host cells underlies many of the early cellular interactions and plays a central role in the rescue of damaged host cells after brain injury [33]. It is expected that intercellular gap junction formation would result in cell-cell communication between host and graft cells and hence increase transplantation success rates as well as the transfer of therapeutic agents. More specifically, connexin-associated gap junction formation and function have been shown to be pivotal for ensuring host cell well-being and potentially mediating a neuroprotective effect [33]. In fact, NSC-mediated rescue of damaged host neurons did not occur when gap junction formation was suppressed by pharmacological and/or RNA-inhibition strategies [33].

Although AF cells have been previously transplanted into several tissues, including the brain, currently there is no information on gap junctions in these cells and whether they form a means of intercellular communication with the host tissue. Therefore, a better understanding of the interactive processes by which AF cells integrate into host neural tissue may provide insights into the interplay between donor and recipient. In this study, we examine the expression of connexins in AF cells at the RNA and protein levels, using in vitro and in vivo techniques. In addition, we determine whether AF cells can form functional gap junctions with other AF cells as well as with cortical cells.

2. Methods

2.1. Cell Culture. Human amniotic fluid (AF) cells were obtained from the Ottawa Hospital, General campus (Ottawa, ON, Canada), following amniocentesis in women at 15 to 35 weeks of gestation (AF15–AF35). The study was approved by the Ottawa Hospital and the National Council Canada-Research Ethics Boards, and a written informed consent was obtained from each donor. AF cells were cultured in Dulbecco’s modified medium (DMEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS, Hyclone) and maintained at 37°C and 5% CO₂ (as described in [37]). AF cells were passaged at 70% confluency every 2-3 days, using 0.05% trypsin/EDTA (Invitrogen) at a 1:3 split ratio.

To generate AF-derived single cell clones [37], a single cell suspension was prepared by gentle trypsinization, and individual AF cells were deposited one cell per well of a 96-well plate, containing 100 μL of DMEM + 20% FBS, using a MoFlo cell sorter (Beckman Coulter). Once the cultures became 70% confluent, clones were subcultured first into 24-well plates, followed by 6-well plates (Nunc), and eventually into 10 cm culture plates (Corning), using the above-mentioned conditions. To purify c-kit-positive AF cells from AF cultures, dissociated single AF cells were stained with c-kit antibody (Santa Cruz, sc-5535) for 30 minutes at 4°C, as previously described [14]. Following the incubation period, the cells were washed twice with cold 2% FBS in PBS and incubated for 30 minutes at 4°C with a secondary phycoerythrin- (PE-) conjugated antibody. The cells were subsequently washed, resuspended in 2 mL of cold 2% FBS in PBS, filtered through a 70 μm filter, and analyzed using a MoFlo Cell Sorter (Beckman Coulter). The clonal (AF-F5) and c-kit-positive AF cells were thereafter expanded serially with a split ratio of 1:3 and cultured in DMEM containing 20% FBS to establish the AF-F5 single-cell-derived clonal line and c-kit-positive AF cell population.

Mouse cortical progenitors were isolated from the E13 ventricular zone, plated onto PLL-coated coverslips (9 × 10⁵ living cells/mL) in DMEM + 10% FBS, and examined within 24 hrs after plating, as previously described [38]. Cortical neurons were generated from neural progenitors by reducing the serum concentration (i.e., 0.5% FBS) during the first 24 hrs, followed by treatment with DMEM + N2 supplement to limit the generation of glial cells. Medium was replenished every 48 hrs for 7 days. Astroglial cultures were generated from E13 neural progenitors (0.5–5 × 10⁵ living cells/mL) cultured in DMEM + 10% FBS. Medium was replenished every 48 hrs for 3 weeks, and cells were passaged several times to eliminate neurons in the cultures [38]. To generate mixed cultures of cortical neurons and astrocytes, E13 neural progenitors were maintained in DMEM + 10% FBS for 2 weeks without passaging.

NT2-D1 progenitor cells (ATCC) were cultured in DMEM (Invitrogen) media supplemented with 10% FBS (Hyclone). Pure cultures of NT2-derived neurons (NT2-N) were prepared as previously described [39]. HaCaT cells were a generous gift from Dr. Kursad Turksen (Sprott Centre for Stem Cell Research, Ottawa, ON, Canada) and were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Hyclone) and split every 2 days.

2.2. RNA Extraction and RT-PCR. Total RNA was extracted from cells, using TRIReagent (Molecular Research Centre), as previously described [37]. Total RNA was quantified with NanoDrop (Thermo Fisher Scientific), and 1 μg was reverse transcribed using Quantitect reverse transcriptase (Qiagen). RT-PCR amplifications were carried out using iQ Supermix (Bio-Rad) in a 20 μL volume containing, 5 μM sense and antisense primers (Table 1), and 15 ng of cDNA. The PCR program consisted of a denaturing step for 3 mins at 94°C, followed by 30 secs at 94°C, 30 secs at 55–58°C, and 30 secs at 72°C for 40 cycles. The final PCR extension period was 5 mins at 72°C. PCR products and 1kb ladder (Invitrogen)
Table 1: Sequence and annealing temperatures for RT-PCR.

<table>
<thead>
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<th>Designation</th>
<th>Sequence (5′-3′)</th>
<th>Annealing temp. (°C)</th>
<th>Amplicon size (bp)</th>
<th>Ref.</th>
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<td>308</td>
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<td>GAPDH-R</td>
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</table>

were separated on a 2% ethidium bromide agarose gel, and the images were captured with FluorChem 8900 Imager (Alpha Innotech). The amplicon size was confirmed by comparison with the ladder (Invitrogen). Expected amplicon sizes are shown in Table 1. B-ACTIN (ACTB) was used as a normalizing gene. NT2/D1, HaCaT, and NT2-neurons (NT2-N) were used as positive controls.

2.3. Antibodies. The following antibodies were used in this study: β-ACTIN (1:5000, WB, Sigma), Cx43 (1:500, ICC; WB, 1:4000, ICC, Sigma), Cx26 (1:100, Zymed), GFAP (1:200, ICC, NeoMarkers), Gβ3 (1:200, ICC, Molecular Probes), MAP2 (1:200, ICC, Sigma), human nuclear antigen (1:100, ICC, antibodies-online), human mitochondrial marker (MTCO2) (1:50, ICC, Abcam), c-kit antibody (1:100, FACS, Santa Cruz sc-5535), and fluorescence-conjugated secondary antibodies (Alexa Fluor 488 anti-rabbit or mouse, rhodamine anti-mouse and Alexa 647 anti-mouse, 1:500, molecular Probes). Hoechst (1:1000, Sigma) was used to stain nuclei.

2.4. Western Blotting. Cells were washed with cold TBS and lysed directly in the culture plate using ice-cold lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton-X, 1% Na deoxycholate), containing a protease inhibitor cocktail (Roche). Cell lysates were incubated for 30 min on ice and clarified by centrifugation at 20 000 g for 20 mins. Protein samples (40 μg) and a molecular weight rainbow marker (Amersham) were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Amersham), using a wet transfer apparatus (Bio-Rad) at 20 V overnight at 4°C. The membranes were incubated in TBS containing 5% nonfat milk with 0.1% Tween-20 (Sigma) for 1 hr at room temperature to block nonspecific binding and then incubated in primary antibodies overnight at 4°C. The membranes were then washed three times for 10 mins with TBS containing 0.1% Tween-20 and incubated with a peroxidase-conjugated secondary antibody for 1 hr at room temperature. Immunoreactivity was visualized, using chemiluminescent substrate (New England Nuclear) and captured by FluorChem 8900 (Alpha Innotech).

2.5. Immunocytochemistry. Cells were grown on coverslips, washed with PBS, and fixed with 65% ethanol containing 0.15 M NaCl for 20 mins [37]. For staining with human nuclear antigen, cells were fixed with 3% paraformaldehyde for 5 mins, washed twice with PBS, and permeabilized for 20 mins in 0.2% Triton-X in PBS (pH 7.0). Following fixation, the coverslips were blocked with serum-free-protein block (Dako) for 30 mins and incubated for 1 hr at room temperature with the primary antibody. Following three subsequent washes (5 mins each) in PBS, the coverslips were incubated with a fluorescence-conjugated secondary antibody for 1 hr, washed, and counter-stained with Hoechst (Sigma). The coverslips were mounted, using Vectashield mounting medium (Vector Laboratories), and immunoreactivity was examined under an Axiovert 200 M fluorescence microscope (Zeiss) and a confocal microscope (Olympus).

For GFAP, Cx43, and Hoechst staining in sections containing implanted AF-DsRed cells, Alexa 488 and Alexa 647 were used as the secondary antibodies to visualize Cx43 and GFAP, respectively. In addition, different filter sets were used for Hoechst (excitation 365, emission 420) and AF-DsRed, cells (excitation 546, emission 575). A separate image was acquired for each fluorophore (Hoechst, Alexa 488, DsRed and Alexa 647), using a laser scanning confocal microscope.
Dye coupling experiments were performed to evaluate the functionality of gap junctions between individual AF cells and other AF cells in culture or mouse neural progenitors, neurons, and astrocytes, as previously described [39, 42]. In brief, AF cells were preloaded with two dyes: 0.1% 1′-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchololate (DiI, Invitrogen) and 0.1% calcine-acetoxyethyl ester (Calcein AM, Invitrogen) for 20 mins at 37°C. DiI, a lipophilic dye that binds to cell membranes and is not transferred to adjacent cells, was used to label donor cells. Calcein AM is in a membrane-permeant form and is taken up by donor cells and hydrolyzed to calcine (MW = 623) by cellular esterases. After cleavage, calcine was readily transferred to adjacent receiving cells through gap junction channels. The Dil- and calcine-loaded cells were washed with isotonic glucose solution to remove excessive dye and dissociated into a single cell suspension, following incubation in trypsin-EDTA (Invitrogen) for 2 mins. Preloaded single AF cells were plated onto cultures of AF or mouse cortical neural progenitors, neurons, or astrocytes, and dye coupling was evaluated after 4 hrs. The level of coupling was determined by counting calcine-positive, Dil-negative cells coupled to a calcine-positive, Dil-positive cell, and the data was presented as mean ± SEM.

2.6. Dye Coupling. Dye coupling experiments were performed to evaluate the functionality of gap junctions between individual AF cells and other AF cells in culture or mouse neural progenitors, neurons, and astrocytes, as previously described [39, 42]. In brief, AF cells were preloaded with two dyes: 0.1% 1′-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchololate (DiI, Invitrogen) and 0.1% calcine-acetoxyethyl ester (Calcein AM, Invitrogen) for 20 mins at 37°C. DiI, a lipophilic dye that binds to cell membranes and is not transferred to adjacent cells, was used to label donor cells. Calcein AM is in a membrane-permeant form and is taken up by donor cells and hydrolyzed to calcine (MW = 623) by cellular esterases. After cleavage, calcine was readily transferred to adjacent receiving cells through gap junction channels. The Dil- and calcine-loaded cells were washed with isotonic glucose solution to remove excessive dye and dissociated into a single cell suspension, following incubation in trypsin-EDTA (Invitrogen) for 2 mins. Preloaded single AF cells were plated onto cultures of AF or mouse cortical neural progenitors, neurons, or astrocytes, and dye coupling was evaluated after 4 hrs. The level of coupling was determined by counting calcine-positive, Dil-negative cells coupled to a calcine-positive, Dil-positive cell, and the data was presented as mean ± SEM.

2.7. Scratch Wound Injury. Scratch wound procedure was performed as previously described [43]. Briefly, scratch wound injury was applied to confluent cortical cultures grown on coverslips, using a 21 G needle (0.8 mm diameter) to create a wound. The cells were washed three times with culture media, fixed with 75% ETOH + 0.15 M NaCl without injury, or 15 mins, 24 hrs, 48 hrs, or 72 hrs after injury. In some experiments, AF cells were plated onto cortical cultures immediately following injury, and the cocultures were fixed at the above-mentioned time points. The labeling of AF cells with EGFP was performed, as previously described [37].

2.8. Motor Cortex Brain Injury. Brain injury studies were approved by the Animal Care Committee at the National Research Council Canada. Briefly, 6-week-old C57 black mice (C57Bl/6, Charles River) weighing about 25 g were anesthetized with isoflurane (Aerrane, Baxter) and placed into equal groups: injury or no injury with cell injection or implant. Prior to injury, mice were placed in a stereotaxic frame, and a midline incision was made in the skin to expose the skull. The bone overlying the motor cortex was removed with a dental drill following mapping, using specific stereotaxic coordinates (from “AP −0.25 mm to −1.0 mm, Lat +0.7 mm”, to “AP +1.25 mm to +3.0 mm, Lat +2.4 mm”) with respect to Bregma (0 mm), as previously described [44]. Injury to the left motor cortex was performed using a sterile graduated needle to remove neural tissue to a depth of 1 mm. The injury site was sealed with bone wax, covered with topical anaesthetic (0.5% marcaret bupivacaine hydrochloride, Sigma), and the skin was sutured.

For transplantation studies, AF cells engineered to express DsRed driven from a CMV promoter by lentiviral infection (Tet07-CMV-DsRed) were injected into three sites within the motor cortex (100,000 cells in 2 μL of PBS per injection), using a 10 μL Hamilton syringe, controlled by an infusion pump at a constant speed (0.5 μL/min) over 4 mins. The syringe was held in place for 5 mins and then gradually withdrawn. After the procedure, bone wax was used to seal the injection sites and the skin was sutured.

The animals were allowed to recover in their cages and sacrificed 2 weeks later. The brains were removed and processed, as described below. The animals were sacrificed after 12 days and the brains were perfused, removed and fixed with 4% paraformaldehyde in PB overnight, washed twice with PB and transferred to 30% sucrose in PB for 2 days. The brains were frozen in O.C.T. compound and sectioned into 8 μm slices (Leica CM 1950). Prior to staining, the sections were thawed at room temperature for 15 mins, washed three times (5 min each) in PBS, and immunostained, as described earlier. Injected AF cells were identified based on DsRed expression (AF-DsRed).

3. Results

3.1. AF Cells Predominantly Express Connexin 43 (CX43). To establish a profile of connexin expression in AF cells, we performed RT-PCR to examine the expression of connexins commonly expressed in the brain (CX26, CX30, CX32, CX36, CX37, CX40, CX43, and CX45). Our results show that AF cells ubiquitously expressed CX43 (GJA1) and CX45 (GJA7) at the gestation periods examined (AF15–AF35) (Figure 1(A)). The expression of CX30, CX32, CX36, CX37, or CX40 was not detected in any of the gestation periods examined (data not shown), whereas CX26 (GJB2) RNA was expressed in the majority of gestation periods (Figure 1(A)) and CX26 protein was only found in a small subset of AF cells in culture (Figure 1(C), (g)-(h)). Of the connexins expressed, CX43 was the most abundant protein in AF cells, as determined by western blotting and immunocytochemistry (Figure 1(B) and 1(C), (a)-(f)). Similar to other connexins, CX43 is assembled into connexins in the trans-Golgi network and transported to the cell membrane where adjacent hemichannels on apposed cells dock to form gap junction plaques [45]. Indeed, we found an intracellular pool of CX43 in the perinuclear Golgi apparatus, as confirmed by trans-Golgi network membrane protein golgin-97-positive staining in AF cells (Figure 1(C), (a)-(b)). Subsequently, CX43 is translocated from the Golgi apparatus to the cell membrane (Figure 1(C), (c)-(d)) and this dynamic process leads to the formation of discrete gap junctions between adjacent cells, as observed by distinct punctate staining at the cell-cell boundaries between individual AF cells.
Figure 1: Expression of connexins in human amniotic fluid (AF) cells. Figure 1(A): RT-PCR analysis of Connexin (CX) expression in AF cells at 15 to 35 weeks (wks) gestation. AF cells expressed CX26, CX43, CX45 in all gestation periods examined. GAPDH transcript and human HaCaT, NT2-D1, and NT2-N cells were used as internal and positive controls, respectively. NTC, No Template Control. HaCaT, Human keratinocyte cell line; NT2-D1, (N)Tera-2 human teratocarcinoma cell line and NT2-derived neurons (NT2-N). Figure 1(B): Western blot analyses confirmed the expression of CX43 protein in AF26 (top panel) and AF30 (middle panel) cells. Embryonic day 18 (E18) mouse brain (Br) and B-ACTIN (ACTB) were used as positive and internal controls, respectively. Figure 1(C) Immunocytochemistry further verified the presence of CX43 and CX26 proteins in AF cultures. CX43 expression (green) was detected as punctate staining at the perinuclear region and the cell membrane. CX43 appeared to be associated with golgi complex in the perinuclear region, as determined by golgin-97 (red) and CX43 double staining (a). The punctate staining pattern ((a), (c), (e)) demonstrated the dynamic translocation of CX43 protein from Golgi complex (a) to the cell membrane (e), arrowheads). Unlike CX43, CX26 protein expression appeared limited to the perinuclear region (g). Nuclei were stained with Hoechst (blue). Panels (b), (d), (f) and (h) represent the corresponding phase contrast images of (a, c, e and g); respectively. Scale bar: 25 μm. Figure 1(D) Dye coupling assessment in AF cells. AF donor cells (AF26, (a); AF16, (d)) were preloaded with Dil (red) and calcine (green) and plated as single cell suspensions onto confluent monolayers of receiving AF cells. Calcine transferred from the donor AF cell to adjacent receiving cells, indicating the formation of functional gap junctions between individual AF cells within 4 hours. Scale bar: 50 μm.
Figure 2: Connexin expression in c-kit-positive and single-cell-derived AF clones. (A) c-kit-positive AF cells were obtained by fluorescence activated cell sorting (FACS). (B) RT-PCR analysis of connexin (CX) expression in c-kit-positive (AF-c-kit) and single-cell-derived (AF-F5) AF clones. GAPDH transcript was used as an internal control. (C) Immunocytochemistry confirmed the expression of CX43 (green) in AF-c-kit and AF-F5 cultures. Hoechst was used as a counter-stain (blue). (b) and (d) represent the corresponding phase contrast images. Scale bar: 25 μm. (D) AF donor cells were preloaded with DiI (red) and calcein (green) and plated as single cells on cultures of AF cells. Calcein transferred from the donor AF cell to neighbouring cells, confirming the formation of functional gap junctions among AF-c-kit cells as well as AF-F5 cells within 4 hours. Scale bar: 50 μm.
Figure 3: Cx43 expression in mouse cortical cells. (a) Immunocytochemistry showed that Cx43 (green) is expressed in Nestin- (red) positive cortical progenitors. (c) Similarly, abundant levels of Cx43 protein were detected at cell-cell boundaries in cortical astrocytes. (e) Only a limited amount of Cx43 was detected in immature neurons (arrow), whereas astrocytes maintained high degrees of Cx43 expression. (b), (d) and (f) are the corresponding phase contrast images of (a), (c) and (e), respectively. Nuclei were stained with Hoechst (blue). Scale bar: 10 μm.

This characteristic was not observed for CX26 in AF cells where protein expression was confined to the perinuclear region (Figure 1(C), (g)-(h)).

In order to examine the functionality of gap junctions, we preloaded individual AF cells with two dyes (DiI and calcein) [39, 42] and plated them onto confluent cultures of AF cells. Dye coupling, consistent with the presence of functional gap junctions, was scored by calcein transfer from labeled AF cells to recipient AF cells 4 hrs after-plating. Coupling was observed in an average of 9 ± 1.06 SEM calcein-positive recipient cells coupled to one DiI-positive labeled cell (Figure 1(D), (a–f)).

3.2. Cx43 Expression in c-kit-Positive and Single-Cell-Derived Clonal AF Cell Populations. Given the heterogeneity of AF cells [37], we used two established protocols to generate a more homogenous cell population based on single cell cloning [37] and c-kit expression [14], as previously reported. Hence, we generated single-cell-derived clonal AF (AF-F5) and c-kit-positive AF (AF-c-kit, Figure 2(A)) cell populations and found a similar RNA expression profile for CX43 and CX45 (Figure 2(B)) and protein expression for Cx43 (Figure 2(C), (a–d)). Dye transfer experiments further confirmed the functionality of gap junctions formed in AF-c-kit (Figure 2(D), (a–c)) and AF-F5 (Figure 2(D), (d–f)) cultures to a similar degree as observed above (data not shown). Hence, AF-F5 cells were used in all subsequent experiments herein.

3.3. Intercellular Communication between AF Cells and Cortical Cultures. Cx43 is considered to be the most ubiquitously expressed member of the connexin family in the mammalian brain and during brain development specifically in neural progenitor cells and astrocytes [46]. Hence, we sought to determine whether AF cells retain Cx43 expression and functional gap junctions in cocultures with cortical cells, in particular, with cortical progenitors and astrocytes which are known to express high levels of Cx43 [46] (Figures 3(a)-3(b)) and 3(c)–3(f), resp.). Indeed, when AF cells were seeded on cortical cultures, Cx43 was detected at the cell-cell boundary between AF cells and GFAP-positive cortical astrocytes (Figures 4(a)–4(c)), arrows). In parallel cocultures, AF cells were distinguished from mouse cortical cells, using a human specific nuclear antigen (hNuc) (Figures 4(d)–4(i)) or in some instances with a human-specific mitochondrial antigen (see Figure 6). Quadruple staining...
confirmed that the cortical cells, which formed gap junctions with AF cells, were GFAP-positive astrocytes (Figures 4(g)–4(i)). Interestingly, when AF cells were cultured alone, CX43 expression was predominantly cytoplasmic and perinuclear (Figure 1(C), (a–d)); however, AF cells cocultured with cortical cultures showed more membrane-bound CX43 staining between adjacent cortical astrocytes (Figure 4(c), 4(f), and 4(i), arrows). Even in the presence of cortical neurons, the majority of CX43 expression was observed at the boundary between AF cells and GFAP-positive astrocytes (Figures 5(a)-5(b)), arrowheads); whereas a negligible amount of Cx43 protein was observed between AF cells and neurons at the interface of neurites and the AF cell membrane (Figures 5(a)-5(b), arrows). Although the staining results suggest that AF cells retain CX43 expression to mediate gap junction formation with target cells, we performed dye transfer experiments (as outlined earlier) to confirm functional gap junction formation and intercellular connections between AF cells and cortical cultures. Coupling was observed in an average 40 ± 15.21 SEM calcein-positive recipient cells coupled to a single calcein-positive, DiI-positive donor AF cell after 4 hours (Figures 5(c)–5(e)). Of note, in earlier cortical cultures (2 days in vitro), AF cells were able to establish functional communication with immature cortical neurons
Figure 5: AF cells selectively establish intercellular communication with cortical astrocytes. (a)-(b) Cocultures of AF cells with cortical cells. Immunostaining showed abundant levels of Cx43 protein (green) at the junction between AF cells and cortical astrocytes (GFAP (red); see arrowheads), whereas there was only a limited amount of staining detected at the boundary with neurons (arrows). Scale bar: 10 μm. (c)–(e) AF cells were preloaded with Dil (red) and calcein (green) and plated as a single cell suspension on cortical cultures to examine metabolic coupling. AF cells readily established functional gap junctional communication with astrocytes within 4 hours, as indicated by calcein transfer, a phenomenon not observed between AF cells and neurons. Scale bar: 10 μm.
Figure 6: AF cells repair the scratch-induced wound injury in cortical cultures. (a)-(b) A low magnification of confluent cortical cultures stained with Hoechst (a and b, blue), GFAP (b, red) and Cx43 (b, green). (c)-(d) Parallel cortical cultures were subjected to scratch injury and stained with the same markers 24 hrs later. GFAP positive astrocytes were seen at the scratch border (d), without repairing the wound. (e)-(f) In contrast, when AF cells were seeded following scratch, they filled the injury site (f, arrows), maintained Cx43 expression (f, green), and facilitated wound repair. (g)–(k) To further identify AF cells located in the injury site, separate cultures were stained with human mitochondrial marker (red), Cx43 (green), and Hoechst counter stain (blue). Cx43 was readily expressed at the boundary between AF cells and cortical cells (k). (l)–(n) Live assays, using GFP-tagged AF cells, were also used to confirm wound repair after scratch injury in cortical cultures. Scale bar: 150 μm (a)–(f), 35 μm (g)-(h), 90 μm (i, l), 10 μm (k), 80 μm (l)–(n).

expressing much lower levels of Cx43 (data not shown); however, functional communication was largely observed between AF cells and cortical astrocytes in later cultures (Figures 5(c)–5(e)). In support of this observation, Cx43 protein expression has been known to decrease significantly following neuronal differentiation [39, 47].

3.4. Cx43 Expression during Injury. As a response to brain injury, astrocytes proliferate and infiltrate the damaged region in an effort to preserve neural tissue and restrict inflammation [48]. Since Cx43 is the main protein expressed in both astrocytes and AF cells and results in functional gap junctional intercellular communication, we examined the interaction of AF cells with host cells in both in vitro and in vivo brain injury models. More specifically, we used an in vitro scratch wound model using cortical cultures, a well-characterized model to investigate the astrocytic response to mechanical injury [49], as well as in an in vivo surgically induced brain injury model targeting the primary motor cortex [50].

Cortical astrocytes expressed abundant levels of Cx43 (Figures 6(a) and 6(b)). Although astrocytes maintained Cx43 expression following a scratch-induced injury, they did not demonstrate the capacity to repair the wound within the first 24 hrs (Figures 6(c) and 6(d)). In fact, glial processes have been shown to protrude into the injured region with astrocytes filling the gap approximately 72 hrs after-injury [51]. In contrast, AF cells seeded onto injured
cortical cultures 15 minutes after scratch were able to adhere to the denuded area and facilitate the repair within 24 hrs (Figures 6(e)-6(f), arrows). In order to confirm that seeded AF cells were able to reestablish connectivity with cortical astrocytes, via Cx43-mediated gap junction formation, we labeled AF cells with a human mitochondrial antibody (hMito) and examined CX43 expression between AF and cortical cells (Figures 6(g)–6(k)). CX43 was readily expressed at the boundary between AF cells and cortical astrocytes (Figure 6(k)). Complementary to these experiments, we performed a live assay by seeding GFP-tagged AF cells following injury and confirmed that AF cells readily filled the injury site, resulting in wound closure (Figures 6(l)–6(n)).

Using a mouse model of brain injury, the motor cortex was injured, as previously described [44, 50], resulting in a cavity that forms as a result of tissue loss and cell death that ensued after the injury (Figures 7(b), 7(d), and 7(f)) compared to sham (uninjured) brains (Figures 7(a), 7(c), and 7(e)). Compared to the organized architecture of neurons and astrocytes in the control brain (Figure 7(e)), the damaged cortex showed significant neuronal loss (approximately 210,000 neurons from a total of 350,000 cells), a disarray of neurite extensions, and a significant infiltration of astrocytes to the injured area (Figure 7(f)). Since astrocytes exhibit a high degree of coupling through gap junctions, composed mainly of Cx43 [52], we examined the immunohistochemical distribution of Cx43 in the injured brain. Indeed, compared to the uninjured brain (Figures 8(a) and 8(c)), abundant levels of Cx43 were detected at the perimeter of the injury site (Figures 8(b) and 8(d)). Semiquantitative immunohistochemistry confirmed increased Cx43 (40%) and GFAP (65%) expression in the damaged motor cortex.
Figure 8: Cx43 expression in the control and injured motor cortices. Immunohistochemistry of Cx43 (green) in the brains of mice subjected to sham surgery (a,c,e) and motor cortex lesion (b, d, f, g). (a)-(b) The stitched confocal images show the expression of Cx43 in the control (a) and injured (b) brains. (c)-(d). Higher magnification images of the areas of motor cortex indicated by insets in (a) and (b) are shown in (c) (sham) and d (lesion). Increased Cx43 staining was observed in the cortex adjacent to the injury site compared to sham cortex. (e)–(g). Double-labeling with GFAP (red) indicated that Cx43 (green) was expressed mainly in astrocytes (red), and more abundant in the injured cortex (f, g), compared to sham cortex (e). Nuclei were stained with Hoechst (blue). All the sections were coronal. Scale bar: 1000 μm (a)-(b), 100 μm (c, d, g); 10 μm (e, f).

(Figures 8(f)-8(g)), compared to the corresponding region in the sham brain (Figure 8(E)). Areas of intense Cx43 puncta were specifically observed in astrocytes within close proximity to the injury site (Figures 8(f)-8(g)). The upregulation of Cx43 enhances intercellular communication in the brain and may facilitate the delivery of beneficial factors to the injured brain.

To determine whether gap junctions form between AF and cortical cells in vivo and hence hold translational relevance, we implanted AF cells labeled with DsRed (AF-DsRed) into the injured motor cortex (Figures 9(a)-9(b)). Immunohistological analysis showed abundant Cx43 expression in the implanted area, as determined by DsRed and CX43 (Figures 9(c)-9(d)), whereas no AF-DsRed cells were found on the contralateral side which did not receive an injury/implantation of cells (Figure 9(a)). At 12 days after implantation, the majority of AF-DsRed cells were located within the injury injection site and needle tracks (Figure 9(c)), accompanied by CX43 expression surrounding the injection site. In fact, Cx43 expression was observed at the junction of AF-DsRed and neighbouring cells, as seen at higher magnification (Figure 9(d)). Although the long-term outcome of AF cell implantation into the motor cortex injury model has not been examined, abundant Cx43 expression between cortical astrocytes and AF cells suggests intercellular communication and potentially reconstruction of neural circuitry after AF cell engraftment.

4. Discussion

The development of functional grafts in the CNS is limited by the potential absence of intercellular communication
Figure 9: Gap junctions between AF cells and astrocytes following cell implantation into the injured motor cortex. (a) Stitched confocal image of mouse brain after motor cortex injury and receiving AF-DsRed (red) cell implant. Immunohistochemistry shows the distribution of Cx43 protein (green) at the interface between the graft and injured host tissue. (b) A higher magnification of the implant confirmed that AF-DsRed cells could be easily traced in the injured brain. (c)-(d) Intense Cx43 expression (green) was observed at the site of implantation. More specifically, distinct Cx43 immunostaining was detected at the junction between AF-DsRed (red) and cortical cells (see arrows). Hoechst was used as a counter stain (blue). All the sections were coronal. Scale bars: 1000 μm (a), 100 μm (b), 50 μm (c), 7 μm (d).

between grafted donor cells and host tissue. Thus, it is expected that enhancement of connexin-mediated intercellular gap junction formation would result in improved cell-cell communication between host and graft cells and increase transplantation success rate. Since AF cells have attracted a great deal of attention as an alternative source of donor cells for cell-based therapies, we examined their potential to form gap junctions and found that AF cells express abundant levels of CX43. Using well-established methods to isolate homogenous c-kit and single-cell-derived AF cell clones, we demonstrated that CX43 may play an important role in intercellular communication among these cells. These results are in agreement with the expression of CX43 in ES cells [53] and other cell lines with stem cell characteristics such as NT2/D1 [42] and P19 [54] cells. This is not surprising, as results from several laboratories have established that CX43 is the most prevalent connexin protein in vertebrates (see [55] for review). Cx43 is expressed in at least 34 tissues and 46 cell types [56, 57], and it plays a critical role in coordinating tissue functions and cellular homeostasis.

In the brain, Cx43 is highly expressed in the developing cortex and maintains its expression in cortical astrocytes throughout adulthood [55, 58, 59]. The presence of active gap junctions in astrocytes allows the regulation of glucose and oxygen delivery to neurons for their energetic and metabolic needs [60, 61]. For instance, Cx43 mediates the transfer of lactate from astrocytes to neurons as an energy substrate and facilitates the synthesis of neurotransmitters for synaptic activity [61, 62]. Similarly, the delivery of glucose and oxygen from the blood to the brain is regulated through an astrocytic network, which is dependent on Cx43, as demonstrated by knock-out experiments [61]. Hence, the ubiquitous expression of CX43 in AF cells also makes these cells suitable to serve as a platform to deliver beneficial factors through direct communication with brain cells. AF cells can potentially help modulate inflammatory cues and buffer pathological stimuli in the brain following injury as well as other neurological diseases. The rapid subcellular translocation of CX43 from the perinuclear compartment to the membrane boundary between AF cells and astrocytes may enhance the reestablishment of a homeostatic state in the brain after injury. Interestingly, Cx43 has also been observed at the borders of AF and cardiac cells, following transplantation into the heart [63], further supporting the application of AF cells in regenerative medicine through the formation of functional gap junctions.

Changes in both spatial and temporal CX43 protein expression are seen following various types of CNS pathologies such as ischemia, neurodegenerative disorders, and traumatic injury [64]. In brain injury an infiltration of
Cx43-positive reactive astrocytes is readily observed in the injured core [65]. The induction of Cx43 expression is a substantial factor in the astroglial response and potentiates intercellular signal transduction via gap junctions following injury [33, 65]. Consistent with these observations, we found Cx43 expression in astrocytes at the site of injury. Following injury, the increased expression of Cx43 in astrocytes and hence the number of gap junction plaques formed at the interface between neighbouring cells may facilitate the formation of gap junctions with graft cells implanted in close proximity to the injury site. Hence, by introducing AF cells, the expression of Cx43 between graft and host cells enables formation of gap-junctions which would aid in establishing communication between AF and CNS cells for delivery of beneficial factors and drugs. GJIC between grafted neural stem (NS) cells and brain cells [33] appears to be an essential participant in the neuroprotective effect associated with NS cell engraftment, particularly in the connexin-associated gap junction interface. Utilizing NS cells grafted into an ex vivo model system for striatal tissue, Jäderstad et al. [66] found that Cx43 expression transiently peaked in host cells following traumatic stimulation, suggesting a window of opportunity for NS cells to establish gap junctions with the host tissue and rescue the damaged cells. Since AF cells express high levels of Cx43 and form functional gap junctions, they have the capacity to mimic a similar connexin-mediated rescue during this critical time frame. In fact, preventing damaged cells from dying has emerged as one of the possible benefits of cell transplantation [1, 2, 25]. Furthermore, cell-cell coupling has been regarded as an early form of communication that precedes and acts as a template to establish electrochemical synapses later on [33]. In this instance, implanted AF cells expressing Cx43 may form gap-junctional coupling with astrocytes to possibly preserve neurons at the injury site. In support of this view, the role of astrocytes in early stages of neuroprotection is gaining more recognition, and initial AF-astrocyte interactions may play an important role during early stages in graft-host interactions [33]. This notion is further substantiated by findings in vitro, which confirm that astrocytic Cx43 gap junctions and hemichannels may remain functionally open following injury and in vivo work, which has shown significant changes in both spatial and temporal Cx43 expression following various models of CNS injury (reviewed in [67]).

It remains to be elucidated how astrocytic gap junctions contribute to neuroprotection in the context of regenerative medicine. Hence, further investigation of intercellular communication between AF and host cells may facilitate the use of these cells for therapeutic purposes.

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Review Article

Amniotic Fluid Stem Cells: Future Perspectives

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1. Introduction

Although human amniotic fluid cells are widely used in routine prenatal diagnosis, the knowledge about these cells remains limited. However, the notion that undifferentiated and differentiated cells of varying origins and lineages are present in amniotic fluid has been supported by several reports over the last three decades. This is not surprising, considering that cells belonging to the amniotic epithelium, fetal skin, and the fetal urogenital, respiratory, and gastrointestinal systems have been detected in amniotic fluid. During prolonged gestation, fetal respiratory, urine, and gut secretions can be found in the amniotic fluid. In addition, it is also known that the composition, the morphology, and the growth properties of amniotic-fluid cell samples are affected by certain fetal pathologies, such as for example, neural tube defects or gastroschisis [1–3].

New interest in amniotic fluid-derived cells was initiated by two independent findings. In 2001, it was suggested that amniotic fluid cells could be used in tissue engineering approaches for the surgical repair of congenital anomalies in the perinatal period. The authors mechanically isolated a subpopulation of cells from amniotic fluid of pregnant ewes with a distinct morphology. The immunocytochemical profile of these cells was very comparable to that of cells of a mesenchymal, fibroblast/myofibroblast lineage. Exhibiting significantly faster proliferation than comparable fetal and adult cells, these amniotic fluid-derived cells could be cultivated on polyglycolic acid polymer scaffolds up to confluent cell layers [4]. It has originally been discussed that such an engineered construct would be optimal to function as a graft for implantation either in the neonatal period or even before birth. This could be of special interest for children born with a body wall defect, who are too young for a graft to be taken from elsewhere in their bodies for reconstructive surgery [4, 5]. The results obtained in animal models are indeed encouraging. However, to the best of our knowledge, we are not aware of a report describing the clinical use of such a cell-based therapy approach in humans until now.

Another finding on amniotic fluid cells initiated a very promising and rapidly growing research field. Almost ten years ago, the first suggestion of human amniotic fluid as a new putative source for stem cells was published [5–7]. The first evidence for the existence of AFS cells was demonstrated by the discovery of a highly proliferative cell type in human amniotic fluid expressing the pluripotent stem cell marker Oct4. Beside the fact that these cells express markers known to be specific for pluripotent stem cells, they were
proven to express cell cycle proteins known to be specific for cycling cells [5–8]. After this first description, many groups have confirmed the existence of these Oct4+/c-Kit+ AFS cells and have reported their potential to differentiate into hematopoietic, neurogenic, osteogenic, chondrogenic, adipogenic, renal, hepatic, and various other lineages [9–19]. Although, regarding their biological properties and marker expression pattern, AFS cells appear to be more similar to embryonic stem (ES) cells than, for example, to trophoblast cells, the precise origin of AFS cells remains elusive. Biochemical, immunocytochemical, biological, and morphological investigations revealed that AFS cells represent a new and specific entity, being distinct from ES cells or other stem cell types, such as the ones which can be isolated from amniotic epithelial or trophoblastic sources. Today it is of great interest to clarify two relevant questions with regard to AFS cells. Where do they come from? Do they have an in vivo biological function? We have already earlier discussed that AFS cells could probably play a role in intrauterine wound healing processes. However, so far there exists no experimental support for this hypothesis. It is obvious that experimental settings allowing to prove this hypothesis are not really easy to imagine or practical at this time (or yet to be developed) [9–19].

Since their first discovery, it was of highest importance to clarify the question whether AFS cells really harbour pluripotent differentiation potential via successfully initiating the differentiation into different lineages starting from one single stem cell. It is relevant to note that many different reports in the literature claiming to describe research on AFS cells did not even clarify what kind of cells they are working with. Quite often, the investigators just used a mixture of cells from amniotic fluid obtained via specific cultivation procedures. However, as mentioned above, such amniotic-fluid-derived cell mixtures contain a wide variety of specific undifferentiated and differentiated cell types. Whenever a study reports a differentiation potential upon specific cell lineages, it is of highest relevance to first clarify which starting cell type was used (by detailed biological and immunocytochemical characterization). Furthermore, the proof that AFS cells really harbour pluripotent differentiation potential can only be obtained starting with one single cell characterized to be a stem cell. In any other case, one could assume that a mixture of amniotic fluid cells, which has been used as starting material, very likely contained a cell type with the potential to differentiate into a specific lineage and other cell types with other differentiation potentials. Or the used in vitro differentiation protocol used in some studies induced a selection (via a growth advantage) for an already (included) differentiated cell type rather than the bona fide differentiation. Single cell approaches are obligatory and practical after minimal dilution experiments.

The first research group, which was really taking that into account, reported that descending from one single Oct4-positive AFS cell, it was possible to induce adipogenic, osteogenic, and neurogenic differentiation [10]. The authors used a two-stage culture protocol followed by a detailed immunocytochemical characterization of the obtained stem cell type [10]. Three years later, another research group isolated monoclonal AFS cells via flow cytometric selection and minimal dilution, which expressed the stem cell markers c-Kit and Oct4 [14]. The authors described the first establishment of monoclonal AFS cell lines, harbouring a high proliferative potential, which could be cultivated for many cycling periods with a stable chromosomal status. Using such AFS cell lines allowed them to demonstrate that adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic cell differentiation could be induced. Importantly, these authors also reported that AFS cells, unlike ES cells, do not induce tumor formation in severe combined immunodeficient (SCID) mice (for a detailed discussion of this aspect see below) [14].

ES cells, when cultivated in the absence of differentiation factors, can spontaneously form three-dimensional multicellular aggregates called embryoid bodies. In the past, embryoid bodies have been widely considered as an optimal starting point for the differentiation of stem cells into various lineages. Accordingly, embryoid body formation followed by different differentiation-inducing approaches is seen as an appropriate way to prove the pluripotent differentiation potential of a specific stem cell type [8, 20]. Consequently, it was of interest to test whether, starting from one single cell, AFS cells are capable of forming embryoid bodies. Indeed, monoclonal human AFS cells can form embryoid bodies, when cultured without antidiifferentiation factors under conditions in which they are unable to attach to the surface of culture dishes and without contact to feeder cells. The formation of such three-dimensional multicellular aggregates is accompanied by a decrease of stem cell marker expression and by the induction of differentiation into different lineages [20]. This study demonstrating the potential to form embryoid bodies was the ultimate proof of AFS cells to be pluripotent. In addition, it now allows the recapitulation and investigation of the three-dimensional structures and tissue-level contexts of many differentiation phenomena during early mammalian embryogenesis [20]. These findings on the pluripotency of AFS cells were obtained using monoclonal cell lines generated via magnetic cell sorting and minimal dilution approaches from human amniocentesis samples. Today, many different established monoclonal lines exist, which can be expanded as immature stem cells with high proliferation rate in culture without the need of feeder cells [14, 20, 21].

Taken together, the current status of knowledge is that AFS cells harbour the potential to differentiate into cell types of the three germ layers (ectoderm, mesoderm, and endoderm) and can form embryoid bodies, known as the principal step in the differentiation of pluripotent stem cells. Compared to other types of stem cells, such as adult stem cells, ES cells, or induced pluripotent stem (iPS) cells, AFS cells, have specific advantages. Adult stem cells are often hard to sample, exhibit lower differentiation potential than AFS cells and cannot be grown with high proliferative activity. The generation of ES cell lines via destroying a human embryo raises a variety of ethical issues, which are discussed differently from country to country. Furthermore, ES cells are tumorigenic, whereas AFS cells, as already mentioned above, do not induce tumor formation in severe...
combined immunodeficient mice. Compared to iPS cells, there is no need for ectopic induction of pluripotency in AFS cells. AFS cells are genomics stable and do not harbour the epigenetic memory nor somatic mutations of already differentiated source cells. Furthermore iPS cells have been reported to accumulate karyotypic abnormalities and gene mutations during propagation in culture. Recently, it has been reported that during the ectopic induction of pluripotency iPS cells only incompletely recapitulate their epigenetic pattern. This important finding must be taken into account when these cells are planned to be used for detailed investigations on differentiation processes as well as when they are considered for new putative therapeutic approaches. AFS cells already exhibit stem cell properties and do not need ectopic induction of pluripotency. Furthermore, AFS cells already exhibit the epigenetic pattern of stem cells. In summary, it is not surprising that many attempts are currently focusing on the question under which conditions AFS cells could be used for stem-cell-based therapies. Furthermore, AFS cells are currently becoming increasingly accepted as an optimal tool for basic research [3, 8, 22–24].

Although ES cells, iPS cells, and AFS cells are considered to harbour a pluripotent differentiation potential, the question of whether they exhibit the same qualitative spectrum of differentiation potential remains unanswered. Pluripotent stem cell types are defined as self-replicating cells (the cells can divide per se) known to have the capacity to develop into cells and tissues of the primary germ layers, ectoderm, mesoderm, and endoderm. These three stem cell types (ES, iPS, and AFS cells) have been demonstrated to harbour the potential to differentiate into cells of the three germ layers. All three can also form embryoid bodies. However, whether they really have comparable potentials to differentiate into a specific cell type with all its known biological functions must be tested from case to case. In fact, we believe that it is necessary to directly examine and compare their differentiation potentials and select the most suitable cell types for basic science projects and for the putative usage in new stem-cell-based therapies. Furthermore, one obvious difference between these three pluripotent stem cell types should be investigated in more detail in future. Since the first description of their in vitro cultivation, ES cells have been known to be tumorigenic. Similarly, iPS cells induce tumor formation, when they are subcutaneously transplanted into nude mice. However, AFS cells have been reported not to form tumors in severe combined immunodeficient mice. Since the latter has so far only been studied in one project analysing a specific set of animal transplantsations, further investigations are warranted to clarify whether AFS cells are really not tumorigenic. Obviously, if it holds true, this would be an important advantage over ES and iPS cells at least with respect to a putative clinical usage [3, 8, 14, 19, 22–25].

2. AFS Cells for Therapy: Future Perspectives

Much of the excitement surrounding human stem cells is connected with the hope of clinicians and patients that these cells can once be used for cell therapies for a wide spectrum of human diseases. Here it must clearly be stated that the work on AFS cell-based therapies is still in its infancy. Many questions are currently under investigation, and so far no therapeutic approach based on AFS cells has reached the level of clinical routine application. However, a variety of new research results provide strong evidence that AFS cells could indeed serve a powerful tool in regenerative medicine [3, 8, 24, 25].

For example, acute and chronic renal failures are disorders with high rates of morbidity and mortality. Kidney transplantation remains the most effective treatment option for a majority of patients with end-stage renal disease. Unfortunately, shortage of compatible organs is a very limiting factor. Treatment strategies are also based upon conventional renal dialysis, but the mortality rate of patients requiring chronic dialysis is high. Accordingly, the putative usage of stem cells in the repair of kidney injury came into focus. Several recently published studies on renal differentiation of AFS cells make it tempting to speculate that these stem cells could once be considered as a new promising source for cell-based therapies to repair kidney injury and warrant further investigations into this direction [24, 26–30]. Using a kidney reaggregation assay, we have recently published that AFS cells harbour the potential to differentiate upon nephrogenic lineages and that this capacity depends on the mammalian target of rapamycin (mTOR) signalling pathway [28] (see also the discussion below). Others have demonstrated that human AFS cells can integrate into renal tissues when injected into isolated murine embryonic kidneys [27] or that injection of AFS cells into damaged kidneys of mice with rhabdomyolysis-related acute tubular necrosis can mediate a protective effect [29]. Although these and other data make it tempting to speculate that AFS cells may provide successful alternative approaches for the treatment of, for example, acute tubular necrosis, many more questions must be answered before such cell-based therapies can be considered for routine applications in humans.

For many different reasons the establishment of new stem-cell-based therapies for heretofore incurable central nervous system pathologies, such as Parkinson’s disease, spinal cord injury, multiple sclerosis, or stroke, is also of great interest. Neural stem cells, which have been investigated for this purpose, can be found in the adult central nervous system and in the developing embryo, but these tissues are not easily available and raise ethical concerns. In the recent years, different groups have reported on the neurogenic differentiation potential of AFS cells. However, before the next steps into the direction of the clinical usage of AFS cell-based approaches can be considered, the proof that AFS really can form mature neurons must be provided. In fact, there is still an ongoing debate in the literature, discussing whether AFS cells are really able to form functional neurons. In the near future, it will be very important to find out what kind of neurogenic cell types can be developed from AFS cells. The question whether AFS cells can differentiate into functional mature neurons must be investigated by analysing the ability to fire tetrodotoxin-sensitive action potentials with the characteristic shape and
duration or by demonstrating synaptic communication by electron microscopy [10, 13–16, 18, 19, 31].

Here, it would be possible to discuss some more examples for putative therapeutic approaches using AFS cells. Sometimes it is argued that many basic questions regarding the origin, the tumorigenicity, the differentiation potential, the epigenetic status, or the genomic stability must be investigated before AFS cells could further be considered as a therapeutic tool. However, we believe all these aspects should be studied in parallel. In addition, for future considerations it is really important to quantitatively and qualitatively compare all these properties of AFS cells to those of other pluripotent or adult stem cell types.

3. AFS Cells in Basic Science: Future Perspectives

Stem cells are very useful tools to study the molecular and cellular regulation of differentiation processes. One approach to learn more about the role of, for example, a specific gene for a certain differentiation process is to knock down the endogenous expression of the gene of interest. Such an approach allows to clarify the role of modulated gene expression for the cell potential to differentiate into a specific lineage. We recently published a protocol for efficient siRNA-mediated prolonged gene silencing in AFS cells [21]. This protocol, which we already tested for a variety of different genes, allows a 96–98% downregulation of the endogenous gene expression over a time period of about 14 days in AFS cells and in a variety of other primary, immortalized, or transformed cells [21].

More recently, we have made use of this approach to study the role of the mTOR pathway in human AFS cells. Deregulation of upstream regulators of mTOR, such as, for example, Wnt, Ras, TNF-α, PI3K, or Akt, is a hallmark in many human cancers. Mutations in the mTOR pathway component genes TSC1, TSC2, LKB1, PTEN, VHL, NF1, and PKD1 trigger the development of the human genetic syndromes: tuberous sclerosis, the Peutz-Jeghers syndrome, the Cowden syndrome, the Bannayan-Riley-Ruvalcaba syndrome, the Lhermitte-Duclos disease, the Proteus syndrome, the von Hippel-Lindau disease, neurofibromatosis type 1, and polycystic kidney disease. Beside a variety of single-gene disorders and tumorigenesis, the mTOR pathway has also been shown to be of relevance for the development of complex diseases, such as cardiac hypertrophy, obesity, or type 2 diabetes. All these pathological consequences of deregulated mTOR activity are explainable, considering that mTOR is the key component of the insulin signalling cascade, which is involved in a wide variety of different processes such as cell growth, proliferation, metabolism, transcription, translation, survival, autophagy, aging, differentiation, and oncogenesis [8, 20, 21, 28, 32]. We found that the entire process of embryoid body formation of AFS cells depends on both mTOR-containing enzymes, mTORC1 and mTORC2 [20]. As mentioned above, modulating mTOR components via specific siRNA approaches revealed that the potential of AFS cells to contribute to renal tissue formation is regulated by this signalling pathway [28]. More recently, the approach to knock-down endogenous gene functions in AFS cells allowed us to detect that the two mTOR regulators, tuberin and PRAS40, are antiapoptotic gatekeepers during early human AFS cell differentiation [32]. Taken together, we strongly believe the approach of siRNA-mediated knockdown of endogenous gene expression in monoclonal human AFS cell lines to be a very powerful tool for future projects dealing with the molecular regulation of differentiation [3, 8].

Another very interesting aspect for future basic research is the banking of AFS cell lines carrying naturally occurring mutations, which are of relevance for certain human pathological phenotypes. In medical genetics the future development of new prophylactic and therapeutic strategies directly depends on a better understanding of the mechanisms by which naturally occurring genetic variation contributes to disease [33]. In countries, where it is legal to use human embryos for research, ES cell lines carrying certain inherited defects are generated from embryos with all kinds of numerical chromosomal abnormalities or specific monogenic disease mutations excluded from transfer into the uterus after preimplantation genetic diagnosis [34]. Also a variety of iPS lines from single-gene disorders, chromosome syndromes, and complex diseases have already been generated, with the aim to use them for basic research projects [35]. Still, as already discussed in detail, both ES cells and iPS cells harbour relevant disadvantages compared to AFS cells. Beside other invasive approaches, amniocentesis is a widely accepted standard procedure of prenatal care since the 1970s. It is almost unpredictable how many amniocenteses are worldwide performed per year. Taken together, we believe that generation and banking of normal human AFS cell lines and of AFS cell lines with chromosomal aberrations, as well as of AFS cell lines with specific monogenic disease mutations could provide very powerful tools for disease modelling in future research. Here it is important to note that banking of AFS cells for non-research purposes, with the aim to protect a child’s health by having stem cells available throughout his or her lifetime, is something else. Some companies in Europe and the USA are already offering to bank AFS cells, when, for example, an amniocentesis is performed for prenatal diagnosis. Their arguments for the preservation of AFS cells are that these cells once could help treating injuries (e.g., repairing cartilage for the knee), healing wounds, or developing skin for specific grafts. As mentioned earlier, in future, extensive research is required to establish the putative clinical use of AFS stem cells in humans. The promising results obtained during the last few years within this still young scientific field clearly warrant further detailed investigations into the direction of putative clinical application of AFS cells. In this paper we would like to emphasize that banking of AFS cells with natural occurring mutations for human genetic research should have started as soon as possible in different laboratories under comparable high-quality standards. It would be worth to encourage different laboratories to sample amniotic fluid from amniocentesis with comparable indications from similar weeks of pregnancy. The protocols to isolate stem
cells, perform minimal dilutions, and characterize the so-obtained monoclonal AFS cell lines should be standardized. Biobanking of AFS cell lines with characterized mutations would allow to jump to the next step of human genetic research using human stem cells [3, 8].

References


Research Article

Comprehensive Characterization of Mesenchymal Stem Cells from Human Placenta and Fetal Membrane and Their Response to Osteoactivin Stimulation

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Mesenchymal stem cells (MSCs) are the most promising seed cells for cell therapy and can be isolated from various sources of human adult tissues such as bone marrow (BM-MSC) and adipose tissue. However, cells from these tissues must be obtained through invasive procedures. We, therefore, characterized MSCs isolated from fresh placenta (Pl-MSC) and fetal membrane (Mb-MSC) through morphological and fluorescent-activated cell sorting (FACS). MSC frequency is higher in membrane than placenta (2.14% ± 0.65 versus 15.67% ± 0.29%). Pl/Mb-MSCs in vitro expansion potential was significantly higher than BM-MSCs. We demonstrated that one of the MSC-specific marker is sufficient for MSC isolation and that culture in specific media is the optimal way for selecting very homogenous MSC population. These MSCs could be differentiated into mesodermal cells expressing cell markers and cytologic staining consistent with mature osteoblasts and adipocytes. Transcriptomic analysis and cytokine arrays demonstrated broad similarity between placenta- and membrane-derived MSCs and only discrete differences with BM-MSCs with enrichment of networks involved in bone differentiation. Pl/Mb-MSCs displayed higher osteogenic differentiation potential than BM-MSC when their response to osteoactivin was evaluated. Fetal-tissue-derived mesenchymal cells may, therefore, be considered as a major source of MSCs to reach clinical scale banking in particular for bone regeneration.

1. Introduction

Multipotent mesenchymal stem cells (MSCs) are able to self-renewed and differentiate into mesodermal lineages such as adipogenic, chondrogenic, osteogenic, myogenic, and angiogenic cells [1]. MSCs were initially isolated from bone marrow by Haynesworth et al. [2]. In the bone marrow, they provide support for hematopoiesis [3]. They also secrete several growth factors important in angiogenesis including vascular endothelial growth factors [4]. Therefore, they represent one of the most promising cell types for cell therapies and tissue engineering or trauma repair. Indeed, different preclinical experiments using MSCs have been performed demonstrating their ability to improve myocardial or cerebral function after ischemic stress, or liver and joint damage after traumatic or surgical injuries [5–8]. They might also be optimal for cellular therapy by inducing immune tolerance. Indeed, they can generally be transplanted even in large outbreed animals across major histocompatibility complex (MHC) barriers without need for immune suppression [9].

The bone marrow is the traditional source of human MSCs, but they have been isolated from a wide variety of human adult tissues such as adipose tissue [10], lung [11], and liver [12]. However, cells from most of these tissues must
be obtained through invasive procedures, and the interindividual variability is hard to control. Several studies describe the isolations of MSCs from fetal tissues such as umbilical cord blood [13], placenta [14–16], amniotic membrane [17, 18], and amniotic fluid [19], and they have described their MSCs characteristics.

Osteoactivin (OA) has the ability to regulate cell proliferation, adhesion, differentiation, and synthesis of extracellular matrix proteins in various cell types [20–30]. OA messenger ribonucleic acid (mRNA) and protein are expressed by human and rodent osteoblasts [29, 30]. OA downregulation decreases osteoblast differentiation and function [31]. Osteoblast cells express increasing levels of OA protein during their differentiation. OA has been demonstrated as essential for the differentiation and functioning of osteoblast cells [32]. We previously demonstrated that OA induces similar osteoblastic differentiation than BMP2 in mice MSC suggesting that OA may be a novel osteoinductive agent [29, 31, 32].

In this study, we optimized the isolation of placental and amniotic membrane MSC and compared their proliferative and differentiation potential to BM-MSCs. We isolated through different methods MSCs from placenta and fetal membranes, and we qualified them according to the standardize protocols from the international society for cellular therapy (ISCT) [33]. We further investigated and demonstrated that OA triggers osteoblastic differentiation in human MSCs and that the differentiation was even more important in fetal MSCs as compared to BM-MSCs. We illustrate that fetal tissues derived MSCs are more prone than BM-MSCs to differentiate into osteoblasts.

2. Materials and Methods

2.1. Placenta and Fetal Membranes Collection. Following approval from the Internal Review Board (HMC-IRB Protocol 9109/09, Weill Cornell Medical College in Qatar), placentas and fetal membranes were collected from donors at Woman’s Hospital at Hamad Medical Corporation immediately after elective caesarean section in the absence of labor, preterm rupture of membrane, chorioamnionitis, preeclampsia, intrauterine growth retardation, or chromosomal abnormalities. The specimens were completely deidentified and considered as biological waste. Therefore, no consent form was taken from the patients.

2.2. Mesenchymal Stem Cell Isolation. Supplementary Figure 1 (available at doi:10.1155/2012/658356) depicts the isolation procedures used in this study. For placenta, the decidua basalis was removed prior to harvesting the placental tissues. The placenta parts were free of any fetal membrane. For fetal membrane, we decided not to separate the amnion and chorion parts to illustrate the most direct workflow for MSCs isolation. The harvested pieces of tissues were washed in phosphate buffer saline (PBS, PH 7.4), mechanically minced into pieces of approximately 1 mm², and subsequently digested with dispase (1 mg/mL, Hyclone), collagenase (300 U/mL) (Hyclone), hyaluronidase (100 U/mL, Hyclone), and DNAse I (80 U/mL, Roche) for 1 h at 37°C under agitation (150 rpm). The homogenate was subsequently washed in PBS. Cells were then filtered on a 100 μm cells strainer. Red blood cells and aggregates were eliminated on a Ficoll gradient. The mononucleated fraction was collected for further analysis.

Two million viable cells were then either directly plated in MSCs culture media (DMEM low glucose with 20% FBS, 2 mM L-Glutamin, and 1% penicillin/streptomycin [16]) or sorted through SORP FACS Aria II (BD Biosciences) and then plated in the MSC culture media in 24 well plates. Cultures were incubated in humidified 5% CO₂ incubators and the media was replaced every 3 days.

Several bone marrows MSCs from different donors (BM-MSCs) were purchased from Stem Cell Inc. (number MSC-001F, Stem Cell Inc.) and PromoCell (number 12974, PromoCell) and maintained in the same culture conditions as placenta-/membrane-derived MSCs (Pl/Mb-MSCs). We performed all analysis at the 4th passages in order to obtain a homogenous cell population and sufficient number of cells to perform all analysis in parallel.

2.3. Immunostaining and Fluorescence-Activated Cell Sorting (FACS) Analysis. For flow analysis of cell surface antigens and cell sorting, MSCs were stained for the expression of CD45, CD34, CD73, CD105, CD90, and CD29 using Mouse anti-human CD45 antibody (BD Biosciences, number 339192, clone 2D1) coupled with Amcyan, Mouse anti-human CD34 (BD Biosciences, number 555821, clone 581) coupled with FITC, Mouse anti-human CD105 (biolegend, number 323212, clone 43A3) coupled with AlexaFlour 647, Mouse anti-human CD73 (BD Biosciences, number 550257, clone AD2) coupled with PE, Mouse anti-human CD29 (biolegend, number 323212, clone TS2/16) coupled with APC-Cy7, and Mouse anti-human CD90 (BD Biosciences, number 550402, clone 5E10) coupled with AF700.

Briefly, 1.10⁶ cells were harvested and nonspecific sites were blocked in PBS-5%FBS-1%BSA-10%FcR Blocking Reagent (Mylenyi Biotec) for 30 minutes on ice. Cell suspension was incubated with specific antibodies for 45 minutes on ice. After washes in PBS and filtration on 45 μm strainer, cells were analyzed by fluorescence activated-cell sorting (FACS) on a SORP FACS Aria II (BD Biosciences) as described later. Data were processed with FACSDiva 6.3 software (BD Biosciences). Doublets were excluded by FSC-W × SSC-W analyses, single-stained channels were used for compensation, and fluorophore minus one (FMO) controls were used for gating, 500,000 events were acquired per sample [34].

2.4. Immunocytochemistry. Cells in culture were grown on 8 chamber slides (BD Falcon, number 354102) and stained as follows.

The antibodies used were Mouse anti-human CD29-FITC (biolegend, number 303016, clone TS2/16), CD73-PE (BD Biosciences, number 550257, clone AD2), CD90-AF568 (BD Biosciences, number 550402, clone 5E10), CD34-PE
Briefly, nonspecific sites and Fc receptors were blocked with PBS/0.3% bovine serum albumin/0.5% HS for 30 minutes and FcR blocking reagent (Milenyi, number 120-000-442). Sections were incubated with primary antibodies (1 hour 30 minutes), washed twice in PBS/0.5% Tween 20 (Sigma-Aldrich), and if necessary incubated with secondary antibodies (1 hour, AF488 goat anti-mouse IgG1 at 0.5 μg/mL). Nuclei were counterstained with 4-6-diamidino-2-phenylindole (Invitrogen). Slides were mounted with the Fluormount Kit (Invitrogen). Sections were analyzed with a Zeiss confocal microscope Laser Scanning Microscope 710 (Carl Zeiss). Pictures were analyzed with Zen 2008 V5.0,0228 software (Carl Zeiss).

2.5. Mesodermal Lineage Differentiation

2.5.1. Adipogenic Lineage. Adipogenic differentiation was induced by culturing 80% confluent MSC for 3 weeks in DMEM-HG, 1 μM dexamethasone, 5 μg/mL insulin (Sigma), 60 μM indomethacin (Sigma; catalogue number: 17378-5G), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma; catalogue number: 15879) [35]. Adipogenic differentiation was assessed by staining cells in wells with Oil Red O.

2.5.2. Osteogenic Lineage. Osteogenic differentiation was induced by culturing 90% confluent MSC for 3 weeks in DMEM-LG, 10% FCS, 0.1 μM dexamethasone (Sigma; Australia Register Number: 16375; Melbourne, Victoria, Australia), 10 mM β-glycerophosphate disodium salt pentahydrate (Sigma; catalogue number: A8960-5G; Castle Hill, New South Wales, Australia), 10 μM indomethacin, 50 μM L-ascorbic acid-2-phosphate (Sigma; catalogue number: A6677-1G), and 0.05 μM M dexamethasone (Sigma; catalogue number: 17378-5G), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma; catalogue number: 15879) [35]. Osteogenic differentiation was assessed by staining cells in wells with Alizarin Red S.

2.5.3. Osteoactivin Stimulation. Three days after MSCs plat- ing (50% confluence), recombinant human osteoactivin was delivered in a single dose of 100 ng/mL in differentiation media DMEM-LG, 10% FCS, 0.1 μM dexamethasone, 50 μM L-ascorbic acid-2-phosphate, 10 mM β-glycerophosphate disodium salt pentahydrate, and 0.3 mM inorganic (sodium) phosphate. Controls were carried out in regular cell culture media described above. The osteogenic differentiation was then assessed at day 7, 14, and 21 after OA treatment by staining with Alizarin Red S. Following treatment, color phase contrast microscopy pictures were acquired at different time points. Analysis of the red channel was performed using image J (NIH). Normalization for cell number was done using the blue channel.

2.6. Transcriptomic Analysis. RNA was isolated using Trizol reagent followed by additional purification using RNeasy extraction kit from Qiagen (QIAGEN, number 74106) with RNA yields that produces satisfactory microarray data. Two quality control measures were carried out: (1) a spectropho-tometric analysis and (2) a size fractionation procedure using a microfluidics instrument (Agilent Technologies). 200 ng of total RNA were analyzed on Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. Data were analyzed using Partek Software (V6.09.1110-6; Affimetrix). Class comparison between BM-MSCs and Pl/Mb-MSCs (three biological replicates of each) was performed to identify gene expression changes with a significant expression differences \( P < 0.05 \) and 2-fold increase or decrease expression. Partek Software gene ontology tools were used to determine gene enrichment [37].

We used Ingenuity Pathway Analysis software (Ingenuity Systems, Redwood City, CA) to identify and analyze relevant pathways from the gene lists obtained after comparison of BM-MSC and Mb/Pl-MSC. Networks were constructed by overlaying the genes in the gene list onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge database using keywords such as organ formation and osteoblast differentiation. Networks of the genes up- or downregulated Pl/Mb- MSCs as compared to BM-MSCs were then algorithmically generated based on their connectivity. A network is a graphical representation of the molecular relationships between genes. Genes are represented as nodes, and the biological relationship between two nodes is represented as a line. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Pathways Knowledge database. \( P \) values for the enrichment of canonical pathways were then generated based on the hypergeometric distribution and calculated with the right-tailed Fisher’s exact \( t \)-test for 2 \( \times \) 2 contingency tables.

2.7. Phospho-Kinase Array. MSCs were cultivated in different-iation or control media in presence or absence of OA for 4 h. Cells were harvested and proteins extracted as recommended and quantified based on sample absorbance at 280 nm using nanodrop device (Thermo-Scientific). 200 μg of protein was loaded on R&D system Human Phospho-kinase Antibody Array (R&D system, number ARY003) according to manufacturer’s instructions.

Arrays were revealed using HorseRadish Peroxidase (HRP) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Data were collected using Geli-ance CCD camera (Perkin Elmer) and extracted using Image J software (NIH). Briefly, arrays’ pictures were inverted and background subtracted. We defined a 120-micron diameter area for signal capture. Median pixel density was used to evaluate the signal. For comparison, independent array values were normalized on their positive control intensity values.

2.8. Cytokine Array. MSCs were cultivated in serum free media for 72 hours as previously published [38], Supernatant was collected and proteins quantified based on sample absorbance at 280 nm using nanodrop device (Thermo-Scientific). 200 μg of protein was loaded on R&D system
Human Cytokine Antibody Array panel A (R&D system, number ARY005) according to manufacturer’s instructions. Arrays were analyzed as described above.

2.9. Statistical Analysis. Student-t, Fisher exact, or chi-square tests were performed as appropriate. All P-values are two-sided with statistical significance evaluated at the 0.05 alpha level. All statistical analysis were done using the data analysis plug-in shipped into the Excel 2008 for Mac (Microsoft). We first calculated the variance of two paired. Mean ± SEM are shown on the graphs. All results are representative of the indicated number of independent experiments.

3. Results

3.1. MSCs Isolation Methods. Supplementary Figure 1 depicts the workflow chart of the enzyme-mediated cell isolation of human term placenta/membrane, by direct culture or cell sorting, for derivation of fibroblast-like cells, that we characterized as multipotent mesenchymal stem cells (MSCs). Two methods were used to isolate MSCs. (i) selection in specific MSC media after direct culture of cell suspension obtained following tissue digestion, (ii) fluorescent-activated cell sorting according to expression of specific MSCs markers as defined by the international society for cellular therapy (ISCT, positive for CD105, CD73, CD90, CD29, and negative for CD45 and CD34) [39]. 15 different amniotic membranes and placentas were used in this study. We were able to differentiate MSCs from all of these specimens, all analyses are representative of 3 different samples.

3.2. MSCs Are More Abundant in Membrane Than Placenta. We quantified the number of MSCs in fetal membrane and placenta using polyvariace flow cytometry. CD45−, CD34− cells were selected and analyzed for the expression of CD105, CD73, CD90, and CD29. MSCs as defined by these 4 markers were significantly more abundant in the membrane, Mb-MSC 15.67% (±0.29%) than the placenta, Pl-MSC 2.14% (±0.65%, Figure 1(a)).

The same results were found when 2 million cells were directly plated after tissue digestion. The number of adherent cells was significantly higher at day 1 in the membrane compare to the placenta (Figure 1(b)).

3.3. Placenta or Membrane Isolated MSCs Have Greater Proliferation Ability Than Bone Marrow MSCs. Growth kinetic of Mb-MSCs and Pl-MSCs were compared with BM-MSCs at the same passage. The proliferation rate of Pl/Mb MSCs was significantly higher than BM-MSCs (Figure 1(c)). Moreover, Mb-MSCs and Pl-MSCs were expandable up to passage 15 without modification of their morphology or proliferation rate as described in other studies however BM-MSC stopped proliferating after passages 7 to 8 [14].

3.4. Analysis of Subpopulations Based on CD90 and CD29 Expression. We first defined our cell population as being negative for CD45 and CD34. In all our independent experiences, the vast majority if not all (85% to 99%) of CD73+, CD105+ cells was also expressing CD90 and CD29 reaching the canonical definition of MSCs [33, 39]. However, the number of CD90+, CD29+ cells positive for CD73 and CD105 was lower ranging from 65% to 85% (Figure 2). We, therefore, decided to further analyze the populations characterized by CD90/CD29 expression. We wondered our ability to derive MSCs from these different cell populations. We sorted 4 subpopulations based on the expression of those markers: CD90+ CD29+; CD90− CD29+; CD90+ CD29−; CD90− CD29− in placentas and fetal membranes (Figure 2 and Supplementary Figure 2). We performed all experiences on 3 independent donors. The purity of the sort was assured by applying the purity mask and controlling for the purity of the different cell populations sorted.

None of the CD90+ CD29+ cells were able to grow in MSCs media. The CD29− CD90− is actually a very homogenous population containing mostly CD73− CD105− cells (Supplementary Figure 2). In contrast, we were able to derive mesenchymal like cells from the 3 other sorted subpopulations. This indicates that expression of at least one of these 2 markers is indispensable for MSCs isolation and qualification.

After 4 passages, the large majority of the cells sorted expressed CD90 and CD29. (Table 1 and Supplementary Figure 3). At this stage, the large majority displayed a CD73+ CD105+ profile. We confirmed the expression of all markers by immunofluorescence staining (Supplementary Figure 4).

3.5. Differentiation Assay of Placenta and Membrane MSCs. Specific induction of adipogenic and osteogenic differentiation was performed on Mb/Pl MSCs sorted based on CD29 and CD90 expression or directly plated after isolation and compared to BM-MSCs differentiation (Figure 3(a)).

All different cell populations from placenta or membrane regardless of the isolation protocol were identically able to differentiate into adipocytes and osteoblasts confirming their phenotypic and functional similarity (data not shown).

3.6. Cytokines Secretion of MSCs. Cytokine secretion profile was highly similar between fetal and BM-MSCs with strong secretion of GROα (CXCL1), IL-6, IL-8 (CXCL8), MCP-1 (CCL2), MIF (GIF, DER6), and serpin E1 (PAI-1), see Figures 3(b) and 3(c). Only discrete differences were noted, GROα (CXCL1) secretion by the BM-MSCs was higher than its expression in the fetal MSCs, whereas the expression of IL-6 and MCP-1 in the BM-MSCs was comparatively lower than in fetal MSCs.

3.7. Transcriptomic Comparison of Fetal and Bone Marrow MSCs. We first analyzed differences between membrane-derived and placenta-derived MSCs. As demonstrated by our PCA analysis, MSCs derived from membrane or placenta could not be differentiated based upon their transcriptomic profile (Figure 4(a)). We then analyzed the different subpopulations defined by CD90 and CD29 expression. They also displayed similar transcriptomic profile (Figures 4(b) and 4(c)).
When compared to BM-MSCs, 145 genes were significantly upregulated and 267 genes were downregulated in Mb-MSCs compared to BM-MSCs (Supplementary Table 1 and Supplementary Figure 5). Similarly, 154 genes were significantly upregulated (133 overlapping with Mb-MSCs upregulated genes) and 272 genes were downregulated (238 overlapping with Mb-MSCs downregulated genes) in the Pl-MSCs compared to BM-MSCs (Supplementary Table 2 and Supplementary Figure 5).

By ingenuity and David analysis, we were able to define several pathways and genes implicated in embryonic morphogenesis and organ development upregulated in the Pl and Mb-MSC compared to BM-MSC (Figure 4(d) and Supplementary Table 3). Several genes implicated in extracellular matrix organization, the skeletal system development and vasculature development were upregulated in the BM-MSC compared to Pl and Mb-MSC (Supplementary Table 4).

We then performed ingenuity pathway analysis building organ formation and osteoblast differentiation molecular networks. We found 14 genes upregulated in Pl/Mb MSC implicated in osteogenic differentiation in literature such as BMP, IGFBP4, IL6, HGF, and PTGS2 (Figure 4(e)).

3.8. Osteoactivin-Derived Osteoblast Differentiation. Amniotic-membrane-derived MSCs (Mb-MSCs) were used for this part of the study as they are similar to the MSCs derived from the placenta and more abundant. In our cell culture and differentiation settings, the Mb/PI MSCs displayed no differences in their ability to differentiate toward osteoblasts compared to BM-MSCs. OA treatment increased the differentiation for both Mb-MSC and BM-MSC at days 14 and 21 as demonstrated by Alizarin red staining, with Mb-MSCs displaying significantly increased osteogenic differentiation (Figures 5(a) and 5(b)). We noticed that the addition of OA to the differentiation media accelerated osteogenic differentiation with positive Alizarin staining from day 7 for Mb-MSC and day 14 for BM-MSC (data not shown).

3.9. Phosphokinase Array Analysis of Differentiating Cells. We analyzed the phosphorylation pattern of a range of phosphokinase after 4 h of OA stimulation in Pl-MSCs and BM-MSCs (Figures 6(a) and 6(b)). For both cell lines, there is a phosphorylation of Chk2 compatible with reduced proliferation during the differentiation process. While CREB is phosphorylated in BM-MSC, OA triggers ERK1/2 phosphorylation in Pl-MSCs. ERK activation was already previously described by Furochi et al. [40] as activated through OA. Those previous findings together with our data lead us to stand for an OA activation role in osteogenic differentiation notably through ERK1/2 pathway activation in fetal-derived MSCs.

4. Discussion

MSCs are thought to have great therapeutic potential due to their capacity for self-renewal and multilineage differentiation [4, 41]. For example, they support hematopoiesis and enhance engraftment of hematopoietic stem cells after cotransplantation [3, 42]. Experimental and clinical data have demonstrated an immune-regulatory function of BM-derived MSCs that may contribute to the reduction of graft-versus-host disease following hematopoietic stem cell transplantation [43, 44]. Furthermore, even if clinical studies remain anecdotal, BM-MSCs have been reported to exert beneficial effects in the healing of a limited number of patients with bone nonunions [45–50]. MSCs initiate the fracture repair process leading to the formation of a cartilaginous template (callus) that is then replaced by new bone that fills the gap [6]. Limitation in MSC number and/or functions is hypothesized to play a critical role in the pathogenesis of post fracture nonunions.

Currently, the bone marrow is perceived as the major source of MSCs for cell therapy. However, aspiration of BM involves invasive procedures. The frequency, differentiation, and growth potential of BM-MSCs decrease significantly with age [51]. Thus, the search for alternative consistent sources of MSCs is of significant value. Indeed, when we consider therapeutic application, it will be mandatory to access cell banks displaying a large variety of HLA types. Among these sources, placenta and membrane may be ideal sources due to their accessibility, painless donor procurement, promising sources for autologous cell therapy, and lower risk of viral contamination. The
Figure 1: Higher proportion of MSCs can be isolated from the membrane compare to the placenta. Fetal tissue derived MSCs display greater proliferation kinetic than bone marrow derived MSCs. (a) MSCs were defined as CD45−, CD34−, CD29+, CD90+, CD73+, and CD105+. Their proportion was then calculated in freshly digested placenta specimens and fetal membranes form 3 different donors. MSCs represented 15.67% (±0.29%) and 2.14% (±0.65%) of cells isolated from the membrane and placenta, respectively (∗∗\(P = 9.25 \cdot 10^{-4}\)). (b) Day 1 phase contrast microscopy of adherent cells from placenta and membrane directly plated after tissue digestion in MSCs media. We can see significantly more adherent cells from digested membrane compared to placenta. (c) MSCs derived from placenta and membranes were expanded up to passage 15 without changes of their morphology or proliferation rate. Proliferation rate was assessed by cell counting at different passages. It was similar in placenta- and membrane-derived MSCs with no differences between early and late passages. However, proliferation rate was significantly higher than proliferation rate of bone-marrow-derived MSCs.
accessibility of these tissues will allow constituting clinically relevant banking program.

In this study, we have isolated MSCs from placenta and fetal membrane using very simple isolation technique with the same great purity yield (more than 95%) than initial FACS sorting methods [53]. Moreover, no difference was found between different MSCs subpopulation of placenta and membrane considering phenotypic characteristics, growth kinetic, markers expression, differentiation assays, and transcriptomic profile. This suggests the plasticity of certain MSC markers. We indeed illustrate that the surface markers used for MSCs cell sorting have limited interest in fetal mesenchymal stem cells isolation. We demonstrate that the yield of MSCs retrieval is 6–8 fold superior in the fetal membranes than in placenta. In addition, others already demonstrated through cytogenetic analysis that placenta-derived MSCs maintained a normal karyotype for 30–40 passages in vitro [54]. Indeed, we demonstrate that the Mb/Pl MSCs retain even at high number of passages significantly better proliferation ability than BM-MSCs. We finally demonstrate that these fetal MSC share close transcriptomic profiles with BM-MSC.

Currently, bone morphogenetic protein-2 and -7 (BMP-2 and -7) are the only biologic modifiers that have received the United States Food and Drug Administration (US-FDA) approval for clinical applications in orthopedic surgery. The BMPs low biologic activity is demonstrated by the doses of tens of milligrams of commercial BMP-2- and -7-containing
Figure 3: Differentiation assay of PlMb-MSCs in comparison to BM-MSCs and cytokines expression. (a) Representative differentiation of PlMb-MSCs passage 4 is shown. Cells were kept in induction medium (differentiation) or control standard medium (control). (a–d) Osteogenic and adipocyte differentiation and control for BM-MSCs. (f–h) Osteogenic and adipocyte differentiation and control for Mb-MSCs. (b) Cytokine expressions of Mb-MSCs and BM-MSCs using the proteome profiler. (c) Quantification of cytokine optical density. Measurements were obtained with ImageJ software (NIH).
Figure 4: PCA representation of transcriptomic comparison of Pl-MSCs, Mb-MSCs and BM-MSCs and ingenuity pathways analysis. (a) Mb-MSCs (red) and Pl-MSCs (green) are overlapping clearly indicating the impossibility to differentiate these MSCs at a transcriptional level. They can clearly be differentiated from bone marrow MSCs (blue). (b) The PCA overlapping between every cell sorted sub-population from membrane (CD29+ CD90− red; CD29− CD90+ blue; CD90− CD29− green) or nonsorted cells (Mb-MSCs purple) indicate the impossibility to discriminate the different MSCs subpopulation at a transcriptional level. (c) PCA of different MSC subpopulation from placenta (CD29+ CD90− red; CD29− CD90+ blue) or directly isolated cells (Pl-MSCs blue) indicate the impossibility to discriminate these MSCs subpopulation at a transcriptional level. (d) Ingenuity pathway analysis was able to define enriched pathways implicated in embryonic morphogenesis and organ development in fetal MSCs compared to BM-MSCs. (e) Ingenuity pathway analysis using organ formation and osteoblast differentiation molecular network shows an enrichment in proosteogenic genes in fetal MSCs compared to BM-MSCs.
products, whereas BMPs concentrations in vivo are around several micrograms per kilogram of bone [55, 56]. BMP therapeutic doses in preclinical and clinical trials varied by factors up to 100 folds, demonstrating low consistency on bone repair [57].

Noteworthy, we have characterized the mesenchymal stem cells by the criteria used by the ISCT [33]. We would like to point a limitation emphasized by the plasticity of the phenotypic markers. First, the true stemness ability (self-renewal) of our MSCs was not demonstrated and should be further documented in studies looking at clonality of the cell lines. Therefore, while they have a real ability to differentiate in different lineage, it is impossible to say if a single cell can indeed differentiate in different lineages. Moreover, the role of this cell types in vivo remains still not clearly define by lack of specific targeting of the mesenchymal stem cells.

We recently demonstrated that OA acts downstream of BMP-2, and our results indicate that OA may have similar osteoinductive effects to BMP-2 in mice.

We investigated the response of fetal MSCs to OA as compared to BM-MSCs. We demonstrate that OA can induce osteogenic differentiation in human MSCs. More interestingly, fetal-derived MSCs display better response to OA than BM-MSCs. We finally demonstrate that OA can also be used as a complement for osteogenic-induced differentiation with fetal MSCs. Finally, in accordance with the literature, we document that the induction of osteogenic differentiation following OA stimulation involved ERK1/2 pathway activation.

Considering that MSCs are way more abundant in membrane compared to placenta, we, therefore, stand that fetal membranes could be used to build MSCs banking.
program in order to meet clinical threshold in bone fracture reparation. Isolation of Mb-MSCs through selective culture in DMEM-low glucose supplemented with 20% serum and antibiotics seems to be the most efficient process. This process is very adapted for automation compatible with large cell banking programs.

Moreover, the increased capacity of response to osteogenic differentiation upon osteoactivin treatment prompts us to study the role of fetal membrane MSCs in experimental preclinical model of bone regeneration. Indeed, critical animal studies would be necessary to determine how MSCs are recruited and survive at the fracture site, their repair effectiveness, and the mechanisms through which they exert their actions.

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References


Amniotic Fluid and Amniotic Membrane Stem Cells: Marker Discovery

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1. Introduction

Both amniotic fluid (AF) and amniotic membrane (AM) represent rich sources of stem cells that can be used in the future for clinical therapeutic applications. Ethical concerns regarding the isolation of stem cells from these sources are minimized [1–3], in contrary to the issues emerging from human embryonic stem cell (ESC) research [4–6]. AF is collected during scheduled amniocenteses between 15th and 19th week of gestation for prenatal diagnosis and the excess of sample can be used for cell sourcing [2, 4–9], whereas AM is usually collected during the caesarean sections of term pregnancies [10, 11]. Given the heterogeneity of the stem cell populations derived from these sources, the isolation of specific cell types is difficult and requires a detailed phenotypic and molecular characterization of the respective cells. Studies that include omics approaches are fundamental in better understanding the mechanisms of molecular expression of these cells and defining the correct methodologies for their isolation, prior to their use in therapeutic approaches.

This paper aims to present the main biological and molecular characteristics of AF- and AM-derived stem cells and also to highlight the recent advances in marker discovery using global methodologies, such as transcriptomics, proteomics, or secretome analyses.

1.1. Amniotic Fluid. AF serves as a protective liquid for the developing embryo, providing mechanical support and the required nutrients during embryogenesis [1, 3]. Amniocentesis has been used for many decades as a routine procedure for fetal karyotyping and prenatal diagnosis, allowing the detection of a variety of genetic diseases [1, 3, 12].

The major component of AF is water; however its overall composition varies throughout pregnancy. At the beginning of pregnancy, the amniotic osmolarity is similar to the fetal plasma. After keratinization of the fetal skin amniotic osmolarity decreases relatively to maternal or fetal plasma, mainly due to the inflow of fetal urine [1]. More interestingly, AF also represents a rich source of a stem cell population deriving from either the fetus or the surrounding...
amniotic membrane [1, 12]. Additional investigations by several groups have been recently focused on the cellular properties of amniotic derived cells and their potential use in preclinical models [13–18] and in transplantation therapies [7, 17, 19–24].

1.1.1. Amniotic Fluid Stem Cells (AFSCs). The amniotic fluid cells (AFCs) represent a heterogeneous population derived from the three germ layers. These cells share an epithelial origin and are derived from either the developing embryo or the inner surface of the amniotic membrane, which are characterized as amniotic membrane stem cells [12]. The AFCs are mainly composed of three groups of adherent cells, categorized based on their morphological, growth, and biochemical characteristics [12]. Epithelial (E-type) cell are cuboidal to columnar cells derived from the fetal skin and urine, amniotic fluid (AF-type) cells are originating from fetal membranes, and fibroblastic (F-type) cells are generated mainly from fibrous connective tissue. Both AF- and F-type cells share a fibroblastoid morphology and the dominant cell type appears to be the AF-type, coexpressing keratins and vimentins [1–3, 8, 9, 25–27]. Several studies have documented that human amniotic fluid stem cells (AFSCs) can be easily obtained from a small amount of second trimester AF, collected during routine amniocenteses [2, 4–9], a procedure with spontaneous abortion rate ranging from 0.06 to 0.5% [2, 28, 29]. Up to date, a number of different cultivation protocols have been reported, leading to enriched stem cell populations. The isolation of AFSC and the respective culture protocols were summarized in a recent review by Klemmt et al. [3] and can be categorized as follows: (i) a single step cultivation protocol, where the primary culture was left undisturbed for 7 days or more until the first colonies appear [2, 3, 30–32], (ii) a two-step cultivation protocol, where amniocytes, not attached after 5 days in culture, were collected and further expanded [3, 5, 33], (iii) cell surface marker selection for CD117 (c-kit receptor) [3, 7, 34, 35], (iv) mechanical isolation of the initial mesenchymal progenitor cell colonies formed in the initial cultures [9], and (v) short-term cultures to isolate fibroblastoid colonies [36]. The majority of the AFSCs, isolated following these methodologies, shared a multipotent mesenchymal phenotype and exhibited higher proliferation potential and a wider differentiation potential compared to adult MSCs [2, 4–7, 9, 24, 37].

1.2. Amniotic Membrane (AM). The amniotic membrane, lacking any vascular tissue, forms most of the inner layer of the fetal membrane [12, 38] and is composed of 3 layers: (i) an epithelial monolayer consisting of epithelial cells, (ii) an acellular intermediate basement layer, and (iii) an outer mesenchymal cell layer, rich in mesenchymal stem cells and placed in close proximity to the chorion [12, 38]. AM was used in clinic for many decades for wound healing in burns, promoting epithelium formation and protecting against infection [39, 40]. Recently, the use of AM has been evaluated as a wound dressing material for surgical defects of the oral mucosa [41], ocular surface reconstruction [40, 42], corneal perforations [43, 44], and bladder augmentation [45].

1.2.1. Amnion Membrane Stem Cells (AMSCs). Amnion membrane stem cells (AMSCs) include two types, the amniotic epithelial cells (AECs) and the amnion membrane mesenchymal stem cells (AM-MSCs) derived from the amniotic epithelial and the amnion mesenchymal layers, respectively [12, 46]. Both cell types are originated during the pregastrulation stages of the developing embryo, before the delineation of the three primary germ layers and are mostly of epithelial nature [38, 47]. A variety of protocols have been established for AECs and AM-MSCs isolation, primarily based on the mechanical separation of the AM from the chorionic membrane and the subsequent enzymatic digestion [47–50]. AM-MSCs exhibited plastic adherence and fibroblastoid morphology, while AECs displayed a cobblestone epithelial phenotype. AM-MSCs shared similar phenotypic characteristics with the ones derived from adult sources. More interestingly, AM-MSCs, similarly to AF-MSCs, exhibited a higher proliferation rate compared to MSCs derived from adult sources [12, 51] and a multilineage differentiation potential into cells derived from the three germ layers [27].

2. Immunophenotype

2.1. Amniotic Fluid Stem Cells. The AF has recently emerged as an alternative fetal source of a variety of cells of stem cell origin [1, 3]. Herein, we aim to summarize the key markers that characterize AFSCs. To date, MSCs represent the best characterized subpopulation of AFSCs. The AFMSCs exhibited typical mesenchymal marker expression, such as CD90, CD73, CD105, CD29, CD166, CD49e, CD58, and CD44, determined by flow cytometry analyses [2, 5–8, 10, 12, 21, 32, 33, 52, 53]. Additionally, these cells expressed the HLA-ABC antigens, whereas the expression of the hematopoietic markers CD34 and CD45, the endothelial marker CD31, and the HLA-DR antigen was undetected [2, 5, 6, 32]. More importantly, the majority of cultured AF-MSCs expressed pluripotency markers such as the octamer binding protein 3/4 (Oct-3/4), the homebox transcription factor Nanog (Nanog), and the stage-specific embryonic antigen 4 (SSEA-4) [2, 5–7, 9, 21, 32, 33, 52].

It was also reported that amniocyte cultures contain a small population of CD117 (a tyrosine kinase specific for stem cell factor present primarily in ESCs and primordial germ cells) positive cells that can be cloned and expanded in culture [7]. The differentiation properties of CD117+ AFSCs were tested for the first time in vivo, proving in this way their stem cell identity [7]. Experimental evidence suggested that AFSCs are derived from spindle-shaped fibroblastoid cells [10].

In an attempt to analyze the AFSCs subpopulations, our group recently identified two morphologically distinct populations of AFSCs of mesenchymal origin, with different proliferation and differentiation properties, termed as spindle shaped (SS) and round shaped (RS) [9]. Both subpopulations were expressing mesenchymal stem cell markers at similar levels. However, it was identified that SS
colonies expressed higher levels of CD90 and CD44 antigens compared to RS colonies [9].

2.2. Amniotic Membrane Stem Cells (AMSCs). A detailed immunophenotype analysis of AMSCs revealed the expression of antigens, such as CD13, CD29, CD44, CD49e, CD54, CD73, CD90, CD105, CD117low, CD166, CD27low, stromal stem cell marker 1 (Stro-1), SSEA-3, SSEA-4, collagen I and III (Col1/Col3), alpha-smooth muscle actin (α-SMA), CD44, vimentin (Vim), fibroblast surface protein (FSP), and HLA-ABC antigen [10, 12, 27]. However, intercellular adhesion molecule 1 (ICAM-1) was expressed in very low levels and proteins TRA-1-60, vascular cell adhesion protein 1 (VCAM-1), von Willebrand factor (vWF), platelet endothelial cell adhesion molecule (PECAM-1), CD3, and HLA-DR were not detected [10, 27]. One of the most abundant proteins found in AM derived cells is laminin, which plays a key role in differentiation, cell shape and migration, and tissue regeneration [54, 55]. RT-PCR analysis further showed that AMSCs expressed genes, such as Oct-3/4, zinc finger protein 42 (zfp42 or Rex-1), stem cell factor protein (SCF), neural cell adhesion molecule (NCAM), nestin (NES), bone morphogenetic protein 4 (BMP-4), GATA binding protein 4 (GATA-4), and hepatocyte nuclear factor 4e (HNF-4e) even in high passages. Brachyury, fibroblast growth factor 5 (FGF5), paired box protein (Pax-6), and bone morphogenetic protein 2 (BMP2) transcripts were not detected [10, 12]. Similarly, AECs were positive for CD10, CD13, CD29, CD44, CD49e, CD73, CD90, CD105, CD117, CD166, Stro-1, HLA-ABC, and HLA-DQlow and negative for CD14, CD34, CD45, CD49d, and HLA-DR expressions, as determined by FACS analyses [27, 47–50]. Further investigation showed that AECs were expressing stem cell markers such as SSEA-1, SSEA-3, SSEA-4, Nanog, sex determining region Y-box 2 (Sox2), Tra-1-60 and Tra-1-80, fibroblast growth factor 4 (FGF4), Rex-1, cryptic protein (CFC-1), and prominin 1 (PROM-1) [38, 50].

3. Transcriptomics

3.1. Amniotic Fluid Stem Cells. A functional analysis of the gene expression signature of AF-MSCs compared to bone-marrow- (BM-), cord-blood- (CB-), and AM-MSCs was initially performed by Tsai et al. [11]. Genes expressed in MSCs from all three sources could be categorized in groups related to (i) extracellular matrix remodeling (CD44, collagen II (COL2), insulin-like growth factor 2 (IGF2), and tissue inhibitor of metalloproteinase 1 (TIMP1)), (ii) cytoskeletal regulation (urokinase-type plasminogen activator (PLAU) and receptor (PLAUR)), (iii) chemokine regulation and adhesion (alpha actinin 1 (ACTN1), actin-related protein complex subunit 1B (ARPC1B) and thrombospondin 1 (THBS1)), (iv) plasmin activation (tissue factor pathway inhibitor 2 (TFPI2)), (v) transforming growth factor β (TGFβ) receptor signaling (caveolin 1 (Cav1), caveolin 2 (Cav2), cyclin-dependent kinase inhibitor 1A (CDKN1A)), and (vi) genes encoding E3 ubiquitin ligases (SMURF) [11]. The upregulated genes in AF-MSCs compared to BM-, CB-, and AM-MSCs included molecules involved in uterine maturation and contraction, such as oxytocin receptor (OXTR) and regulation of prostaglandin synthesis, such as phospholipase A2 (PLA2G10). Other upregulated genes in this group were involved in signal transduction related to (i) thrombin triggered response ((F2R and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-protein-related pathways (rho-related GTP-binding protein (RHOF)), regulator of G protein signaling 5 and 7 (RGS5, RGS7), and phospholipase C beta 4 (PLCB4) [11].

In recent studies on AFSCs, Kim et al. described for the first time the gene expression changes in total AFSC population during different passages by illumina microarray analysis. 1970 differentially expressed genes were detected and categorized according to their expression profiles into 9 distinct clusters [56]. Genes with gradually increasing expression levels included chemokine (C-X-C motif) ligand 12 (CXCL12), cadherin 6 (CDH6), and folate receptor 3 (FOLR3). Down-regulated genes were among others, cyclin D2 (CCND2), keratin 8 (K8), IGF2, natriuretic peptide precursor (BNP) B, and cellular retinoic acid binding protein 2 (CRABPII) [56]. To obtain further information, chip data analysis on aging genes was performed and revealed upregulation of gene transcripts, such as nerve growth factor beta (NGFβ), insulin receptor substrate 2 (IRS-2), insulin-like growth factor binding protein 3 (IGFBP-3), and apolipoprotein E (APOE). Expression of genes, such as PLAU, E2F transcription factor 1 (E2F1), IGF2, breast cancer type 1 susceptibility gene (BRCA1), DNA topoisomerase 2-alpha (TOP2A), transforming growth factor β (TGFβ), and regulator of G protein signaling 5 and 7 (RGS5, RGS7), were involved in signal transduction related to (i) thrombin triggered response ((F2R and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-protein-triggered response ((F2 and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-protein-related pathways (rho-related GTP-binding protein (RHOF)), regulator of G protein signaling 5 and 7 (RGS5, RGS7), and phospholipase C beta 4 (PLCB4) [11].

Wolfrum et al. performed a global gene expression analysis of AFSCs compared to iPSCs derived from AF (AFiPSC) and ESCs [57]. Among these, genes related to self renewal and pluripotency (1299 genes e.g., POU class 5 homeobox 1 (POU5F1), Sox2, Nanog, microRNA-binding protein LIN28) and AFSCs-specificity (665 genes, e.g., OXTR, HHT, RGS5, neurofibromatosis type 2 (NF2), p53 tumor suppressor (TP53), tumor necrosis factor superfamily member 10 (TNFSF10), 5′-nucleotidase (NT5E)) were detected in AFSCs [57]. Furthermore, the authors examined the expression of senescence and telomere associated genes in AFSCs of early and later passage, in order to study the effect of reprogramming on bypassing senescence observed in AFSC cultures. Sixty-four genes were identified as differentially expressed in AFSCs compared to AFlPSC lines. Of these, telomere-associated genes and genes involved in regulating cell cycle, such as the mitotic arrest deficient-like 2 (MAD2L2), the poly ADP-ribose polymerase 1 (PARP1), replication protein A3 (RPA3), the dystrokarsitogenina 1 (DKC1), the mutS homolog 6 (MSH6), the CHK1 checkpoint homolog (CHEK1), the polo-like kinase 1 (PLK1), the POU class 2 homeobox 1 (POU2F1), the CDC2, the Bloom syndrome gene RecQ helicase-like (BLM), the Werner syndrome RecQ helicase-like (WRN), the DNA methyltransferase 1 (DNMT1), the DNA methyltransferase 3 beta (DNMT3B), the lamin B1 (LMNB1), and the DNA replication factor 1 (MCM2) were analyzed. Further studies included chemokine (C-X-C motif) ligand 12 (CXCL12), cadherin 6 (CDH6), and folate receptor 3 (FOLR3). Down-regulated genes were among others, cyclin D2 (CCND2), keratin 8 (K8), IGF2, natriuretic peptide precursor (BNP) B, and cellular retinoic acid binding protein 2 (CRABPII) [56]. To obtain further information, chip data analysis on aging genes was performed and revealed upregulation of gene transcripts, such as nerve growth factor beta (NGFβ), insulin receptor substrate 2 (IRS-2), insulin-like growth factor binding protein 3 (IGFBP-3), and apolipoprotein E (APOE). Expression of genes, such as PLAU, E2F transcription factor 1 (E2F1), IGF2, breast cancer type 1 susceptibility gene (BRCA1), DNA topoisomerase 2-alpha (TOP2A), transforming growth factor β (TGFβ), and regulator of G protein signaling 5 and 7 (RGS5, RGS7), were involved in signal transduction related to (i) thrombin triggered response ((F2R and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-protein-triggered response ((F2 and F2RL)), (ii) hedgehog signaling ((hedgehog acyltransferase (HHAT)), and (iii) G-protein-related pathways (rho-related GTP-binding protein (RHOF)), regulator of G protein signaling 5 and 7 (RGS5, RGS7), and phospholipase C beta 4 (PLCB4) [11].
(CDT1), were downregulated in AFSCs compared to AfIPSCs and ESCs. In contrast, peptidylprolyl cis/trans isomerase (PIN1), lamin A/C (LMNA), growth arrest and DNA damage inducible alpha (GADD45A), chromobox homolog 6 (CBX6), NAPDH oxidase 4 (NOX4), endoglin (ENG), histone H2B type 2-E (HIST2H2BE), CKDN1A, CKDN2A growth differentiation factor 15 (GDF15), and serine protease inhibitor 1 (SERPINE1), among others, were upregulated in AFSCs compared to AfIPSCs and ESCs [57].

3.2. Amniotic Membrane Stem Cells. Transcriptomic analysis using DNA microarrays has been reported for AM-MSCs [11]. These experimental data provided information on the AM-MSC gene expression pattern compared to gene expression profiles of AF, CB, and BM-MSCs. Several upregulated genes in AM-MSCs involved in immune adaptation regulation between the maternoplacental interface were identified. Among others, spondin 2 (SPON2), interferon, alpha inducible protein 27 (IFI27), bradykinin receptor B1 (BDKRB1), small inducible cytokine subfamily B member 5 and 6 (SCYB5, SCYB6), and Yamaguchi sarcoma viral-related oncogene homolog (LYN) were found to be upregulated [11]. In addition, other genes with increased expression in AM-MSCs compared to AF, CB, and BM-MSCs included (i) transcription factors, such as forkhead box F1 (FOXF1), heart and neural crest derivatives expressed 2 (HAND2), and transcription factor 21 (TCF21) and (ii) metabolic enzymes, such as dipeptidyl-peptidase 6 (DPP6), tryptophan 2,3-dioxygenase (TDO2), and sialyltransferases (STs) [11].

4. Proteomics

4.1. Amniotic Fluid Stem Cells. Proteomic studies on the total AFSC population, including epithelial (E-type), amniotic fluid specific (AF-type), and fibroblastic (F-type) cells, revealed 2400 spots that resulted in the identification of 432 different gene products. The majority of the proteins was localized in cytoplasm (33%), mitochondria (16%), and nucleus (15%) and represented mainly enzymes (174 proteins) and structural proteins (75 proteins). A relatively high percentage of membrane and membrane-associated proteins were also present (7%) [58]. Among the detected proteins, 9 were corresponding to epithelial cells, such as ATP synthase D chain (ATP5H), NADH-ubiquinone oxidoreductase 30 kDa subunit (NUIM), annexin II (Anx2), annexin IV (Anx4), 40S ribosomal protein SA (Rpsa), glutathione S-transferase P (GSTP), major vault protein, and cytochromes 19 and 7 (CK-19, CK-7), whereas 12 proteins were reported to be expressed in fibroblasts, including fibronectins, tropomyosins, transgelin (TAGLN), arp2/3 complex 34 kDa subunit (P34-arp), gelsolin (Gsn), elongation factor 1-β (EF-1β), and others. Eight proteins were found to be expressed in keratinocytes, including keratins, ribonucleoproteins, Anx2, aetyl-CoA acetyl-transferase (ACAT1), and others. Three to be expressed in epidermis, including tropomyosins and keratins and one in mesenchymal cell type (vimentin 1 (Vim 1)) [58].

Recent studies provided evidence that a diversity of metabolic enzyme expression in the amnion cells is involved in metabolic and genetic syndromes, and thus, their detection might be important for prenatal diagnosis. A more detailed analysis for determining specific metabolic enzymes present in AFSCs was reported by Oh et al. [59]. Ninety-nine proteins had been identified, such as carbohydrate handling enzymes, amino acid handling enzymes, proteins of purine metabolism, and enzymes of intermediary metabolism [59, 60].

A proteomic analysis was also performed on different culture passages of CD117+ AFSCs, exhibiting variations in protein expression that mainly occurred in early passages [35]. Twenty-three proteins were differentially expressed between early and late passages with the most sticking downregulated proteins, the Col1, the Col2, the vinculin (Vcl), the CRABP II, the statamin (STMN1), and the cofillin-1 (CFL1). In contrast, TAGLN and Col3 are increased during passages [35]. Proteins that showed dysregulated levels along the passages were the 265 protease regulatory subunit 7 (PSMD7), the ubiquitin carboxyl terminal hydrolase isoenzyme L1 (UCH-L1), the heterogeneous nuclear ribonuclear protein H (hnRNP H), and the TAR DNA-binding protein 43 (TDP-43) [35].

In 2007, the proteomic map of human AF-MSCs was constructed and directly compared to the one derived from BM-MSCs [2]. 261 different proteins were identified in AF-MSCs with the majority of the proteins localized in the cytoplasm (41%), whereas others were found in the endoplasmic reticulum (8%), nucleus (13%), mitochondria (12%), ribosomes (1%), cytoskeleton (6%), cytoplasm and the nucleus (5%), and secreted (2%) proteins [2]. AF-MSCs expressed a number of proteins related to proliferation and cell maintenance, such as ubiquilin-1 (UBQLN1), which is known to control cell cycle progression and cell growth, the proliferation associated protein 2G4 (PA2G4), a nucleolar growth-regulating protein, the secreted protein acidic and rich in cysteine (SPARC), which is regulated during embryogenesis and is involved in the control of the cell cycle and cell adhesion, and the enhancer of rudimentary homolog (ERH) that also regulates cell cycle [2]. TAGLN and gaeleton 1 (Gal 1), both present in stem cells and related to differentiation, were also abundantly expressed in AF-MSCs. Other proteins expressed in high levels in AF-MSCs were related to development, such as Deltex-3-like (DTX3L), and ii) cytoskeletal organization and movement, such as CFL1, the coactosin-like protein (CLP), and the cofilin-1 (Vcl), the CRABP II, the statamin (STMN1), and the cofilin-1 (CFL1). In contrast, TAGLN and Col3 are increased during passages [35]. Proteins that showed dysregulated levels along the passages were the 265 protease regulatory subunit 7 (PSMD7), the ubiquitin carboxyl terminal hydrolase isoenzyme L1 (UCH-L1), the heterogeneous nuclear ribonuclear protein H (hnRNP H), and the TAR DNA-binding protein 43 (TDP-43) [35].

In our later study [9], we established the proteomic map of the two morphologically distinct AF mesenchymal progenitor cell types (SS and RS) by 2-DE. Twenty-five proteins were differentially expressed in the two subpopulations. Proteins upregulated in SS-AF-MSCs compared to RS-AF-MSCs included reticulocalbin-3 precursor (RCN3), collagen α1 (I) (COL1A1), FK506-binding protein 9 precursor (FKBP9), Rho GDP-dissociation inhibitor 1 (RhoGD1), chloride intracellular channel protein 4 (CLIC4), tryptophanyl-tRNA synthetase (TrpRS), and 70 kD heat
4.2. Amniotic Membrane Stem Cells. A detailed approach for studying human AM proteins was described by Hopkinson et al. [61]. In this study, the authors performed a proteomic analysis of AM samples that were prepared for human transplantation, by using 2-DE gels. The wash media from the AM samples were also examined and the secreted proteins were identified. Proteins detected in both AM and the wash media suggested that partial protein release had occurred. These proteins were mostly soluble cytoplasmic proteins and were categorized according to their subcellular localization and function [61]. One example of the most abundant and consistent proteins in AM is THBS1 which is reported to play a role in wound repair, inflammatory response, and angiogenesis [62, 63]. Mimetcan (also named osteoglycin/OGN) is another protein detected in AM that represents a small leucine-rich proteoglycan, found in the ECM of connective tissue. Mimetcan is reported to maintain the tensile strength and hydration of the tissue [61, 64–66]. In addition, the larger form of mimetcan was expressed in AM cells and was susceptible to proteolytic cleavage [65]. TGF-β-induced protein ig-h3 (βIG-H3), an ECM adhesive molecule acting as a membrane-associated growth factor during cell differentiation and wound healing, and integrin α6 (CD49f), a component of α6β4 integrin, were also present in significant amounts in AM cells [61, 67, 68]. It is well known that α6β4-βIG-H3 interaction plays an important role in mediating cell adhesion and wound repair signaling pathways [69].

Another important study by Baharvand et al. [70] was focused on the analysis of epithelium-denuded human AM showing both quantitative and qualitative differences compared to nontreated AM [61]. They investigated the proteome of the human AM epithelium, which was used as a limbal stem cell niche for treating ocular surface reconstruction [71, 72]. 515 spots were detected in all the 2-DE gels and 43 proteins were identified using MALDI TOF/TOF MS in AM. The most abundant proteins were different isoforms of lumican (LUM) and OGN, both members of the proteoglycan (PG) family. In particular, OGN might play role in many biological processes including cell growth, angiogenesis, and inflammation [66]. Other proteins detected included collagen VI α1/α2 (Col6a1/Col6a2), fibrinogen beta chain (FGB), transglutaminase 2 isof orm A (TGM2A), b-actin variant (ACTB), 70 kD heat shock protein 5 (HSP5), nidogen 2 (NID2), CD49f, βIG-H3, and tubulointerstitial nephritis (TIN) [70]. Some of the proteins identified in this study were also related to extracellular matrix (ECM). Among the detected ones, fibronectin (FN), laminins, and collagen IV (Col4) and VII were reported to promote epithelial adhesion and migration [73, 74].

5. Secretome

Recently, significant progress has been made regarding the analysis of the secreted proteins from AFSCs. It has been documented that AFSC secretome was responsible for enhancing vasculogenesis and was capable of evoking a strong angiogenic response in murine recipients [75]. According to this study, a detailed analysis of the AFSC-conditioned media revealed the presence of known proangiogenic and antiangiogenic factors using luminex's MAP Technology. Vascular endothelial growth factor (VEGF), stromal cell-derived factor 1 (SDF-1), interleukin 8 (IL-8), monocyte chemotactic protein 1 (MCP-1), and two angiogenesis inhibitors, interferon-gamma (IFNγ) and interferon gamma-induced protein 10 (IP-10), were identified as secreted proteins [75–77]. It was also demonstrated that a relative small number of AFSC was enough to secrete a detectable amount of proangiogenic growth factors and cytokines. The secretion of these can be regulated in a dose-dependent manner according to the initial cell number of the cells used [24, 75].

A systematic study on AFSC-secreted proteins led to the conclusion that proangiogenic soluble factors from AFSCs can mediate the recruitment of endothelial progenitors in an ischemic rat model [78]. In particular, conditioned medium derived from AFSCs could potentially deliver angiogenic growth factors and cytokines into the skin flap of the ischemic rat model and was responsible for triggering the endogenous repair by recruiting endothelial progenitor cells [78].

In our recent studies, we examined the therapeutic potential of an AF-MSCs and their secreted molecules in mice with acute hepatic failure [24]. A variety of cytokines and growth factor were detected in AF-MSC conditioned medium. Cytokines such as interleukin 10 (IL-10), interleukin 27 (IL-27), interleukin 17 family (IL-17E), interleukin 12p70 (IL-12p70), interleukin-1 beta (IL-1β), and interleukin-1 receptor antagonist (IL-1ra), responsible for inducing local and systemic downregulation of pro-inflammatory mediators, were detected. SERPINE1, MCP-1, and SDF-1, responsible for promoting tissue repair, were also secreted [24, 79, 80]. Interestingly, among the highly expressed growth factors were platelet-derived endothelial cell growth factor (PD-ECGF), endostatin/collagen XVII (EN/Col17), urinary plasminogen activator (uPA), TIMP1, TIMP2, heparin-binding EGF-like growth factor (HB-EGF), fibroblast growth factor 7 (FGF7), and epidermal growth factor (EGF), responsible for liver regeneration and tissue repair [24, 81].

6. Summary

The current data so far suggest that amniotic fluid and amniotic membrane may represent promising sources for stem cells of mesenchymal origin. Indeed, MSCs are more abundant and a wide range of protocols has been described for their isolation. However, it is reported that different culture conditions of the same type of cells may affect their differential gene expression pattern, which represents a limitation for their isolation and expansion in vitro. Studies
including phenotypic analysis, using methodologies such as flow cytometry and immunohistochemistry, as well as transcriptomics, proteomics, and secretome analyses approaches, aim to determine the protein profile of these cells (Figure 1). Data generated by such studies are expected to clarify their differential repertoire and validate the molecular profile of these stem cells. However, the main issue urged to be addressed is the isolation of a homogenous population that may facilitate systematic studies for the elucidation of the function of these multipotent cells.

Such approaches may lead to the identification of key antigens that mirror the phenotype of these cells and explain their distinct features properties. This type of studies will open the way for a systematic and efficient isolation of these cells prior to their use at the clinical setting.

**Appendix**

**Questions for Further Investigation**

Which are the appropriate isolation methods and culture conditions of AFSCs or AMSCs that will allow the identification of a consistent phenotype?

Is there a single marker that can be used for AFSCs or AMSCs isolation?

The AFSC and AMSC populations are heterogeneous and differ in their phenotypic and molecular properties. Methods of isolation can result in a homogeneous cell population.

AFSCs or AMSCs can be used as tools in regenerative medicine: establishment of culture conditions with minimal or no animal substances.

**Marker Discovery.** The AFSCs and the AMSCs initial characterization can be performed by immunophenotype analysis by using well-characterized cell surface markers such as AFSCs: CD90, CD73, CD105, CD29, CD166, CD49e, CD58, CD44, HLA-ABC, SSEA-4; AMSCs: CD13, CD29, CD44, CD49e, CD73, CD105, CD117, CD166, Stro-1, HLA-ABC, SSEA-3, SSEA-4, Nanog, Sox2, Tra1-60, Tra1-80, FGF-4, CFC-1, PROM1 [37, 49, 50].

**Transcriptomics and Proteomics Revealed the Identification of Key Markers Expressed such as.** AFSCs: Nanog, Sox2, Tra1, 80, FGF-4, CFC-1, PROM1. AMSCs: CD90, CD73, CD105, CD29, CD166, CD49e, CD58, CD44, HLA-ABC, SSEA-4, Nanog, Sox2, Tra1-60, Tra1-80, FGF-4, CFC-1, PROM1.
Since there is no common marker available for AFSC and AMSC, a wider panel of markers needs to be employed. This also urges the conduction of further detailed array and functional analyses in order to define the most appropriate markers for AFSC and AMSC characterization.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Amnion Epithelial Cells as a Candidate Therapy for Acute and Chronic Lung Injury

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Acute and chronic lung injury represents a major and growing global burden of disease. For many of these lung diseases, the damage is irreparable, exhausting the host’s ability to regenerate new lung, and current therapies are simply supportive rather than restorative. Cell-based therapies offer the promise of tissue regeneration for many organs. In this paper, we examine the potential application of amnion epithelial cells, derived from the term placenta, to lung regeneration. We discuss their unique properties of plasticity and immunomodulation, reviewing the experimental evidence that amnion epithelial cells can prevent and repair lung injury, offering the potential to be applied to both neonatal, childhood, and adult lung disease. It is amazing to suggest that the placenta may offer renewed life after birth as well as securing new life before.

1. Introduction

Chronic lung diseases, in both children and adults, are leading causes of morbidity and mortality worldwide, estimated to account for about 10% of global mortality [1]. It has been estimated by the World Health Organization that, by 2030, chronic lung disease, mainly caused by tobacco smoking, occupational irritant exposure and pollution, will become the third most common cause of death worldwide [1]. However, mortality is just the tip of the iceberg. A recent economic analysis of the burden of chronic lung disease in Australia revealed that almost 1 in 5 adults aged 40 or older have chronic obstructive pulmonary disease (COPD) to some degree, with half of these individuals having advanced disease [2]. The estimated financial cost of COPD in Australia in 2008 was nearly $9 billion. Further, chronic lung disease does not only affect adults. About 1 in 80 children aged under 10 suffer morbidity from COPD. In particular, over recent decades advances in perinatal care have greatly improved the survival chances of very preterm babies, principally through the reduction of acute respiratory distress syndrome (RDS) by antenatal corticosteroids and postnatal surfactant therapies [3, 4]. However, almost a third of these survivors develop chronic neonatal lung disease, so called bronchopulmonary dysplasia (BPD), a disease with the consequent long-term burdens of childhood respiratory dysfunction and neurodevelopmental delay [5]. Unfortunately, both neonatal BPD and adult COPD have an important feature in common. Neither have an effective treatment.

Accordingly, together, these childhood and adult chronic lung conditions represent a significant and growing burden of disease for which there is no targeted intervention that might restore lung function and thereby reduce morbidity and mortality. However, while the causes of childhood and adult COPD differ, the fundamental lung injury is similar—chronic inflammation, fibrosis, and scarring [6–8]—and the clinical end result—loss of functional lung tissue—is identical. As such these chronic lung diseases may be amenable to regeneration, which may be afforded by cell-based therapies. In this paper we review the recent advances in the application of placenta-derived cells as a potential therapy for human lung disease. Specifically, we will review the unique properties of amnion, the effect of amnion cells on different models of lung injury and explore the likely mechanisms of action of amnion cells in lung repair with a view to human clinical trials.
2. Unique Therapeutic Properties of Amnion Epithelial Cells

At the first international workshop on placenta-derived stem cells, convened in Brescia, Italy in 2007, two key properties of placental cells that make them attractive for regenerative medicine were highlighted: plasticity and immunomodulation [9]. Human amnion epithelial cells (hAECs) are a subset of placental-derived stem cells that display both of these key features and, perhaps, possess advantages over the other populations of stem cell-like cells in the placental tissues. First, the amnion itself is derived from the embryonic epiblast prior to gastrulation. This is important because cells derived from the epiblast prior to gastrulation are thought to retain multipotent memory or plasticity, reflecting the capability of the epiblast itself to differentiate into the ectoderm, endoderm, and mesoderm of the definitive embryo. Thus, at least theoretically, amnion cells should be capable of differentiation down each primary lineage. Over the past 6 years or so we, and others, have carefully characterized human amnion epithelial cells (hAECs), defining cell types and asking whether they share any transcriptional factors with embryonic stem cells that might confer pluripotentiality. First, cells isolated from amniotic membranes by simple digest are essentially exclusively epithelial cells [10]. These human amnion epithelial cells (hAECs) do not express mesenchymal or haematopoietic cell markers and differ from cells derived from amniotic fluid in early to midpregnancy [10–12]. This distinction between amniotic membrane-derived amnion epithelial cells, and amniotic fluid stem cells is that the former are a pure population of epithelial cells while the latter are a mixed cell population of mesenchymal, stromal, and epithelial cells. This difference is important to keep in mind when assessing possible therapeutic and regenerative medicine applications for each of these cell populations, as will be discussed later. However, while hAECs are all epithelial cells they are still a heterogeneous population of epithelial cells with diverse cell marker expression. Importantly, these cell lineage markers include early “stem cell” markers such as the POU domain, class 5, transcription factor, Nanog homeobox; SRY-2 box, the stage-specific embryonic antigen-4 (SSEA4) [10, 13]. For example, in one report 44% of hAECs expressed SSEA4, 5–15% of cells expressed Oct-3/4, and 5–15% expressed Nanong and/or Sox-2 [10]. Consistent with the expression of such early lineage markers, the differentiation repertoire of hAECs has been confirmed in vitro using various techniques (phenotypic, mRNA expression, immunocytochemical, and/or ultrastructural characteristics), demonstrating that hAECs derived from term placental membranes can be successfully differentiated into cardiomyocytic, myocytic, osteocytic, adipocytic (mesodermal), pancreatic, hepatic, lung (endodermal), neural, and astrocytic (neuroectodermal) cells [13, 14]. With regard to the lung, hAECs express thyroid transcription factor or Nkx 2.1 mRNA, one of the earliest lineage markers of the developing lung that is essential for branching lung morphogenesis and type II alveolar cell formation [14].

However, while hAECs express many markers of early stem cells, they are not omnipotent like embryonic stem cells. Indeed, there are a number of key differences between hAECs and embryonic stem cells that suggest that hAECs may be more suitable for clinical application. For example, unlike embryonic stem cells (ESCs) and human induced pluripotent stem (IPS) cell lines [15, 16], hAECs do not form teratomas when injected into the testes of mice with severe combined immunodeficiency (SCID) [13, 17] and they maintain a normal karyotype and cell cycle distribution with telomere stability over prolonged in vitro passage [10]. These observations suggest that the more limited pluripotency displayed by hAECs, compared with ESCs or IPS cells, will pose less risks for in vivo tumour formation after-transplantation than those other stem cells. Furthermore, hAECs express no, or very little, class IA and class II human leukocyte antigens (HLAs) [13, 18]. In fact, likely reflecting their functions during pregnancy, hAECs express the immunosuppressive human leukocyte antigen G (HLA-G) that confers a degree of immune privilege by suppressing NK cells, inducing apoptosis of activated CD8+ T-cells and inhibiting CD4+ T cell proliferation [18, 19]. Such findings are consistent with promoting maternal tolerance of a fetal allograft (including its membranes) for the nine months of human pregnancy. Such a property might also suggest a low risk of tissue rejection when given therapeutically. So far this indeed appears true. Following xenotransplantation hAECs can survive for prolonged periods in immune competent monkeys, rabbits, guinea pigs, rats, and swine without immunorejection, albeit without confirmed in vivo differentiation [19–22]. In one study hAECs transplanted into neonatal swine and rats resulted in human microchimerism in various organs and tissues without immune clearance [19]. Furthermore, hAECs injected into healthy human volunteers did not elicit any clinical signs of acute rejection and recipients did not produce antibodies against HLA antigens [23]. However, while undifferentiated hAECs do not express HLAs, apart from HLA-G, it would appear that as they are made to differentiate, at least in vitro, this immune privileged state may be lost. For example, recently we showed that, following differentiation into hepatic and pancreatic lineages, significant numbers of hAECs began to express Class IA, but not Class II HLA [13]. The clinical significance of this finding for future cell transplantation remains unclear but suggests that cells differentiated in vitro prior to transplantation may be less suitable for allogeneic use than primary undifferentiated cells. We will revisit this theme later.

3. Endogenous Lung Stem Cells

Before any discussion of stem-cell-mediated lung repair, it is useful to distinguish the roles and activities of exogenous stem cells, such as hAECs, from those of resident endogenous lung stem cells. While the very slow natural turnover of lung and bronchial epithelia and the multiple distinct anatomical zones of the lung have made the identification of lung stem cells difficult [24–26], a number of different resident lung stem or progenitor cells have been identified
of these cells was from a mixed population isolated from cell type that had been injured. Importantly, while the origin of the lung insult itself, di sites of injury and their plasticity to respond derived from amniotic fluid: their ability to track to specific than the alveoli—and expressed the Clara cell 10 kDa pro- bronchoalveolar junction and bronchial distribution rather the hAFSCs tra the airways. As with the hyperoxia alveolar injury model, hAFSCs were administered to mice which had undergone the type II alveolar cell product surfactant protein C. Next, migrated to the distal lung and expressed both TTF1 and hAFSCs to repair alveolar lung injury they administered the lung-type cell, as evidenced by the expression of thyroid... engraftment, albeit without confirmed di...ect repair is via immunomodulation [29] and by supporting endogenous lung stem cell activity. Indeed, while the lung contains its own population of resident endogenous stem cells, it is thought that their regenerative efforts become exhausted during severe injury, leading to both acute and chronic respiratory embarrassment. It is this feature that makes the lung a particularly receptive organ for exogenous cell therapy.

4. Amnion and Models of Lung Injury

The first report of using amnion cells for repairing lung injury was by Carraro and his colleagues [30] who used amniotic fluid stem cells (hAFSCs) obtained from amnio- censis in midpregnancy. They assessed this mixed population of cells that included hAECs, other epithelial cells, and mesenchymal cells with regard to their reparative abilities in two different murine models of lung injury. First, they demonstrated that hAFSCs could engraft into mouse embryonic lung explants in vitro and differentiate into a lung-type cell, as evidenced by the expression of thyroid transcription factor 1 (TTF1). Then, to assess the ability of hAFSCs to repair alveolar lung injury they administered the cells to mice following short-term hyperoxia. The hAFSCs migrated to the distal lung and expressed both TTF1 and the type II alveolar cell product surfactant protein C. Next, hAFSCs were administered to mice which had undergone naphthalene lung injury. Naphthalene targets Clara cells in the airways. As with the hyperoxia alveolar injury model, the hAFSCs trafficked to the sites of injury—this time in a bronchoalveolar junction and bronchial distribution rather than the alveoli—and expressed the Clara cell 10 kDa pro-tein. This first report highlighted two key properties of cells derived from amniotic fluid: their ability to track to specific sites of injury and their plasticity to respond specifically to the nature of the lung insult itself, differentiating into the cell type that had been injured. Importantly, while the origin of these cells was from a mixed population isolated from amniotic fluid, the cells had been sorted by c-kit positivity—a stem cell marker—and derived from clonal cultures to further select for stem cell-like behaviour. Therefore, it was not surprising that the cells displayed pluripotency. However, in neither of the two injury models was there evidence of amelioration of injury. This suggests that integration and differentiation in vivo are not sufficient for exogenous stem cells to effect repair. Furthermore, translating this cell therapy into clinical practice may have some limitations. The cells were derived from amniotic fluid by amniocentesis, selected by c-kit expression and then expanded and purified through clonal isolation. Such a source is not likely to be a ready source of sufficient cells for widespread application because amniocentesis is an invasive procedure that carries a risk of miscarriage. It is unlikely that women will be prepared to expose their pregnancy to such risks for the benefits of others. It is also unlikely that sufficient numbers of cells will be able to be derived for widespread application, although expansion would be feasible.

Utilising placentae from term births is one strategy to circumvent these problems entirely. Cargnoni and her coworkers [31] transplanted a mixed population of fetal membrane-derived cells from the amnion and chorioamnion from both allogeneic and xenogeneic (50% human mesenchymal cells, 50% hAECs) sources, to a murine bleomycin model of adult idiopathic pulmonary fibrosis (IPF). Importantly, unlike the amniotic fluid derived cells used by Carraro and colleagues, the cells used by Cargnoni were primary cells that had not been selected for either c-kit expression or clonal activity. Bleomycin produces many of the histological hallmarks of IPF such as intra-alveolar buds, mural incorporation of collagen, and obliteration of the alveolar space [32]. Both allogeneic and xenogeneic populations of mixed primary cells, administered either systemically or intraperitoneally, mitigated lung fibrosis to a similar extent and markedly reduced neutrophil infiltration, a key prognostic determinant of IPF. Persistence of both cell populations was detected in the lung 14 days after administration confirming engraftment, albeit without confirmed differentiation into a lung phenotype. Indeed, it is unclear if engraftment is even essential for their therapeutic effect. The same group had earlier demonstrated microchimerism in the lung at 90 days only by PCR after intraperitoneal injection of a similar xenogeneic population of cells. That finding suggests that the significance of the engraftment seen in the second study was likely to be modest [19, 31].

Extending these early reports, Moodley and colleagues [14] also used the bleomycin model of lung injury in SCID mice with the aim of examining differentiation of the cells into lung phenotypes in more detail. In this study a pure population of primary, unselected hAECs derived from term placental membranes were used. First, primary undifferentiated hAECs were cultured in small airway growth media (SAGM), known to induce differentiation of umbilical mesenchymal cells and embryonic stem cells into type II pneumocytes [33, 34], to explore whether hAECs could be directed down an alveolar epithelial phenotype lineage. After prolonged culture in SAGM, hAECs appeared to
mechanism(s) by which hAECs may work. Based on this observation, some doubt was cast on the likely engraftment of hAECs in the lung [29].

- **5. Mechanisms of Action of hAECs: Engraftment versus Immunomodulation**

It is fair to say that, at present, there remains some uncertainty about the primary mechanism(s) by which hAECs affect lung injury prevention/repair. Specifically, whether in vivo engraftment and differentiation are necessary or whether modulation of the host response to injury that then reduces inflammation and fibrosis, either directly or indirectly, is key. At this stage it would appear that the latter is the more likely. Our recent report [29] clearly demonstrated that hAECs can exert a reparative effect without the need for engraftment or differentiation and the work of Carraro and his colleagues showed that engraftment and differentiation per se was not sufficient for injury prevention/repair [30].

The apparent inconsistencies between studies regarding whether in vivo integration and differentiation of hAECs following injury actually occurs may be explained by the methods used to identify hAECs in vivo. Murphy et al. [29] chose fluorescence-activated cell sorting (FACS) with gating applied to exclude dead cells to detect only live resident human cells. Previous studies relied on PCR or in situ hybridization for human DNA or immunohistochemistry. These latter methods are all unable to discern living from dead cells [14, 31] and so it is possible that those studies were simply reporting dead cells. Of course, this does not explain the in vivo identification of surfactant protein-expressing hAECs [14]. Nonetheless, we have suggested that the primary mechanism of injury repair in their study was likely to be paracrine signaling to the surrounding tissues to reduce proinflammatory and profibrotic mediators [29]. This is consistent with previous reports of a beneficial effect of amnion where cellular differentiation has not been confirmed, such as in brain ischaemia, Parkinson’s disease, spinal cord injury, myocardial ischaemia, critical limb ischemia, burns and skin wounds [20, 35–39].

The exact identity of antiinflammatory and anti-fibrotic factors that might be released by amnion cells remains to be elucidated. However, the ophthalmology literature has described for some time the beneficial effect of amnion for corneal ulcers mediated through a reduction in HLA Class II antigen presenting cells at the site of injury, reduction in apoptosis and inflammation [40]. This literature suggests hAECs are able to inhibit the chemotactic migration of neutrophils and macrophages to the site of injury, possibly via MIF and suppression of IL-1α, IL-1β, and proteinase [41], similar to that shown by Moodley et al. [14]. Indeed, hAEC-induced suppression of macrophage and neutrophil migration into the injured lung has been a consistent finding [29, 31]. This is supported by the observation that hAECs decrease macrophage migration in vitro [42]. That hAECs modulate macrophage and/or neutrophil migration is likely to be important in the context of lung injury because both macrophages [43] and neutrophils [44] play important roles in mediating such injury. In this regard, very recently we showed that hAECs were unable to mitigate bleomycin-induced lung injury in the lung [42]. This strain of mouse is known to have deficient macrophage function.
with macrophages unable to switch from an M1 (pro-

fibrotic) phenotype to an M2 (reparative) phenotype. We

suggested that this observation was consistent with hAECs

exerting their reparative effects via macrophages rather than
directly [42].

Of course, hAECs may operate through other mech-

anisms too. For example, they express the anti-inflamma-
tory IL-1 receptor antagonist, IL-10, collagen XVIII, throm-

bospondin-1 and all four tissue inhibitors of metallopro-
teinase (TIMPs) [45]. Apoptosis of leucocytes has been

reported and hAECs express apoptosis-inducing genes Fas

L, TNF, and TRAIL [41, 46]. Furthermore, there is evidence
to support an anti-angiogenic effect through release of
endostatin, TSP-1 and TIMPs and the antibacterial protein
lactoferrin [47]. The relative contributions of these pathways

that be undi-
to amnion cell-mediated tissue repair will clearly require a

considerable amount of working through.

In relation to fibrosis, human corneal and limbal fibro-

blasts grown on the matrix surface of amniotic membranes
displayed marked down regulation of TGFβ-signalling sys-
tem with decreased expression of TGFβ-1, β-2 and β-3 iso-
forms and reduced expression of TGF-Receptor II preventing
fibroblast activation into myofibroblasts [48]. Furthermore,
amniotic membrane is also capable of reversing already
differentiated myofibroblasts back into a fibroblast pheno-
type, which may be particularly useful for ameliorating
more established disease [49]. Since TGF-β signalling plays a
central role in pulmonary fibrosis [50] the ability of hAECs to
decrease TGF-β signalling and prevent fibroblast activation
is likely to be an important effector mechanism in their
reparative properties.

6. Towards Clinical Trials

Before hAECs can be effectively translated into a future

cellular therapy for lung injury a number of questions need
resolving. It would be useful if the identity of the anti-
inflammatory and anti-fibrotic signaling pathways at play are
characterized. It may be possible that these pathways can
be manipulated pharmacologically rather than necessitate
cell delivery. In this way, the reparative abilities of hAECs
would have been used to develop new drug-based therapies
by revealing the key pathways that need targeting. However,
such an endeavour will be considerable and more immediate
therapy may be afforded by simply administering cells. If
this is the case then it will also be necessary to deter-
mine the optimum cell type for transplantation, whether
that be undifferentiated hAECs, or hAEC-derived partially
differentiated lung progenitor cells, or even a mixed cell
population. As detailed earlier, the hAECs used in the studies
to date have been unselected, primary cells—a heterogeneous
population of epithelial cells. It is possible that only a subset
of these cells are reparative and that by identifying and
purifying those cells more effective regenerative therapies
can be developed. Of course, it is also possible that it is
the heterogeneous nature of the population that is a
key attribute of the therapy, providing diverse cells to
undertake diverse roles at different stages of tissue injury-
repair-resolution. If this is so then purified subpopulations

of cells may prove to be less, rather than more, effective. It
is also unknown whether both undifferentiated and partially
differentiated cells equally effect immunomodulation. This
would be important to define before embarking upon clinical
trials. From a pragmatic perspective, regulatory authorities
are more likely to approve primary, unmanipulated cells than
cells that have undergone extensive purification, selection,
and differentiation. However, if differentiated cells are more
effective then these should form the basis of future therapies.

With regard to regulatory approval and cell handling,
it will also be necessary for future studies to adhere to
good manufacturing practice (GMP) processes suitable for
clinical use, as has already been described [10, 51], to readily
ensure standardization and clinical applicability moving
forward. Should hAECs prove useful clinically, such stand-
ardization will be a foundation of cell banks. We believe
that while autologous use of hAECs may offer a safe first
step application, for example in preterm neonates with
bronchopulmonary dysplasia [26], ultimately widespread
use of hAECs will require the development of biobanks of
high quality cells for allogeneic application, most likely in an
alpha clinic setting [52]. The clinics and cell banks will be
necessary simply because the majority of patients do not have
their own amnion cells in storage.

Finally, and possibly most importantly, all of the studies
performed to date have administered hAECs very early
during the injurious process in the lung. There has been
no comprehensive assessment of whether hAECs are able
to repair established and long-standing lung injury. This is
clearly critical because the most common clinical application
of cell therapy for lung disease will be to those individuals
with chronic, established and extensive lung injury. This is
where the current clinical burden lies and where current
therapies desperately fail.

7. Conclusion

There is no question that acute and chronic lung disease
require novel therapies. Preclinical studies have shown that
amnion cells are able to reduce fibrosis and inflamma-
tion, and thereby improve lung function. However, several
questions remain unanswered, including whether these
cells work, whether there are subpopulations of cells that
are most effective, and whether amnion cells are able to
repair established disease. We are indeed hopeful that the
immunomodulatory concert that is present at the maternal-

fetal interface during pregnancy may soon extend long after
birth to offer new therapies for sufferers of chronic lung
disease.

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