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Editorial

From fetal development and beyond: A continued role for placenta in sustaining life?

One of the most important contributors to fetal life is the placenta, a fetomaternal organ which acts as a cradle to the developing fetus, providing protection, nutrition and respiration, whilst also participating in fetomaternal tolerance, a phenomenon which is both fascinating yet still not entirely understood. For years, this important organ has been studied from scientific and clinical perspectives in order to gain a better understanding of its structure, development and functions, as well as its occasional abnormalities, which provide insight into fetal dysfunction and pregnancy complications such as miscarriage, fetal growth restriction and preeclampsia.

Intriguingly, in recent years, the placenta has also attracted wide attention as a source of stem cells for research and development of cell therapy strategies. In particular, two main characteristics of this organ have led to these studies. The first of these is the fact that placental tissues arise early in gestation, and in particular, the fetal membranes originate during the pre-gastrulation stages of embryological development, suggesting that these tissues may harbor stem/progenitor cells which display plasticity and some degree of stemness that is typical of embryonic cells. Secondly, considering the essential role of placenta in maintaining fetomaternal tolerance during pregnancy, it is conceivable that placental cells may have immunomodulatory properties which could make them useful in allo-transplantation settings.

Over recent years, mounting evidence has accumulated in support of these hypotheses. Indeed, different cell populations which harbor both properties of stem/progenitor cells, as well as immunomodulatory properties, have been isolated and characterized from both human and animal placental tissues. Advances in this field have also encouraged researches to explore the potential effects of these cells in preclinical animal models of different diseases, with the hope of being able to utilize these cells for future clinical applications. To this end, promising results have been achieved, in particular using cells isolated from the amnion. Indeed, this fetal membrane, which for many years was known for its protective role in the womb, and subsequently as a surgical dressing material, is now proving to be something of a treasure chest for placental stem/progenitor cells.

All of these considerations inspired the organization of the EMBO workshop “From fetomaternal tolerance to immunomodulatory properties of placenta-derived cells in cell therapy”, which was held in Brescia, Italy, from October 3–6, 2010, with the aim of providing an update on the properties and clinical potential of stem/progenitor cells isolated from different parts of placenta.

This meeting also marked the first official appointment of the International Placenta Stem Cell Society (IPLASS; www.iplass.net), and was conceived as a stepping stone in its ongoing Aim to achieve constant improvement in scientific awareness regarding worldwide research into placental stem cells. In particular, the founders and members of the scientific working group that originally conceived the idea to form IPLASS aim to create a network of researchers, skills and ideas to promote and foster research on all aspects related to knowledge, experimentation and clinical uses of placenta-derived stem cells, in order to achieve safe therapeutic applications using these cells as soon as possible. Therefore, IPLASS strongly encourages continuous exchange between basic, translational and clinical researchers, all with the final aim of applying placental cells for novel therapeutic purposes in the developing field of regenerative medicine. Keeping in mind the importance of interaction between the members, IPLASS promotes staff mobility through exchange programs and the organization of a scientific meeting every two years.

This issue of Placenta includes the abstracts of the workshop participants, the meeting report and some selected reviews which arose from this meeting. As this volume will testify, the meeting provided an opportunity to learn the most important new developments and insights which have been gained regarding the induction of fetomaternal tolerance, microchimerism of fetal progenitor cells in the mother, and immunology of transplantation, in order to discuss on basic mechanisms key to successful transplantation. Futhermore, we also explored the differentiation potential and immunological characteristics of cells isolated from different placental regions, and the mechanisms underlying the immunomodulatory effects of these cells. The meeting also focused on basic research into placental cells and addressed the preclinical and initial clinical studies that have been performed,

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or are currently in progress, in which placental cells or fragments of the entire amniotic membrane are utilized to repair or regenerate injured tissues in a variety of diseases, in particular those associated with degenerative processes induced by inflammatory and fibrotic mechanisms. Several broad themes emerged from the meeting, highlighting the fact that the range of potential clinical applications of placenta-derived cells is continuously widening and evolving, even if, in many cases, the mechanisms underlying the beneficial effects which can be gained remain to be better elucidated.

Therefore, the placenta should never be seen as a waste material but, in addition to claiming its important role during pregnancy, it should be regarded as a great gift from nature as a source of cells and bioactive molecules for therapeutic applications. Therefore, if we consider the placenta as "the tree of life", a representation that is often used to depict this organ, we are now beginning to see that the branches of this tree continue to extend from fetal development and beyond, and that placenta may continue to sustain our life even outside of the womb.

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Meeting report of the first conference of the International Placenta Stem Cell Society (IPLASS)

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ABSTRACT

The International Placenta Stem Cell Society (IPLASS) was founded in June 2010. Its goal is to serve as a network for advancing research and clinical applications of stem/progenitor cells isolated from human term placental tissues, including the amnio-chorionic fetal membranes and Wharton's jelly. The commitment of the Society to champion placenta as a stem cell source was realized with the inaugural meeting of IPLASS held in Brescia, Italy, in October 2010.

Officially designated as an EMBO-endorsed scientific activity, international experts in the field gathered for a 3-day meeting, which commenced with "Meet with the experts" sessions, IPLASS member and board meetings, and welcome remarks by Dr. Ornella Parolini, President of IPLASS. The evening's highlight was a keynote plenary lecture by Dr. Diana Bianchi. The subsequent scientific program consisted of morning and afternoon oral and poster presentations, followed by social events. Both provided many opportunities for intellectual exchange among the 120 multi-national participants.

This allowed a methodical and deliberate evaluation of the status of placental cells in research in regenerative and reparative medicine.

The meeting concluded with Dr. Parolini summarizing the meeting's highlights. This further prepared the fertile ground on which to build the promising potential of placental cell research. The second IPLASS meeting will take place in September 2012 in Vienna, Austria.

This meeting report summarizes the thought-provoking lectures delivered at the first meeting of IPLASS.

1. Introduction

For many years immunologists have been intrigued by the placenta due to the fact that this organ contributes to the maintenance of fetomaternal tolerance. Nevertheless, the mechanisms underlying maternal acceptance of the fetal allograft are not yet entirely understood and remain a major challenge.

In 1953 Medawar hypothesized that: i) there is a physical separation between the mother and the fetus; ii) the fetus is

Abbreviations: AM, amniotic membrane; BM, bone marrow; CLI, critical limb ischemia; DCs, dendritic cells; GVHD, graft-versus-host disease; hAECs, human amniotic epithelial cells; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-gamma; IPLASS, international placenta stem cell society; MSCs, mesenchymal stem/stromal cells; MCA, middle cerebral artery; NSC, embryonic brain-derived neural cells; PDACs®, placenta-derived cell therapy products; PLX-PAD, placenta derived adherent stromal cells; Tregs, regulatory T-cells; WJCs, Wharton's jelly mesenchymal stromal cells.

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antigenically immature, and iii) that the mother possesses an immunological inertness [1]. Since that time, research advances have led to a revision, and in some instances, a retraction of these assumptions. New data have emerged that suggest that several mechanisms may contribute to the induction of maternal-fetal tolerance [2,3]. Considering the important role played by the placenta in modulating maternal immune responses, placental cells (*e.g.* cells isolated from amnion and chorionic fetal membranes) are likely to be ideal tools for cell based therapeutic applications.

With these concepts in mind, the keynote lecture of Dr. Diana W. Bianchi, and the first two sessions of the meeting were dedicated to cutting-edge research on mechanisms that contribute to or are correlated with fetomaternal tolerance and transplant immunology. The subsequent sessions focused on the biological and immunological properties of cell populations that can be isolated from different placental regions. The meeting participants also explored the characteristics of these cells that may make them valuable candidates for therapy. They also addressed research advances with a view to potential clinical applications.

1.1. Fetomaternal tolerance and transplant immunology

1.1.1. Fetal-maternal cell trafficking

During pregnancy, transplacental trafficking of cells from the fetus to the mother leads to a persistence of fetal cells in the maternal circulation and/or tissues without evidence of graft rejection or graft versus host disease (GVHD) [4].

A pioneer in the field of fetal-maternal stem cell trafficking, Dr. Diana W. Bianchi of the Mother Infant Research Institute at Tufts Medical Center, USA, graciously delivered the keynote speech on "Fetal cells in the adult female following pregnancy: an under-appreciated source of progenitor cells." This lecture highlighted her landmark findings of fetal cell microchimerism. The Bianchi laboratory was the first to demonstrate the long-term persistence of fetal $CD34^+$ $CD38^+$ nucleated cells in maternal blood [5]. Subsequently, using fluorescence *in situ* hybridization and Y-chromosome-specific probes, her laboratory showed that fetal cells also persist and trans-differentiate in maternal organs [6]. Most notably, the demonstration of a male thyroid follicle in a surgically-removed thyroid specimen from a post-partum woman suggested that fetal cells had stem cell-like properties [7] and could repair maternal organs. Although many autoimmune diseases are associated with fetal cell microchimerism, Dr. Bianchi's presentation focused on the naturally acquired pregnancy-associated progenitor cells that may have regenerative properties [8] Using a transgenic mouse model that expresses green fluorescent protein, she described her laboratory's efforts to understand the genes, cell surface antigens, and functions expressed by fetal cells in murine maternal organs [9]. Dr. Bianchi elegantly captured the intimate fetomaternal interactions at both a cellular and molecular level. She offered insightful research directions on how to advance scientific and clinical applications of these fetal cells.

1.1.2. Fetomaternal tolerance

Several mechanisms have been proposed to explain fetomaternal tolerance and immunomodulation. Among these mechanisms, regulatory T-cells (Tregs) can suppress maternal allo-responses targeted against the fetus [10].

In his presentation entitled "Regulatory T-cells in pregnancy," Dr. Alexander G. Betz from the Medical Research Council in Cambridge, UK, and his colleagues explored the possibility of using Tregs from pregnancy for the therapy of autoimmune diseases. These cells show marked proliferation in pregnant women. They may even contribute to clinical improvement of autoimmune conditions in pregnant females. This response seems to be limited to the period of the pregnancy. Investigating the behavior of polyclonal Tregs can provide insights into potential clinical applications in autoimmune diseases.

Another mechanism involved in protecting the allogeneic fetus from maternal T-cells is the activity of the tryptophan catabolizing enzyme IDO (indoleamine 2,3 dioxygenase) [11]. Dr. Andrew L. Mellor of the Immunotherapy Center at the Medical College of Georgia, USA, addressed the potential use of IDO as an immunoregulatory drug in his speech "Indoleamine 2,3 dioxygenase (IDO): a pivotal counter-regulatory switch at sites of inflammation." IDO has been shown to have a significant role in the regulation of T-cell mediated immune responses. Importantly, fetal tissues were actively rejected by maternal T cells only when fetal tissues were allogeneic. This revealed that IDO was essential in wild-type mice to protect fetal allografts [12]. Genetic ablation of IDO did not show the same results. This suggested that other mechanisms are involved in regulating maternal T-cell mediated responses [13]. In humans and mice, some dendritic cells (DCs) express IDO in response to inflammatory stimuli, which causes T-cell suppression [14]. This effect is also seen in some pathogens that lead to immunologic attenuation and reduced pathogen specific immunity. In mice, tumor growth promoters stimulate DCs to express IDO. Genetic ablation causes enhanced anti-pathogen and tumor response, especially when coupled with immunization strategies. Dr. Mellor will further investigate the role of IDO in T-cell and immune response regulation and its potential therapeutic applications.

Mesenchymal stem/stromal cells (MSCs) are multipotent, non-hematopoietic cells, capable of differentiation toward multiple cell lineages [15]. The relationship between MSCs from the fetus or mother and their role in fetomaternal tolerance was discussed by Dr. Sicco Scherjon of the Department of Immunohematology and Bloodbank at Leiden University Medical Center, Leiden, Netherlands, in his presentation "MSCs and the possible role in fetomaternal tolerance: a paradigm for transplantation tolerance." Growth characteristics of maternal and fetal MSCs do not differ [16]. The low immunogenicity of these cells was realized after engraftment in immune-competent sheep. These properties are partially explained by the fact that MSCs do not express HLA class II and co-stimulatory molecules in vitro, Both autologous and allogeneic MSCs inhibit the mixed lymphocyte reaction [17]. Using a trans-well technique, inhibition was shown to be both by cell-to-cell contact and the production of immunosuppressive cytokines by MSCs. Dr. Scherjon viewed this to be of potential therapeutic value in preventing solid organ rejection.

1.1.3. Transplantation tolerance

A major obstacle in transplantation is GVHD [18]. Dr. Kathryn J. Wood from the University of Oxford, UK, used her talk "Translating transplantation tolerance in the clinic: where are we, where do we go?" to address the current research progress in immunological tolerance in transplantation. Her emphasis was on cutting-edge approaches that allow deletion and immunoregulation to reduce or prevent immune response to donor antigens. In particular, Dr. Wood presented her series of investigations demonstrating the unique role of interferon-gamma (IFN-γ) in the functional activity of $CD25^+CD4^+$ Tregs [19]. These discoveries open the door for new immunological tolerance-based therapies in transplantation medicine.

1.1.4. MSCs and transplantation tolerance

Finding a suitable cell source, likely with low immunogenicity and immunomodulatory properties, is an important factor for successful transplantation outcome. In her presentation, "Immunomodulation by mesenchymal stem cells and clinical experiences,"

Dr. Cecilia Götherström from the Karolinska Institute, in Stockholm, Sweden, explored the possibility of using MSCs isolated from fetal tissues. Fetal MSCs have low immunogenicity and high differentiation potential in combination with a low in vivo oncogenic risk [20,21]. These cells do not induce an immune response and differ in many ways from adult cells that derive from bone marrow (BM). The differences include a higher capacity for expansion, and a higher differentiation ability; these cells more readily differentiate into bone [22]. This is believed to be due to the more primitive nature of the cells. They have longer telomeres, higher telomerase activity and exhibition of pluripotent embryonic markers, such as Nanog and Oct-4 (octamer-binding transcription factor 4) [20]. Fetal MSCs also have potential future applications. In prenatal transplantation for type III osteogenesis imperfecta, allogeneic fetal MSCs migrated from the intravascular space and demonstrated site-specific differentiation in bone and long-term persistence in an immunocompetent recipient across major histo-incompatibility barriers [23].

1.2. Recent pre-clinical and clinical studies using placenta-derived cells

1.2.1. Rationale and advances in pre-clinical studies

Human amniotic membrane (AM) has a long history of clinical utility. Experimental and clinical studies have demonstrated that AM transplantation promotes re-epithelialization, decreases inflammation and fibrosis, and modulates angiogenesis [24]. The applications of AM in surgery include treatment of skin wounds, burn injuries, chronic leg ulcers, head and neck surgery, and prevention of tissue adhesion in surgical procedures [24]. These ongoing applications are also enriched by studies aimed at expanding the use of AM-based therapy for other pathological conditions [25].

The use of the entire AM, was addressed by Dr. Susanne Wolbank from the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, AUVA Research Center in Vienna, Austria. In her presentation, "Suitability of amniotic membrane and cells thereof for tissue regeneration approaches", Dr. Wolbank discussed her evaluation of the differentiation potential of the entire AM *in toto* [26]. In vitro culture of AM under conditions that induce osteogenesis was shown by immunohistochemistry to result in mineralization and osteopontin expression by its sessile cells, coupled with a significant rise in calcium content and mRNA expression of multiple bone specific proteins. Taking into account these positive results, Dr. Wolbank concluded that stem cells within human AM can successfully differentiate along the osteogenic pathway, a finding she believes may enhance or replace current bone tissue engineering protocols.

The talk from Dr. Ornella Parolini from the Centro di Ricerca E. Menni, Fondazione Poliambulanza – Istituto Ospedaliero in Brescia, Italy, entitled, "Placenta generalities: structure and immunomodulatory properties- in vitro and in vivo studies", initiated an in-depth discussion regarding the structure of the placenta, and the biological and immunomodulatory properties of the different cell populations that can be isolated from placental tissues [2,3,25]. She addressed the use of placental cells for the treatment of different pathological conditions, mainly for those involving inflammatory and fibrotic mechanisms. In previous in vitro studies, Parolini's team demonstrated that AM-derived cells do not induce a T-cell response, actively suppress T-cell mediated immunity, and block differentiation and maturation of monocytes [27–29]. By in vivo studies, this group also showed that amniotic and chorionic cells can successfully engraft long-term in newborn swine and rat models. This indicates active tolerance of these cells [27]. After both allogeneic and xenogenic transplantation into mice with bleomycin-induced lung injury, fetal membrane-derived cells reduced lung fibrosis, despite the rare presence of donor cells in host lungs [30]. Successful outcomes were also obtained when fragments of the entire AM were applied as patches to treat rats with cardiac ischemia and rats with liver fibrosis induced by bile duct ligation [31,32]. In all of these applications, the beneficial effects observed seemed most likely related to bioactive molecules produced by placenta-derived cells that act by paracrine actions to promote the repair of host tissues.

It is therefore evident that the AM is an attractive, high-throughput source of stem cells, with features that encompass broad differentiation potential, important immunomodulatory properties and paracrine activities [25].

These concepts were reinforced by Dr. Ursula Manuelpillai of the Monash Institute of Medical Research, Monash University, Victoria, Australia, in her presentation entitled "Human amniotic epithelial cells (hAECs): a cellular therapy for inflammatory diseases?". Dr. Manuelpillai highlighted her group's recent findings in experimental mouse models of lung and liver fibrosis using bleomycin and carbon tetrachloride (CCl_4) treatments, respectively. In the lungs of bleomycin-injured mice, a small percentage of injected hAECs persisted for longer periods compared to Wharton's jelly-derived MSCs. These cells produced surfactant proteins A-D following transplantation. This suggests hAECs differentiate into type II alveolar epithelium [33,34]. Overt immune responses to the xenotransplanted cells were not evident. Mice with lung and liver injuries that were treated with hAECs showed reduced apoptosis, inflammation, and fibrosis [34,35]. Dr. Manuelpillai believes that immunosuppressive mediators from the hAECs could modulate the activity of T-cells, DCs, and natural killer cells. Decreased fibrosis may be due to a reduction in pro-fibrotic cytokines, and induction of collagen degrading matrix metalloproteinases in the injured lungs and livers. hAEC treatment may also inhibit monocyte recruitment. In vitro studies showed that treatment with hAEC-conditioned medium did not stimulate proliferation of collagen depositing hepatic stellate cells, but enhanced apoptosis and altered cytokines secreted by these cells. She concluded that hAEC transplantation may be useful for targeting tissue inflammation, and in some instances, may also contribute to cell replacement.

Dr. Francesco Alviano and his colleagues of the Department of Histology, Embryology and Applied Biology at the University of Bologna, Italy, explored the use of AM-derived stem cells in treatment of diabetes mellitus. His speech was entitled "Amniotic membrane-derived stem cells and pancreatic islet-cell differentiation." Dr. Alviano chose the AM as the source for these cells due to the ability of hAECs to express beta cell-markers, as well as the angiogenic and immunomodulatory properties of the AM-MSCs [36,37]. He compared the in vitro pancreatic differentiation of hAECs to that of human derived pancreatic cells. His group confirmed the pancreatic differentiation ability of hAECs. These cells exhibited increased glucagon and insulin expression. Preliminary studies in vivo using streptozotocin-diabetic rats showed that rats treated with hAECs and pancreatic-MSCs underwent a partial and transient correction of the altered phenotype.

Dr. Mark L. Weiss and colleagues in the Department of Anatomy and Physiology at Kansas State University, USA have been investigating the hypothesis that Wharton's jelly-derived MSCs may have clinical application in GVHD. In his presentation "Wharton's jelly mesenchymal stromal cells (WJCs) as immunoregulators in allogeneic transplantation," Dr. Weiss outlined the status of current clinical trials that have used MSCs for treating or preventing GVHD. Dr. Weiss then outlined work that revealed plasticity in the immune properties of MSCs in response to cytokines such as INFγ (which has been called licensing of the MSCs), and suggested how this may apply in clinical treatment of GVHD. WJCs are MSCs that are obtained easily, safely and pain-free from donors of a consistent

age, in contrast to BM derived- or adipose derived- MSCs that involve painful and invasive collection methods [38]. At first glance, WJCs and BM-derived MSCs have similar immune modulation properties and do not stimulate immune cell proliferation. Recent literature has revealed subtle differences in the in vitro immune modulation properties of WJCs with respect to MSCs derived from adipose tissue or from BM [39–42]. This literature suggests that WJCs and adipose-derived MSCs may be superior to BM-derived MSCs as therapy for GVHD. In conclusion, Dr. Weiss suggested that: licensed MSCs have superior therapeutic effects in animal models of GVHD. This should be considered when designing future clinical trials for GVHD.

Dr. Peter Ponsaerts's laboratory team in *Experimental Hematology at the University of Antwerp, in Belgium, has* previously studied the use of various autologous and allogeneic stem cell populations in animal models of neurotrauma, including spinal cord injury and experimental autoimmune encephalomyelitis. In course of their studies, they did not (yet) observe any significant results to indicate potential in vivo benefits of stem cell transplantation for neurological diseases [43]. In his presentation entitled "Physiological comparison of autologous and allogeneic cell implantation in the central nervous system: defining and regulating immune cell activity against mesenchymal and neural stem cell grafts", Dr. Ponsaerts further focused on optimizing the therapeutic procedures undertaken. Through culture and imaging studies, his group determined survival, differentiation, and immunogenicity of autologous and allogeneic cellular implants in the central nervous system of immunocompetent mice. These studies used murine BM-derived MSCs and embryonic brain-derived neural cells (NSC). While autologous transplantation of MSCs resulted in graft survival for at least four weeks, extensive microglial infiltration and astrocytic scar formation was observed [44]. In contrast, allogeneic transplantation of MSCs resulted in graft rejection two weeks post-transplantation [45,46]. Further autologous transplantation of NSC resulted in two week graft survival, followed by a progressive decrease in survival and increased infiltration by glial and astrocytic scar tissue [43]. Given the low survival percentage (less than 2%) of grafted autologous and allogeneic cells at week 2 post-grafting, the direct contribution of NSC to regeneration can be questioned. Potential benefits should be determined from the secreted factors and/or the reaction of endogenous stem cells towards the grafted cells. Interestingly, from this meeting, it appears that – in case of the use of placenta-derived cells – it seems to be easier to treat diseased mice using human cells instead of autologous or allogeneic cells. During the discussion of his presentation, it was therefore suggested that future experiments in regenerative medicine should use both human and autologous mouse or rat cells to demonstrate proof-of-principle, to compare results, and to understand the relative therapeutic efficacies.

To address whether placenta stem cell-based therapy may offer a neuro-restorative treatment, in the presentation, "Cell therapy for stroke: towards clinical application of Celgene human placenta-derived cells," Dr. Cesar V. Borlongan of the Department of Neurosurgery and Brain Repair at the University of South Florida College of Medicine, USA, in collaboration with Celgene Cellular Therapeutics (New Jersey, USA), explored the safety and efficacy of human placenta-derived cell therapy products (PDACs®) in adult rat models of stroke [47,48]. His work is based on an intravenous transplantation model that occurs two days after transient occlusion of the middle cerebral artery (MCA). Results showed significant improvement in behavioral and neurological recovery from stroke. The response was seen to be dose dependent. Similar results were seen in cases of permanent MCA ligation. Significant improvement was seen with positive graft survival up to six months post-transplantation. It was also shown, due to absence of human specific vimentin staining, that graft survival was not essential for functional recovery in PDACs® transplanted stroke animals. Dr. Borlongan and his collaborators at Celgene Cellular Therapeutics also confirmed the safety of the intravenous cell transplants as evidenced by the lack of tumor or ectopic tissue formation in all PDACs® transplanted stroke models.

1.3. Clinical applications

In the presentation, "Placenta derived adherent stromal cells for the treatment of critical limb ischemia (CLI) - Lessons from first clinical trial," Dr. Racheli Ofir from Pluristem Therapeutics Ltd. (Haifa, Israel), presented data derived from phase I clinical trials performed in parallel in the US and in Europe supporting the angiogenic and anti-inflammatory properties of placenta derived adherent stromal cells, indicated as PLX-PAD. These cells are derived from the human decidua and are expanded using Pluristem's 3D proprietary technology [49]. Based on the accumulated in vitro and in vivo data, it was suggested that the anti-inflammatory and angiogenic properties of the PLX-PAD are mediated largely through paracrine effects. Pluristem has two ongoing phase I trials for the allogeneic use of these cells in critical limb ischemia (CLI) in patients who have exhausted all current therapies. The three-month follow-up data, including 21 patients afflicted with CLI, was presented. No immunologic reactions or other adverse effects related to the investigational product were observed in both trials, suggesting a promising safety profile. Furthermore, statistically significantly positive efficacy data was obtained using hemodynamic parameters, Ankle-Brachial Index, Toe-Brachial Index and Transcutaneous Oxygen Tension, as well as Quality of Life, pain and wound healing in patients treated in the trial. The data derived from these clinical trials suggests that allogeneic PLX-PAD administration to humans is safe and effective. This paves the way for further clinical studies.

2. Conclusions

In summary, the first meeting of IPLASS was a celebration of novel conceptual and technical ideas directed towards advancing placenta stem cell research at multiple levels, from basic and translational to clinical investigation. Meanwhile, this meeting was also an occasion to point out the many unanswered issues on placental cells. These issues include the need for better definition of these cells in terms of their precise location in the placental tissues, their phenotype and stem cell potential, as well as the need to verify whether placental cells are better than those isolated from other sources in terms of therapeutic applicability. In this regard, only comparative studies using in parallel cells isolated from different sources in the same pre-clinical models will address this issue. The advantages of placenta with respect to other sources include the wide availability of discarded material, the possibility of banking placental cells and preparing "off-the-shelf" placenta-derived products (*e.g.* cells, fragment of AM).

However, the mechanisms whereby placental cell-based treatments exert therapeutic effects still remain poorly elucidated. In particular, it is increasingly accepted that improvement in tissue function observed after placental cell-based therapies are most likely due to paracrine action of these cells at the site of injury, rather than on their tissue-specific differentiation (*i.e.* regeneration of host tissues). The relative contributions of each of these two mechanisms, repair versus regeneration, as well as of the molecular pathways involved, remain to be determined.

Notably, seamless collaborative efforts between academic and industry-based scientists and regulatory authorities were apparent in most of the presentations. Altogether, this meeting achieved the IPLASS's goal of establishing a solid foundation for a "no-barrier" multi-disciplinary, multi-institutional, and multi-national scientific endeavor on which to build the future of placenta stem cell research and therapeutic applications.

Conflict of interest

CVB is the Vice President-Elect of IPLASS, receives research funds from Celgene Cellular Therapeutics, and has patent applications relating to placenta cells. OP is the elected IPLASS President and has patent applications related to placental cells.

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Fetus specific T cell modulation during fertilization, implantation and pregnancy

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ABSTRACT

Recently there is an increasing interest in aspects of a more specific immunoregulation during pregnancy. Understanding these mechanism might have a broader application not only for reproductive immunology but also in general for biology and medicine. Especially the induction, already before conception, of feto-specific T cells with a possibly regulatory function gives a biological explanation of local immunotolerance at the maternal fetal interface, supporting the epidemiological evidence of a feto/paternal-specific immuneregulation. Understanding the expression of specific HLA-classes on trophoblast and the crosstalk of these antigens with various cell types, specifically modulated in the decidua, resulting in the secretion of cytokines and (angiogenic) chemokines has given us a more and more detailed understanding of this regulation. This regulation could be induced by fetal cells circulating in the mother (microchimerism) and from the interaction with fetal subcellular fractions as exosomes, but also from paternal antigens present in seminal fluid. Molecular interaction between paternal and fetal antigens and receptors in endometrium and the decidua are discussed.

This review highlights besides uNK cells, especially the function of $CD4^+$ and $CD8^+$ T cells with a regulatory function in the context of recurrent miscarriage and pre-eclampsia. Besides HLA, also male-specific minor histocompatibility antigens and the genetic background for these pregnancy complications are discussed.

1. Introduction

The uncomplicated acceptance by the mother of her immunologically foreign, semi-allogeneic fetus, is one of the enigmas of human reproduction. The immunology of pregnancy is not only intriguing but also an interesting model for transplantation. The understanding of immunological mechanism involved in tolerance induction during pregnancy might help to design future strategies for the prevention of graft rejection and complications associated with the need of long-term immunosuppression.

Recent experimental evidence support epidemiological data [1] that already before implantation fetus specific tolerance is induced for paternally derived antigens. The importance of T cells with a regulatory function, locally active in the decidual lining of the uterus, is only recently appreciated [2,3]. The role of immunomodulatory T cells in reproductive biology is of central importance also for the understanding of immunological memory, a well appreciated phenomenon in human reproduction, however, far from completely understood. The expression of paternal antigens on invading fetal trophoblast and their interaction with not only uterine NK cells but also with T cells, might not only be responsible for both non-specific- and specific-suppression of the anti-fetal cytotoxic T cell responses, but also for long-term immunological memory. Also the possible role of macrophages, being part of this immunological network in the uterus during (human) pregnancy, in these immunomodulatory processes is not yet fully appreciated [4]. Studies on the actual presence and phenotypes of these T cells and their function in relation to genetic differences (polymorphisms) between mother and her child will help to understand the immune mechanisms involved in fetal-maternal immunoregulation.

2. Human leuckocyte antigens (HLA), immune modulation and angiogenesis

The key mechanism for the escape of maternal rejection and for the induction of tolerance lies in the aberrant expression of HLA molecules by trophoblast cells. Extravillous trophoblast (EVT) cells, migrating into the decidua, express an unusual

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combination of classical polymorphic HLA-C and the non-classical, monomorphic HLA-E, -F and -G. Already in the early 1980s it was shown that EVT cells are positive for W6/32, an antibody common for the HLA-A, -B and -C antigen, although it was not realized – as it later turned out – that it was only HLA-C which is expressed on EVT [5,6]. Villous syncytiotrophoblast does not express HLA class I and – as EVT cells – HLA class II. Limited information is available on the expression of soluble class I MHC antigens during pregnancy with the exception of s-HLA-G [7]. Soluble HLA-G5 (sG1) and soluble HLA-G6 (sG2) induce apoptosis of activated T cells, especially $CD8^+$ T cells [8,9]. HLA-DR expression in villous stroma, amnion and in the decidua is restricted to cells with a macrophage morphology [10] and does not include trophoblast cells. At the fetal-maternal interface the invading trophoblast encounters a wide range of maternal tissue leukocytes: including specific uterine NK cells (uNK), macrophages, dendritic cells and T cells. HLA molecules are able to interact with specific receptors on these leukocytes, stimulating the production of several cytokines (i.e. TGF-β), chemokines and angiogenic factors, such as VEGF, PlGF, IL-8, angiopoietins (Ang1 and 2) [11]. By these mechanism the maternal immune response is modulated in a characteristic way, promoting trophoblast invasion which is essential for normal implantation and for changes in blood vessel development (e.q. the spiral arteries). Invasion of trophoblast is a highly delicate balance. A deep trophoblast invasion is associated with a placenta increta; an inadequate trophoblast invasion and insufficient remodeling of the spiral arteries is seen in pre-eclampsia (PE), one of the most severe complications of pregnancy. Pathogenesis of complications of pregnancy such as recurrent spontaneous abortions (RSA) – not in the context of chromosomal abnormalities –, implantation failure or intrauterine growth restriction (IUGR) is shared with pre-eclampsia, thought to be all resulting from abnormal trophoblast invasion. As one of the possible common immunological pathways, the effects of interaction between maternal KIR receptors and fetal HLA-C polymorphisms is well established. Inhibition of maternal uNK cell function will prevent a proper invasion of trophoblast into the decidua. As we recently demonstrated the presence of KIR receptors on T cells, especially on decidual tissue derived T cells, this mechanism might also be functional in T cell regulation [12].

2.1. HLA sharing

HLA sharing between partners will lead to HLA homozygosity in the fetus and as a result less HLA incompatibility with the mother. This can affect the maternal immune response already at the time of mating and conception as well as during implantation and throughout pregnancy. HLA compatibility is thought to be more prevalent in women with implantation failure. This might partly explain RSA and PE. However also genetic explanations are suggested, whereby homozygosity of lethal recessive genes, in linkage disequilibrium with specific HLA haplotypes, might be implied [13]. Genes coding for some of the molecules involved in the immune response in human, such as TNF-α, are located within the HLA complex on chromosome 6. As a consequence HLA haplotypes could be just indicator genes for linked genes coding for cytokines such as TNF-α.

An association between fetal-maternal HLA compatibility, decreased anti-paternal antibodies levels, specific HLA alleles, or combinations of KIR receptors in the mother and HLA-C alleles in the fetus and inadequate trophoblast invasion suggest that these are risk factors associated with implantation failure and pregnancy complications.

2.2. Microchimerism

Epidemiological studies have demonstrated that rheumatoid arthritis patients tend to show clinical improvements during pregnancy, especially if the level of class II matches between mother and child is higher [14]. This suggests a role for paternal antigens in the modulation of the maternal immune response during pregnancy. These antigens must be derived from fetal cells trafficking into the mother during pregnancy, which will persist life long [15]. Even fully HLA mismatched, allogeneic fetal cells entering during donor egg pregnancies persist for many years after delivery in the circulation of healthy women, suggesting an immunomodulatory mechanism preventing the deletion of these cells from the host [16]. The persistence of a small population of foreign cells in another individual, microchimerism, is well recognized both after organ transplantation [17] and after pregnancy, whereby fetal microchimerism during pregnancy is a natural consequence of pregnancy. There is a potential important role of feto-maternal cell trafficking, fetal microchimerism or placental exosomes or microparticles and (the level of) anti-paternal antibodies in the induction of host tolerance (in the mother) to her pregnancy (graft tissue) [17–20]. The immunogenicity of these paternal HLA antigens is dependent on the HLA phenotype of the mother: on average 15–30% of women who have been pregnant produce antibodies but certain combinations of fetal and maternal HLA generate significantly more antibodies [21]. This sensitization is associated with the generation of cytotoxic T cells specific against paternal antigens [22]. Successful pregnancy could be characterized by the development in the mother of some degree of leukocyte chimerism-dependent, donor specific tolerance. Central tolerance induction via positive and negative selection of chimeric T cells in the thymus could modify the generation of specific T cell clones and could be important in controlling the immune response. In the mouse model is shown that maternal peripheral T cells become tolerant to specific paternal antigens. However, this immunological tolerance is transient, and the situation is reversed directly after delivery [23]. As after delivery pregnancy induced immunomodulation is lost, these activated fetal cells in maternal tissue might trigger the later development auto-immune disease in the mother such as auto-immune thyroid disease and systemic sclerosis.

2.3. T cells

Studies in the mouse model gave strong arguments that fetal/placental antigen presentation and recognition of fetal antigens by $CD4^+$ T cells is exclusively by indirect recognition. This is not surprising, as the placenta has no MHC class II expression, making the direct antigen presentation via $CD4^+$ cells impossible. In human pregnancy a HLA-C mismatch leads to an increase in $CD4^+CD25^{dim}$ activated T cells and the presence of functional $CD4^+CD25^{bright}$ regulatory T cells [24]; in HLA-C *matched* pregnancies no such proliferation is found. As $CD4^+$ cells are involved, this supports that also in human pregnancy (peptides processed from) HLA-C alleles are recognized *indirectly* and presented in the context of self MHC class II on maternal APCs. HLA-C, which is highly polymorphic, can elicit (in- and outside) pregnancy allogeneic T cell responses; one of the mechanism explaining tolerance and absence of rejection of the fetal allograft in uncomplicated pregnancies. In the decidua $CD8^+$ cells is the most abundant T cell type [25]. However, $CD8^+$ cells are MHC class I restricted and may directly recognize intact HLA-C on allogeneic fetal cells. HLA-C specific receptors have been demonstrated on decidual T cells [12], most possibly affecting their cytoxic potential. Potential other targets for $CD8^+$ cells are HLA-E and minor histocompatibility antigens. Also the decidual $CD8^+CD28^-$ subset includes besides activated effector and effector-memory

cells also T suppressor cells, while unprimed, naïve $CD8^+$ T cells in the decidua are almost absent [26]. In decidual $CD8^+CD28^-$ a substantial reduced expression of the cytolytic molecules perforin and granzyme B were demonstrated precluding cytotoxic responses of these cells in human decidua [26,27]. $CD8^+CD28^-CD103^+$ which are present in decidual isolates have a potential regulatory function can be induced *directly* by fetal trophoblast [27].

Besides T cells, several other immunocompetent cells – interacting with T cells – are active in human decidual tissue. In early decidua around 40% of the stromal cells are leukocytes, of which 45–70% are NK cells, 30% are macrophages and fewer than 20–30% are $CD3^+$ T lymphocytes [28]. Although through pregnancy the $CD3^+$ fraction remains stable, the largest immune cell population in the 3rd trimester is $CD3^+$, because the number of NK cells is decreasing [28].

T-helper cells (T_H) can be classified by the cytokines they are producing as T_H1 cells, involved in cellular immunity and cells involved in humoral immunity, the T_H2 cells. More recently T regulatory cells (Tregs) and T_H17 subsets were described. Wegman et al. were the first who suggested that in normal pregnant mice, pregnancy is characterized by a dominance of the T_H2 over the T_H1 type immune response [29]. In human endometrial samples taken peri-implantation a T_H2 dominance was found; however no local T_H2 dominance could be demonstrated in term decidual samples acquired before or after labor. Because of this and although there is evidence that, because of this T_H2 dominance, e.g. infectious defends mechanism in peripheral blood of normal pregnant women is reduced, the concept is debated. A T_H2 dominance shift is probably less extreme or could even not be found in women with complicated pregnancies [30]. Using lymphocyte function markers in – unstimulated – peripheral blood samples, no change in pre-eclampsia patients in the T_H1/T_H2 ratio could be demonstrated, although there was an increase in T_H2 cells in normal pregnancies.

2.4. Regulatory T cells

Regulatory T cells are cells with immune suppressive properties of which at least four different subsets have been described: Tr1 (type I regulatory T cells), T_H3 (T-helper 3) cells, the natural arising regulatory T cells (Tregs), and $CD8^+$ cells with regulatory properties. Non-specific circumstances, such as sub-optimal antigen presentation and hormonal factors (elevated estrogen levels around ovulation and a specific hormonal milieu e.g. high progesterone levels), together with paternal alloantigens [31], result in expansion of these regulatory cells [32].

Tregs are essential for natural self-tolerance and characterized by the surface expression of CD4 and CD25 (IL-2R α-chain). In addition to a high expression of CD25 they express cytotoxic T lymphocyte antigen (CTLA-4 = CD152) and other surface markers such as GITR, OX40, CD62, CD38, CD122, CD132. Intracellularly the transcription factor Foxp3 is expressed. The important role of especially Tregs in the development of maternal tolerance is shown both in animal models and in human tissues; the role of the other 3 subsets is probably more redundant or has further to be elucidated [32].

It is most likely that the Tregs also in humans are induced in peripheral lymphoid tissues and not in the thymus and that e.g. fetal antigens can drive the expansion of the Treg pool. Foxp3 expression is critical for their development and for their regulatory function [33]. A mutation in the Foxp3 gene results in mice in the loss of regulatory function. It was shown in early human pregnancy decidual tissue, that these Tregs also have a high expression of CTLA-4. They mediated – most probably via cell–cell contact – T cell inhibition in a dose dependent manner, most probably via membrane bound TGF-β and IL-10, but possibly by other regulatory candidate binding molecules such as Lag-3 and galectin-1. IDO expression has been shown in many studies to be of importance in tolerance induction, although this role is also disputed [34]. CTLA-4 expression by Tregs might play a role in competing with the co-stimulatory molecule CD28 via binding B7-1 (CD80) and B7-2 (CD86) and in the upregulation of IDO expression by dendritic cells and macrophages.

In humans the subset of $CD4^+CD25^{bright}$ has a regulatory potential as shown by the suppression of proliferation of $CD4^+$ cells in vitro [3].

2.5. Semen

The induction of pre-conception, pre-implantation T cell mediated immune modulation via the expansion of paternal specific $CD4^+$ and $CD8^+$ cells by paternal antigens present in seminal fluid is demonstrated in the mouse model [35]. Surprisingly there is only suppression after intravaginal immunization, while subcutaneous immunization remains ineffective. Semen contains both MHC class Ia, Ib and class II, possibly expressed on seminal leukocytes or cellular fractions (exosomes). In combination with immunomodulatory factors such as TGF-β or PGE_2 in the seminal fluid, paternal specific immunomodulation is induced. Paternal antigens can be presented directly via paternal APCs in semen or indirectly via maternal APC to maternal T cells. As no $Foxp3^+$ cells were present in the vagina mucus these APC probably travel to peripheral lymph node where Tregs are generated. Naïve $CD4^+CD25^-$ T cells become Tregs, when they are stimulated in the presence of TGF-β or PGE_2. Estrogen leads to increased production of TGF-β, which especially during the secretory phase, modulates anti-paternal T cells responses [36] (Fig. 1). In mice Tregs are already found in draining lymph nodes within two days after mating [37], combined with an upregulation of Foxp3 expressing cells and Foxp3 mRNA in the uterus. Interestingly, seminal fluid is able to inhibit rejection of tumor cells with a same MHC background [38].

3. Endometrium and decidua

3.1. Preimplantation changes in endometrium

As Tregs are expanded already before implantation, specific T cell receptors important for pregnancy regulation, like immunoglobulin-like transcript–2 (ILT-2), killer cell immunoglobulin-like receptors (KIR) and receptors for e.g. HLA-G (NKG receptors) should be triggered by circulating molecules around conception. These receptors are found on T cells [12] and soluble class I MHC (sMHC-I), found in serum and semen of healthy individuals, can trigger these receptors. Tregs express the chemokine receptor CCR4 and CCR8 [39] and CCR5, which are of importance for trafficking of Tregs into the decidual tissue. Uterine endometrium does express the chemotactic factor CCL4 which attracts Tregs via its ligand CCR5. CCL4 is increasing during the estrus mouse cycle and there is a direct association between CCL4 levels and the expression of Foxp3 in the uterus, which is explaining by a selective attraction of $CCR5^+$ Tregs in the endometrium (Fig. 2a). CCL17 which is highly expressed in the uterus is important for the interaction with macrophages, which then produce the ligand CCL22, of importance for the homing of Tregs into the decidua [40]. Local production of other chemokines such as CXCL9, CXCL10 (and CCL4) trigger the influx of uNK cells; CXCL16 is a factor attracting monocytes and T cells into the endometrium/decidua [41]. CXCR6 -which is the sole receptor for CXCL16- is expressed by T cells and monocytes. Tregs accumulate periodically in the endometrium and peak during estrus [31]. In human

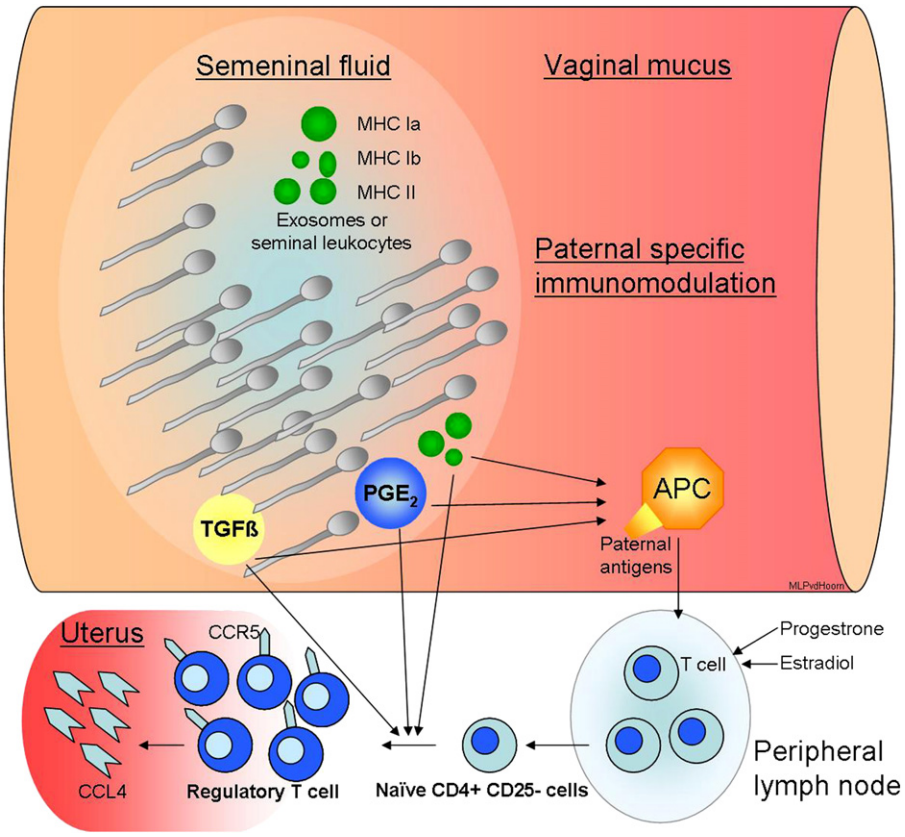

Fig. 1. Schematic representation of factors influencing the induction of Tolerogenic T cells during mating and pregnancy.

endometrium it is confirmed that $CD4^+CD25^+$ and $FOXP3^+$ Tregs increase already in the late follicular phase of the menstrual cycle, possibly related to serum estradiol levels followed by a substantial decrease of Tregs in the luteal phase [42].

3.2. Postimplantation changes in the decidua

Increases of the $CD4^+CD25^+$ subsets in early human decidua pregnancy [43], with a further increase in the second [44,45] and third trimester [45] (Fig. 2c) were described by several groups [46]. They gradually return to lower levels postpartum [47]. No such increases are found in the systemic circulation as Tregs in non-pregnant individuals and pregnant controls are the same and significantly lower than percentage found in decidual tissue (Fig. 2b).

4. Immunology and pregnancy complications

4.1. Miscarriage

RSA is affecting 1% of couples attempting pregnancy. Meta-analysis does not show that sharing between couples of any specific HLA allele is a significant or important risk factor for RSA [13]. An increased risk of sharing is suggested for HLA-A (OR 1.4; 95% CI 0.95–2.05) and for HLA-DR, sharing of least one allele is associated with an increased risk (OR 1.3; 95% CI 1.01–1.75). Carriage of HLA-DR1 or HLA-DR2 in combination with HLA-G*010102 allele is associated with an increased risk (OR 4) for RSA [48]. This association possibly reflects a genetic interaction with other genes, e.g. for cytokines, MBL2 [49] or thrombotic disease, in the same genetic region. Also studies of HLA-G sharing and of

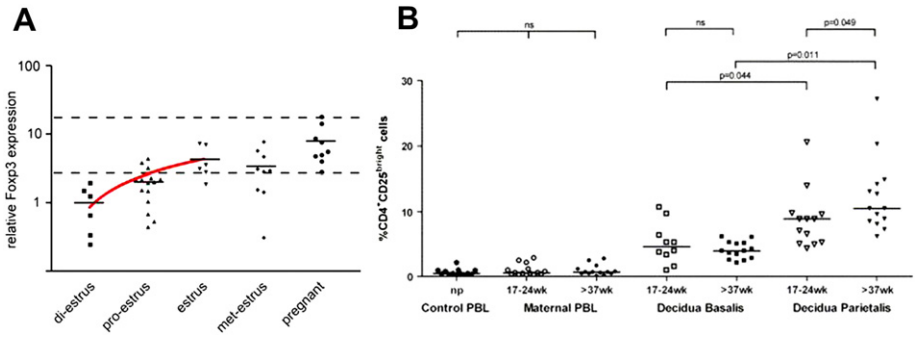

Fig. 2. Changes in endometrial-decidual contribution of cells with a regular phenotype during (Fig. 2a) menstrual cycle and pregnancy in mice (from Ref. [31]) and in human pregnancy (Fig. 2b) compared to peripheral samples from non-pregnant and pregnant control samples (from Ref. [45]).

frequency of HLA-G alleles do not support HLA-G histocompatibility as a factor for RSA. In women having an abortion after IVF treatment lower sHLA class I and class II levels compared to control intact pregnancies were found [50], whereby a T_H2 cytokine response is associated with increased sHLA class II levels. Maternal SNP variations in the promoter of HLA-G might influence HLA-G expression, associated with RSA [51]. Immunohistochemically no differences in HLA-G or HLA-E expression was demonstrated between tissue samples from RSA patients and controls [52]. Both in RSA couples and PE parents the frequency of HLA-C2 in the fetus is increased while the KIR AA (inhibiting) genotype frequencies in the women is also increased [53,54]. Activating KIR genotypes for HLA-C2 such as KIR2DS1 are reduced (OR 2.63 95% CI 1.54–4.49) [54]. Also having a male first born might induce a maternal immune response against the male-specific minor histocompatibility (HY) antigens associated with a negative effect on pregnancy success. This is found only in secondary RSA women carrying HY-restricting HLA class II alleles (OR 0.35; 95% CI 0.2–0.7) [55,56]. This suggests a possible role for $CD4^+$ T cells in providing help for $CD8^+$ cytotoxic T cells against minor H antigens.

Although possibly harmful during mid- and late- gestation, the inflammatory response associated with small amounts of T_H1 cytokines might be of advantage during implantation. Gonadotrophins induce cytokines such as TNF-α and IFN-γ, which are both also produced by the blastocyst. IL-10 production, which is known to reduce spontaneous abortion in the CBA x DBA/a abortion model, is only increased after implantation and is produced by the trophectoderm and the inner cell mass.

Tregs are needed for a successful allogeneic pregnancy in mice. These Tregs expand during pregnancy, induced by paternal antigens, and prevent rejection of the fetus [37]. In an abortion prone mouse model decidual tissue was shown to contain IFN-γ producing T cells (T_H1 cells), specific for paternal alloantigen. Their presence is possibly related to an insufficient induction of Tregs during pregnancy. Tregs from normal pregnant mice could inhibit proliferation [57] and IFN-γ secretion and other T_H1 cytokines such as IL-2 [39,58] by these T_H1 cells. Transfer of Tregs was shown to prevent fetal resorption in this mice model [34,57] in an alloantigen specific manner, most probably by enhancing T_H2 cytokine production (IL-4 and IL-10) [58]. This specific finding was not supported by other studies, where especially the induction of TGF-β, neurophilin-1 [34,59], leukemia inhibiting factor and heme-oxygenase-1 [34], created a tolerant microenvironment, while the levels of IDO remained unchanged. In this mouse model the fetus seems also to be protected via linked immunosuppression for minor antigens [59] Fig. 2.

In RSA patients, both in peripheral blood samples and in the decidua reduced levels of $CD4^+CD25^+$ Tregs were found [60]. Both in peripheral blood and in the decidua the proportion of Tregs were found to be lower in RSA patients and in non-pregnant controls compared to normal pregnant controls [43,61].

RSA patients have substantial lower percentage of Tregs in the systemic circulation [42] and in the decidua [39], comparable to postmenopausal levels, both in the follicular phase and in the luteal phase. Also the functional capacity of these suppressor cells is lower [42]. The ratio of $CTLA4^+/CD28^+$ in peripheral blood and in the decidua is significantly lower in miscarriage compared to controls and is higher in Tregs ($CD4^+CD25^{++}$) suggesting that Tregs support pregnancy via upregulation of CTLA-4 [39].

4.2. Pre-eclampsia

Epidemiological studies strongly suggested an immunological basis for PE [62]. Changing partner, artificial donor insemination and oocyte donation all increase the risk of hypertensive disorders in pregnancy, while there is a protective effect of prolonged period of semen exposure. It is now evident that interaction between HLA-C ligands on fetal trophoblast cells and KIR receptors on maternal cells is of crucial importance for remodelling of spiral arteries and immune modulation. Evidence for a common genetic etiology contributing to the pathogenesis of PE, suggesting that recessive mutations may play a role, is supported by the finding of the higher incidence in women related to the patient compared to controls [63]. It remains unclear if any specific HLA allele, haplotype or gene in linkage disequilibrium with the HLA region is associated with PE, although cumulative evidence is suggesting that (homozygosity at) the HLA-DR locus [64], especially HLA-DR4 [65], might be associated with PE [66]. Other genetic backgrounds such as heterozygosity of the CTLA-4 allele, associations with the 1082G allele of the IL-10 gene and a decrease in the AA homozygous genotype in the promoter region of the IL-10 gene might predispose to PE [67], while it is also likely that women who carry certain cytokine polymorphisms, such as the upregulating TNFα-308 A/A genotype or the IL-1α-producing-4845 G/G genotype make them more susceptible for PE [68].

In peripheral blood samples from patients with PE lower levels of $CD4^+FOXP3^+$ Treg cells have been found, although no differences in the $CD4^+CD25^{bright}$ subset could be demonstrated [69,70]. Also no differences in $CD4^+$ cells staining positive either for GITR or CTLA-4 could be demonstrated [69]. This is suggesting that even in severe pregnancies complications there are no large differences in systemic Tregs and that for the immunological changes especially local mechanism should be important. This is shown in PE patients having decreased numbers of $CD4^+CD25^{high}$ T cells in decidual tissue – and in peripheral blood [71,72].

5. Conclusion

Understanding of fetus specific tolerance induction during pregnancy may lead to new tools also for the induction of donor specific tolerance in the transplantation setting. A potential problem is the fact that heavy post-transplant immunosuppression for conventional organ transplantation might be anti-tolerogenic as it is also effecting the function and level of Tregs.

In pregnancy complications, so far the important issue of differences between local immuneregulation versus systemic changes in the immune response have insufficiently been taken into account. As PE has pathophysiological pathways resembling a systemic inflammatory response, it is reasonable to accept changes in the systemic regulation during PE. However, these changes might be different locally, at the fetal-maternal interface and systemic changes might be rather the consequences of the disease than the cause. Epidemiological findings in PE suggest a more prominent role for T cells in the disease and especially the putative role of Tregs in modulating the immune response and understanding also immunological memory in this disease may lead to important new biological concepts.

Conflict of interest

The authors state they have no conflict of interest.

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Pregnancy-associated progenitor cells: An under-recognized potential source of stem cells in maternal lung

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ABSTRACT

Novel therapies are needed for the treatment of acute and chronic lung diseases, many of which are incurable. The use of exogenous stem cells has shown promise in both animal models and clinical trials. However, to date, the stem cell literature has under-recognized naturally acquired pregnancy-associated progenitor cells (PAPCs). These cells are found at sites of injury or disease in female tissues. They persist for decades after parturition in maternal blood and organs, with the largest number being found in the maternal lungs. Their presence there may be one explanation for the sex differences observed in the prevalence and prognosis of some lung diseases. Although the clinical significance of these cells is as yet unknown, the literature suggests that some of the PAPCs are stem cells or have stem cell-like properties. PAPCs harvested from the blood or organs of parous women could potentially be used as an alternate source of cells with regenerative properties for the woman herself or her children. Because PAPCs preferentially traffic to the maternal lung they may play a significant role in recovery or protection from lung disease. In this review article, we discuss ongoing research investigating the administration of both adult and placenta-derived stem cells to treat lung disease, and how PAPCs may also play an important future therapeutic role.

1. Introduction

Transplacental bidirectional trafficking of cells occurs in all human pregnancies [1–3]. Although the exact purpose of this cellular exchange is unknown, it is thought to be important in development of immune tolerance of the mother to the fetus and vice versa [3–5]. Substantial numbers of maternal cells cross the placenta and travel to the fetal lymph nodes where they induce production of fetal T-regulatory cells (T-regs). The anti-maternal fetal T-regs persist into adulthood [3].

Similarly, microchimeric fetal cells persist in the maternal circulation and/or tissue without evidence of graft rejection. This has given rise to the term *fetal cell microchimerism* [6]. Fetal cells can be identified for decades after the pregnancy [2,7,8]. Therefore, as a result of pregnancy, females acquire populations of cells that have unknown effects on their health. One hypothesis is that fetal cells might trigger a graft-versus-host reaction leading to autoimmune disease. This offers a potential explanation for why many autoimmune diseases are more prevalent in middle-aged women [9]. The other main theory is that fetal cells home to injured or diseased maternal tissue where they act as stem cells and participate in repair [10,11]. It is also possible that the fetal cells are merely innocent bystanders and have no effect on maternal health [12].

Despite the fact that the specific health implications of fetal cell microchimerism have yet to be definitively determined, a growing body of literature points towards disproportionately increased fetal cell presence at sites of injury. Khosrotehrani et al. [13] showed in a pregnant murine model that the number of fetal cells in the maternal liver increased in response to a chemical injury induced by carbon tetrachloride. Other researchers showed that skin and spinal cord injuries in pregnant mice resulted in significantly more fetal cells at the site of injury [14].

Taken together, the current literature suggests that a subpopulation of microchimeric fetal cells possess properties similar to stem cells. They have been called “pregnancy-associated progenitor cells,” or PAPCs [10]. Evidence exists to suggest that at least some of the fetal cells are hematopoietic stem cells, while other research suggests that some are mesenchymal stem cells [15]. If such studies are validated, fetal cells could potentially be harvested, expanded *in vitro*, and reintroduced to the mother to aid in tissue repair. Together with the exogenous stem cell therapies currently being

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studied, PAPCs should be explored as a novel source of stem cells. Because PAPCs preferentially traffic to the maternal lung [16] they may play an especially important role in lung disease.

Many acute and chronic lung diseases are currently incurable. Despite significant advances in symptomatic care, the only option for many patients is transplantation. This is not a guaranteed cure, as lung transplantation has a 50% mortality rate at 5 years [17]. Innovative therapies could have a significant impact on the morbidity and mortality of lung diseases. Exogenously administered stem cells are currently being investigated as novel therapeutic approaches for many lung diseases, including chronic obstructive pulmonary disease (COPD), emphysema, pulmonary fibrosis, pulmonary hypertension and acute respiratory distress syndrome [18]. Although there is currently little experience using stem cells for treatment of lung diseases, preliminary results from animal models and clinical trials are promising.

Physicians and researchers alike have long recognized the presence of sex differences in lung diseases. Idiopathic pulmonary fibrosis, idiopathic pulmonary arterial hypertension and lymphangiomyomatosis (LAM) are all more prevalent in females [18]. Emphysema in males is typically more extensive and characterized by greater peripheral involvement and larger emphysematous areas compared to females [19]. Additionally, there are sex differences in the prognosis of acute respiratory distress syndrome, with men having a much higher mortality rate [20]. Most studies addressing this issue have focused on the effects of sex hormones on lung development and progression of disease. Although differences in sex hormones may be responsible for some of these discrepancies, the field has overlooked the potential role of pregnancy and fetal cell microchimerism. This is especially important, given the high concentration of PAPCs found in the maternal lung [16]. In this review we present the current state of stem cell therapies and discuss PAPCs, an under-appreciated and potentially powerful alternative type of stem cell.

2. Current knowledge regarding stem cell therapy in the lung

Studies performed in numerous animal models suggest that stem cell therapies may be a promising approach for the treatment of lung diseases [21]. Intravenous delivery of stem cells results in significant trapping of cells within the lung, making cell-based therapies even more attractive for treatment [22]. In developing potential therapies, the type of stem cell to be used must be considered, as each has different characteristics and potential uses.

2.1. Hematopoietic stem cells

Hematopoietic stem cells (HSCs) differentiate into all blood cell lineages [23]. HSCs have been used for the treatment of cancer, myelodysplastic syndromes, and hereditary immunodeficiency disorders [24]. It is generally accepted that HSCs are $CD34^+$, $CD38^-$, $CD133^+$ and negative for lineage-specific markers (lin^-) [25].

Bone marrow-derived stem cells (BMSC) can reconstitute the hematopoietic system of an irradiated mouse, and contribute to the epithelium of the liver, lung, GI tract and skin [23]. In early studies that administered exogenous BMSCs to individuals, researchers noticed increased engraftment at sites of chronic lung injury. This led them to hypothesize that the injured lung was actively recruiting bone marrow-derived stem cells in order to aid repair [26].

Subsequent studies had conflicting results, with some demonstrating substantial engraftment while others showed none [26]. Transplantation of BMSCs into emphysematous mice resulted in decrease, and even reversal, of emphysematous structural changes within the damaged lungs, despite lack of apparent engraftment. This suggests that a paracrine effect may play a critical role [17]. BMSCs interact with endothelial cells in the lung to prevent thrombin-induced endothelial hyperpermeability. The mechanism involves activation of Cdc42, resulting in increased integrity of adherens junctions, leading to decreased inflammation and edema [27].

2.2. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are stromal cells that adhere to plastic, are negative for hematopoietic lineage antigens, and have the potential for differentiation into adipocytes, chondrocytes and osteocytes in culture [28]. More recently MSCs have been shown to be capable of neuronal, epithelial and muscular differentiation *in vitro* [29]. Because MSCs possess the features of stromal cells that support growth and maintenance of a variety of cell types in tissues, they are good candidates for cell-based therapies for lung disease. Additionally, MSCs have decreased immunogenicity due to low expression of major histocompatibility (MHC) I proteins, and lack of MHC II proteins and T-cell co-stimulatory molecules, such as CD80, CD86 and CD40. This allows administration of allogenic MSCs without generation of a significant host immune response [28].

Administration of bone marrow derived MSCs (BM-MSCs) has already demonstrated potential clinical benefits in mouse models of asthma, acute lung injury, fibrotic lung disease, chronic obstructive pulmonary disease (COPD), and pulmonary hypertension [17]. Similarly to HSCs, MSCs are suspected to work through a paracrine effect. For example, MSC administration was shown to reduce the extent of fibrosis in bleomycin-induced lung injury with minimal engraftment [30]. It has also been demonstrated that the MSC culture medium can replicate the beneficial effect [28].

Rather than engraftment, modulation of inflammation and immune cells may be the primary cause of the beneficial effects of BM-MSCs. MSCs are known to be able to alter the functions of B and T lymphocytes as well as neutrophils, in part by the release of cytokines. Compared to controls, mice that receive BM-MSCs after lung injury have decreased levels of pro-inflammatory cytokines, increased levels of anti-inflammatory cytokines (such as IL-10 and IL-13) [28], and an overall decrease in histological evidence of inflammation. Similar results are seen in murine models for asthma, pulmonary fibrosis, radiation-induced injury, and pulmonary vascular disease [17]. The use of allogenic MSCs in patients with moderate to severe COPD is already in Phase II clinical trials in the United States [17].

2.3. Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are bone marrow-derived cells that contribute to vascular repair and homeostasis [31]. EPCs express surface markers of both hematopoietic and endothelial cell lineages, such as CD31 (PECAM), CD34, CD133 (Prominin), VEGFR-2 (KDR, Flk-1), and vWf. These cells expand in culture and differentiate into mature, functional endothelial cells [31,32].

There are several reasons to believe that EPCs may be useful in the treatment of lung diseases. Through maintenance of the necessary vascular scaffold, these cells contribute to the homeostasis of the lung parenchyma [32]. Additionally, EPCs can be transduced to express pro-angiogenic factors or inhibitors of smooth muscle cell proliferation to minimize disease progression [33].

EPCs play a role in vasculogenesis and vascular repair in rodent models of pulmonary hypertension. These cells may act via a paracrine effect to diminish inflammation and by secretion of angiogenic and other growth factors such as SDF-1 (CXCL12), VEGF and PDGF [26,32]. As with other types of stem cells, EPCs home to areas of injury after systemic administration [17].

A human clinical trial testing the effect of administration of autologous EPCs for the treatment of pulmonary hypertension is

already underway. Preliminary results show improved clinical measures such as 6-min walk, mean pulmonary artery pressure, pulmonary vascular resistance and cardiac output [34].

2.4. Placenta-derived stem cells

The placenta and fetal membranes have recently been demonstrated to contain cells with stem cell-like properties, including amniotic epithelial and mesenchymal stromal cells (hAEC and hAMSC, respectively), chorionic mesenchymal stromal and trophoblastic cells (hCMSC and hCTC, respectively) and HSCs [22].

Placenta-derived stem cells have significant plasticity [21] and low immunogenicity [35]. They are able to engraft in solid organs, including the lung, brain and bone marrow [35]. The placenta can be readily obtained without invasive procedures and it does not have many of the ethical concerns that are associated with other sources of stem cells [36].

The use of placenta-derived stem cells for the treatment of pulmonary diseases is being tested in animal models. In a bleomycin-induced model of pulmonary fibrosis, investigators showed that either intratracheal or intraperitoneal administration of placenta-derived stem cells, whether allogenic or xenogenic, reduced the amount of fibrosis when compared to controls [21]. There was also decreased neutrophil infiltration and attenuated expression of pro-inflammatory cytokines [37]. This is consistent with existing hypotheses regarding the immunomodulatory properties of fetal membrane-derived cells [22].

Fetal membrane- and tissue-derived stem cells may have other benefits. For example, fetal MSCs have growth advantages over their adult counterparts, including expression of pluripotency markers, more rapid growth and longer telomeres maintained during passaging [38]. Isolation of stem cells directly from fetuses, however, would require performing unnecessary procedures on pregnant women and their fetuses. Remarkably, fetal cells are naturally present in the blood and organs of women who have been pregnant.

3. Pregnancy associated progenitor cells (PAPCs)

The presence of fetal cells in maternal organs was first reported in 1893 by the German pathologist Georg Schmorl. He observed multi-nucleated syncytial giant cells in the pulmonary circulation of women who had died of eclampsia. He correctly hypothesized that fetomaternal cell trafficking might also occur in normal pregnancies [39] although this was not demonstrated for another 76 years [40].

In the human, the number of fetal cells in the maternal circulation increases throughout gestation and decreases rapidly after parturition [1], although a population persists for several decades [2,7,8]. Male cells with an osteocyte-like morphology can also be found in the lamellae of cortical bone in postpartum females. These results suggest that PAPCs enter the maternal blood as progenitors and engraft within bone where they persist for decades [2]. PAPCs are also present in healthy lung, thyroid, skin and lymph node tissues [10,41,42].

Due to the challenges associated with human research, including limited access to appropriate tissues in living subjects, and lack of a complete reproductive history in deceased subjects, more recent efforts have focused on murine models. Animal studies typically involve mating a wild-type female to a male transgenic for green fluorescent protein (GFP). The fetal cells can be easily identified by their green color. This model system was employed to perform histological studies of the placenta to observe fetal cell trafficking. Fetal cells could be visualized in the decidua as early as 10 days post conception [43]. The frequency of PAPC detection increased during the second half of gestation. In other experiments, maternal lung, liver and spleen were used to trace the natural history of fetal cell trafficking [16]. PAPCs are detectable at day e11, increase to a maximum at day e18-19, and then are cleared rapidly in the immediate postpartum period.

3.1. PAPCs in the maternal lung

PAPCs may have a unique impact on lung disease. In conjunction with the GFP mouse model described above, researchers use a variety of methods to detect PAPCs in the maternal lung. Fetal cells can be directly visualized by fluorescent stereomicroscopy as discrete green fluorescent foci that are widely distributed throughout the lung [16]. They can also be detected using flow cytometry, PCR and fluorescence microcopy [16,44] (Fig. 1). No matter which method is used, the highest frequency of PAPCs is consistently observed in the lung as opposed to other maternal organs. This is also independent of gestational age or whether the mating was allogenic or congenic [16,44]. Fetal and placental cells have also been consistently detected in human lungs, starting with the initial discovery of syncytiotrophoblasts in the lungs of pregnant women who died of eclampsia [39]. More recent reports show that fetal cells are present even in the healthy lungs of women at autopsy [41]. In diseased lungs, fetal cells preferentially cluster at

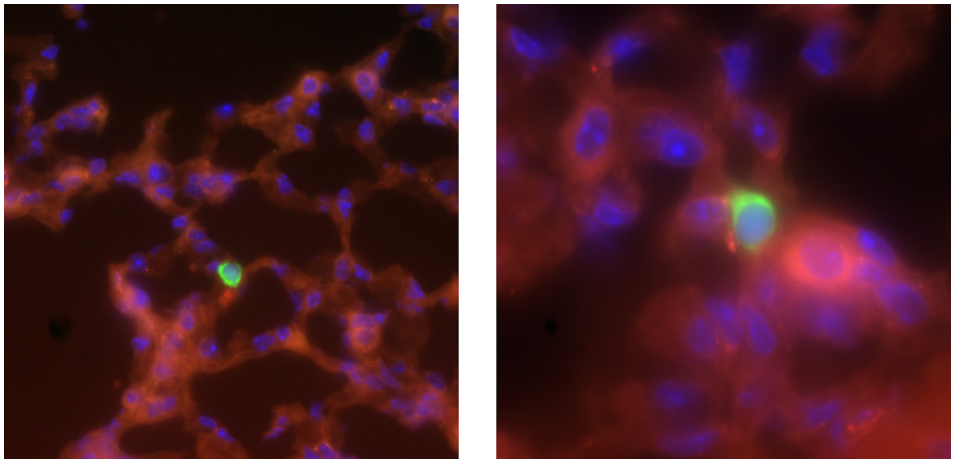

Fig. 1. Fetal cells expressing the green fluorescent protein (GFP) transgene are clearly visible within the murine maternal lung at gestational day 18. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. *Left: 400×, Right: 1000× magnification.*

Table 1
Evidence from studies in both humans and mice suggests that PAPCs may be stem cells or have stem-like properties. It is currently unknown whether one type of cell crosses the placenta and then differentiates, or whether the trafficking fetal cell population consists of multiple different progenitors.

	HSC	MSC	EPC	Placental
Human studies	Male cells in CD34-enriched apheresis products from non-pregnant female bone marrow donors [15] Fetal HSCs in the placenta could migrate to the mother [15]	Male cells detected in maternal bone marrow identified as MSCs by morphology, immunophenotype, self-renewal *in vitro* and osteogenic and adipogenic differentiation [2]	Fetal cells expressing vWF, CD34 and CD31 in the intervillous space [54]	Trophoblasts, as identified by morphology, in the lungs of women with eclampsia [39]
Murine studies	$CD45^+$ fetal cells within the maternal blood vessels [46] $CD34^+$ fetal cells in the maternal lung and liver [53]	Fetal cells expressing CD29 (integrin b1), CD44, and CD105 (endoglin) in the maternal lung [53]	$CD31^+$ (PECAM) fetal cells within the vascular wall in areas of inflammation [46] $CD31^+$ fetal cells in the maternal lung, liver, spleen and kidney [53]	Fetal cells with large nuclei in the placental decidua [43]

the site of lung tumors when compared to healthy surrounding lung [42].

The reason for the high concentration of PAPCs in the maternal lung is currently unknown. It might be due to the high rate of blood flow through the lung or that the pulmonary capillary bed is the first encountered by PAPCs after flowing through the uterine vein into the inferior vena cava (i.e. passive mechanisms). Alternatively, it is possible that the lungs (e.g. capillary endothelium) present a receptive microenvironment for retention and engraftment of these cells, or that homing to the lungs is stimulated by an as yet undetermined chemical axis [45], such as SDF-1/CXCR4, which promotes retention of a variety of cells in tissues.

3.2. PAPCs increase at the site of injury and differentiate

Previously, researchers hypothesized that fetal cells might be capable of triggering a graft-versus-host disease, leading to autoimmune disease [9]. This was supported by the fact that the number of fetal cells is often increased in tissues affected by autoimmune diseases [12]. More recent research suggests that the fetal cells home to areas of injury and inflammation [10]. In a murine model of contact dermatitis initiated during pregnancy, fetal cells preferentially trafficked to the injured skin [46]. Using histochemistry, fetal cells were identified as $CD45^+$ leukocytes in the maternal blood vessels and $CD31^+$ cells that contributed to angiogenesis. This last finding supports the hypothesis that fetal cells cross as progenitors and differentiate within the maternal tissues.

Other studies have demonstrated similar results in the murine brain. PAPCs crossed the blood–brain barrier and integrated within the maternal brain for up to 7 months postpartum [47]. The number of cells increased significantly during the postpartum period, with the highest frequency of cells detected at 60 days after parturition. PAPCs had morphologies similar to neurons, showed axonal and dendritic projections that became more complex over time, and expressed neuron-specific genes such as NeuN and β3-tubulin. The number of PAPCs in the brain was higher in a model of Parkinson's disease.

Analogous results have been observed in human studies. Fetal cells have been demonstrated to contribute to thyroid follicles in a postpartum woman with an adenomatous goiter [48], the liver parenchyma in a postpartum woman with hepatitis C [49], and the appendices of pregnant women with appendicitis [50]. In all of these reports, the fetal cells were morphologically indistinguishable from and continuous with the maternal tissue, identifiable as fetal only by the presence of a Y chromosome.

One of the biggest questions surrounding fetal cell microchimerism is the type or types of cells that make up the population. It was originally hypothesized that PAPCs are a uniform population of cells that have characteristics somewhere between that of embryonic and adult stem cells [51,52]. Since then it has been demonstrated that fetal cells, especially within the lungs of pregnant female mice, are a diverse group, expressing a variety of surface markers found on both immature and mature cell types [53] (Table 1). The differentiation state of these cells may have implications for maternal health. More mature cells would be more likely to initiate a host immune response, while immature cells would be more likely to contribute to tissue repair [12]. Further research is needed to clarify whether these cells cross the placenta as a non-uniform population with respect to lineage, a homogenous population at various stages of differentiation (e.g. fibroblast versus myofibroblast), or if they trans-differentiate once in they arrive maternal organs.

4. Conclusions

Fetomaternal cell trafficking is a well-described phenomenon occurring during all pregnancies. Fetal cells are present in maternal organs; the largest number is in the maternal lungs. PAPCs can be detected for decades after parturition. The clinical significance of these cells is unknown, but they include a population of progenitor cells that may contribute to tissue repair and regeneration. Their presence in the maternal lung may be one explanation for the sex differences observed in the prevalence and prognosis of lung disease.

Novel therapies are needed for the treatment of acute and chronic lung diseases. The use of exogenous stem cells has shown promise in both animal models and clinical trials. With stem cell research there is controversy surrounding the use of embryos as a stem cell source, while the use of adult stem cells is restricted by their limited plasticity. The use of placenta-derived stem cells has been recently described. These cells have significant plasticity and are readily obtained without invasive procedures. To date, the stem cell literature has under-emphasized the contribution of PAPCs, which concentrate in the maternal lung and are disproportionately present in areas of injury. Fetal cells harvested from the blood or organs of parous women could potentially be used as an alternate source of stem cells with potential therapeutic properties for the woman herself or her children.

Since they are naturally-acquired, fetal progenitor cells may have advantages over other types of stem cells with therapeutic promise. Induced pluripotent stem (iPS) cells, which require cell culture and exposure to viruses and transcription factors, embryonic stem cells, which require destruction of embryos, and adult stem cells, which have limited plasticity, all have significant disadvantages not shared by PAPCs [6]. Because PAPCs are haploidentical with the mother, they are less likely to be rejected than exogenously administered stem cells. They may also be less likely to be engulfed by macrophages.

Future research should focus on clarifying the identity of PAPCs in the maternal lung, their isolation and expansion in culture, and determining if parous females experience health benefits or consequences from the presence of these cells.

Conflict of interest

The authors have no conflicts of interest to disclose.

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Promising cellular therapeutics for prevention or management of graft-versus-host disease (a review)

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ABSTRACT

Graft-versus-host disease (GVHD) frequently occurs following allogeneic hematopoietic stem cell transplantation. The primary treatment for GVHD involves immune suppression by glucocorticoids. If patients become refractory to steroids, they have a poor prognosis. Therefore, there is a pressing need for alternative therapies to treat GVHD. Here, we review clinical data which demonstrate that a cellular therapy using mesenchymal stromal cells (MSCs) is safe and effective for GVHD. Since MSCs derived from bone marrow present certain limitations (such as time lag for expansion to clinical dose, expansion failure *in vitro*, painful and invasive bone marrow MSC isolation procedures), alternative sources of MSCs for cellular therapy are being sought. Here, we review data which support the notion that MSCs derived from Wharton's jelly (WJ) may be a safe and effective cellular therapy for GVHD. Many laboratories have investigated the immune properties of these discarded MSCs with an eye towards their potential use in cellular therapy. We also review data which support the notion that the licensing of MSCs (meaning the activation of MSCs by prior exposure to cytokines such as interferon-γ) may enhance their effectiveness for treatment of GVHD. In conclusion, WJCs can be collected safely and painlessly from individuals at birth, similar to the collection of cord blood, and stored cryogenically for later clinical use. Therefore, WJCs should be tested as a second generation, off-the-shelf cell therapy for the prevention or treatment of immune disorders such as GVHD.

1. Graft-versus-host disease (GVHD) is a significant clinical problem

Allogeneic hematopoietic stem cell transplantation (allo HCT) is increasingly utilized successfully as a potentially curative treatment in the management of hematologic malignancies, bone marrow failure syndromes, and inborn errors of metabolism [1]. In the treatment of hematologic malignancies, a critically important component of the efficacy of allo HCT comes from a donor-derived, immunologically driven, graft-versus-tumor effect which lowers risk for relapse when compared to high dose chemo-radio therapy and autologous HCT. Correlated with the positive graft-versus-tumor effect is the occurrence of graft-versus-host disease (GVHD). GVHD is a transplant-related complication mediated by donor-derived T-cells and affects 25–75% of patients receiving allo HCT [2,3,4]. GVHD is a principle contributor to transplant-related non-relapse morbidity and mortality following allo HCT and represents the major non-relapse barrier to the success of this otherwise potentially curative treatment approach [2].

GVHD occurs as an acute (a GVHD) or chronic (cGVHD) clinical syndrome, somewhat arbitrarily defined as occurring prior to or after 100 days post transplant, respectively. Although significant overlap exists, aGVHD and cGVHD have very distinct clinical manifestations, natural histories, treatment responses and prognosis [5,6]. Specifically, aGVHD manifest most commonly as an acute inflammatory process principally involving the integument, intestinal tract and liver, and frequently presents as a maculopapular rash, nausea, vomiting and diarrhea and hepatic cholestasis, respectively. In contrast, cGVHD is a chronic inflammatory process leading to fibrosis of involved organs and frequently presents clinically with SICCA syndrome-like features, scleradermatous-like skin changes, chronic fibrosing pulmonary, hepatic and intestinal manifestations and cytopenias. The severity of aGVHD is determined by a staging/grading system grade I–IV, with higher grades related to a worsening prognosis and likelihood of response to any therapy [7]. Following allo HCT, patients with aGVHD grades I–II experience 5 year leukemia-free survival of 44–51%; in contrast, survival decreases to 26% for patients with

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grade III and 7% for grade IV aGVHD [8]. Chronic GVHD historically has been determined as limited or extensive; however, a National Institute of Health consensus criteria model has been established recently which will facilitate better prognostication and treatment response determinations [9]. Chronic GVHD represents the leading cause of late treatment related deaths among recipients of allo HCT. Despite its adverse effects, cGVHD is associated with a decreased risk for relapse of hematologic malignancies.

Several well-defined risk factors associated with the development and severity of GVHD include human leukocyte antigen (HLA) mismatching between donor and recipient, sex mismatching, advanced recipient and/or donor age, stem cell source, and methodology of GVHD prophylaxis [10,11]. Despite optimal HLA matching, GVHD commonly occurs and this fact is attributed to the likely presence of donor-recipient mismatching of minor histocompatability antigens not currently accounted for in routine HLA typing [12,13]. Further, given that only approximately 25% of patients in need of an allo HCT will have an HLAmatched sibling donor, alternative graft sources have increasingly been utilized. The use of new conditioning regimens has resulted in a remarkable growth in the use of HLAmatched and mismatched unrelated adult and cord blood stem cell sources, as well as haploidentical related donors, especially in older patients. The increased use of these alternative donor stem cell sources in allo HCT is accompanied by increased transplant-related complications, including GVHD [14]. The long time-course of GVHD which may follow allo HCT produces a huge financial burden to our health care system and a significant time demand upon the health care team.

2. Standard care for GVHD patients

Two principal approaches to the management of GVHD include prevention and treatment. The most commonly employed strategies to prevent GVHD include optimal HLA matching at MHC class I and II loci between donor and recipient, and blocking T-cell antigen recognition and resultant proliferation during the early initiating phases of GVHD through pharmacologic prophylaxis, most commonly consistent of a calcineurin inhibitor in combination with methotrexate or mycophenolate mofetil or an MTOR inhibitor such as rapamycin. Less common, but increasingly utilized approaches include graft manipulation through *in vivo* or *ex vivo* T-cell depletion strategies and limiting tissue damage caused by the preparative regimen. Once an inflammatory cascade is triggered and donor T-cells begin destroying host tissues, the treatment regimens for GVHD ensue. GVHD treatment involves various immunosuppressive therapies. The standard initial treatment is steroid therapy. However, a significant percentage of patients will prove to be resistant to steroid therapy and will subsequently be treated with second-line immunosuppressive agents [5,6]. Steroid-resistant aGVHD portends a very poor prognosis, second-line agents frequently prove ineffective and as a result survival is $<10\%$ at 5 years. Therefore, alternative therapies are needed to prevent and treat GVHD following allo HCT.

3. MSCs for treating GVHD

One promising treatment for GVHD involves the infusion of third party, HLA-disparate, unrelated bone marrow derived mesenchymal stromal cells (BM-MSC). The *in vivo* and *in vitro* properties of BM-MSC suggest their potential use in a broad range of inflammatory and immune-mediated conditions, such as GVHD. BM-MSC are a population of undifferentiated multipotent mesenchymal stromal cells which express HLA class I and do not express HLA class II or costimulatory molecules CD40, CD80 or CD86 [15–19] and have been demonstrated to modulate immune and inflammatory response in animal models of inflammatory disease including GVHD [19–23], and to facilitate repair of connective tissues [24–27].

MSCs inhibit the activation of and proliferation of activated T-cells that have been induced by a variety of stimuli [18,28] and down-regulate inflammatory cytokine expression such as tumor necrosis factor (TNF)-α, IL2R-α, elafin, and interferon-γ (IFN-γ) [29,30]. Dander et al. investigated the effects of MSC infusion on lymphocyte counts in transplanted patients with steroid-refractory GVHD [30]. Interestingly, $CD4^+$ T-cell subsets changed significantly after MSC infusion. Specifically, Tregs increased and Th1 and Th17 populations decreased significantly in patients whose symptoms improved. Le Blanc et al. [31] reported the first case of successful treatment of severe refractory aGVHD using *ex vivo* expanded haploidentical MSCs. In a subsequent report, these investigators demonstrated a positive therapeutic effect with allogeneic MSCs in patients experiencing steroid-refractory aGVHD with no significant adverse events attributed to the cells [32].

Additional studies have reported very encouraging clinical results and confirmed the safety of MSCs in the treatment of steroid-refractory aGVHD [31–45]. Specifically, Kurtzberg et al. presented at the 2010 American Society of Blood and Marrow Transplant meeting [46] that using allogeneic MSCs as a rescue agent for severe treatment resistant aGVHD demonstrated a 64% response rate in 59 children by day 28, and that response to MSCs correlated with improved overall survival at 100 days. This work suggests that MSC therapy has an excellent risk/benefit profile [46]. Martin et al. presented at the same meeting the results of a randomized, placebo-controlled, multicenter phase 3 trial of MSCs in the treatment of steroid-refractory aGVHD which involved 244 patients [47]. Although the principle endpoint of durable complete response >28 days was not significantly better in the MSC-treated population, significant differences in response for patients with multi-organ involvement, liver and intestinal involvement were realized for the MSC-treated cohort.

A summary of the published reports describing the clinical outcomes of patients treated with MSCs in the management of both aGVHD and cGVHD with varying results is shown in Table 1 [31–47]. These reports included patients that received a variety of conditioning regimens including myeloablative, or non-myeloablative, or reduced intensity conditioning (RIC), with no apparent differences in the response to MSC treatment. Furthermore, the patients received MSCs from a variety of sources including HLA identical, haploidentical, or third party, unrelated and unmatched donors. Most clinical data came from MSCs derived from bone marrow, but work from Fang et al. used MSCs derived from adipose tissue [35–37], with no apparent differences in response. Important for the availability of off-the-shelf cell therapy, MSCs from freshly expanded samples or from cryogenically stored/thawed cell preparations have been used with no apparent differences in response. MSCs have been shown to be safe: no ectopic tissue formation has been derived from infused MSCs. Finally, MSCs did no harm: no clearly defined increased incidence of opportunistic infections or relapse of malignancy were reported. In summary, the data support the concept of MSCs as a safe, well-tolerated and variably effective treatment for GVHD. Importantly, MSCs can be cryogenically banked, thawed and given without the need for donor-recipient matching.

4. Improving MSCs for clinical use in GVHD

BM-MSCs have specific problems that limit their usefulness. For example, BM-MSC isolation requiresaspiration from the marrow cavity which is a painful, invasive procedure, with certain risks. Several studies indicate that adult-derived MSCs have limited expansion potential or slower expansion *in vitro* compared to fetal-derived MSCs and that adult MSCs may be less-responsive than fetal or neonatal MSCs in certain applications [48–53]. Therefore,

Table 1
Review of clinical reports and trials that evaluated mesenchymal stromal cells (MSCs) to treat graft versus host disease (GVHD). This table is an update from the table that appeared in Toubai T et al., Curr Stem Cell Res & Ther, 2009.

Citation	MSC Source	MSC Donor	Number of patients/ GVHD grade	HCT Conditioning	Dose of MSCs (/kg)	Effect on GVHD	Ref. #
LeBlanc K et al., 2004	BM	Haploidentical (mother)	1 (9 yr boy) Grade 4	Myeloablative	2×10^6 first 1×10^6 second	Improvements	[31]
Ringden O et al., 2006	BM	HLA identical sib $n = 2$ Haploidentical $n = 6$ HLAmismatch $n = 4$	9 (12 infusions) steroid refractory aGVHD 8 cGVHD 1	Myeloablative $n = 5$ RIC $n = 3$ ATG only $n = 4$	$0.6–9 \times 10^6$	Complete response 6 Response 1 Slight effect 1 No response 4	[32]
Fang B et al., 2006,2007	Adipose	Unrelated mismatch Haploidnetical Haploidentical Unrelated mismatch $n = 4$	1 (38 yr) 2 steroid refractory 1 chronic hepatic 6-steroid refractory aGVHD	Myeloablative Myeloablative Myeloablative Myeloablative	2×10^6 first 1×10^6 second	Complete response Complete response Complete response Complete response 5/6 complete response	[35–38]
Muller I et al., 2008	BM	Mismatch family $n = 8$ HLA identical $n = 2$ HLA matched unrelated $n = 1$	7 (11 infusions) aGVHD $n = 2$ cGVHD $n = 3$ Hemophagocytosis $n = 1$ Graft rejection prophylaxis $n = 1$	Myeloablative $n = 5$ RIC $n = 2$	$0.4–3 \times 10^6$	aGVHD 1/2 alive and well cGVHD 1/3 slide improvement Hemophagocytosis, good response Graft rejection prophylaxis alive and well	[42]
Le Blanc K et al., 2008	BM	HLA identical sib $n = 5$ Haploidentical $n = 18$ Unrelated mismatch $n = 69$	55 (92 infusions) Grade 2 $n = 5$ Grade 3 $n = 25$ Grade 4 $n = 25$	Unknown	$0.4–9 \times 10^6$	Children 17/25 complete response, 4/25 partial response Adult 13/30 complete response, 5/30 partial response total 30/55 complete response (54%), partial response 9/55 (16%) Overall 2 yr survival 53% for complete response vs 16% for partial or non-response	[40]
von Bonin M et al., 2008	BM	Unrelated mismatched	13 (32 infusions) Grade 3 $n = 2$ Grade 4 $n = 11$	Myeloablative $n = 1$ RIC $n = 12$	0.6×10^6 (0.6–1.1)	2 patients (15%) complete response 5/11 (45%) partial response	[43]
Zhou H et al., 2010	BM	HLAmatched, unrelated	4 cGVHD	Nonmyeloablative $n = 4$	$1–2 \times 10^7$ (4–8 infusions)	4/4 Complete response	[45]
Kebriaei P et al., 2009	BM	Osiris unrelated unmatched $n = 6$	31 (62 infusions) Grade 2 $n = 21$ Grade 3 $n = 7$ Grade 4 $n = 3$	Myeloablative $n = 15$ RIC $n = 8$ Nonmyloablative $n = 4$ DLI $n = 4$	2×10^6 $n = 16$ 8×10^6 $n = 15$	24/31 Complete response, 5 partial response 2 No response	[39]
Arima N et al., 2010	BM	Related, HLA identical $n = 1$ Unknown	3 Grade 3 $n = 3$	RIC $n = 1$ unknown $n = 2$	$0.5–2 \times 10^6$ intra-arterial injection into GVHD sites	1/3 partial response	[33]
Baron F et al., 2010	BM	third party, mismatch	20 patients (19 historic controls)	Nonmyeloablative coinfusion w/MSC $n = 20$	Unknown	MSC coinfusion appears safe MSC coinfusion might prevent death from GVHD without impacting GVT	[34]
Lucchini G et al., 2010	BM	Single donor unrelated HLA mismatch	aGVHD Grade 1–4 or cGVHD $n = 11$	Variable: TBI, RIC, etc	$0.7–3.7 \times 10^6$ 1–5 infusions	8/11 Complete (23%) or partial (47%) response 3 No response	[41]
Weng JY et al., 2010	BM	HLAmatched third party, mismatched	cGVHD 73% severe, 26% moderate) $n = 19$	Variable: TBI, RIC, etc	$0.23–1.42 \times 10^6$ 1–5 infusions	14/19 Complete (4) or partial (10) response 5 Died	[44]

Updated from Toubai T, Paczesny S, Shono Y et al. Curr Stem Cell Res & Ther 2009.

alternative tissue sources, such as discarded tissues resulting from pregnancy, which contain fetal-derived MSCs have been considered as an alternative MSC source. Here, we focus upon the potential of these tissues, with the aim to improve the next iteration of clinical trials. Two notions are presented. First, we review the literature that suggests that MSCs from discarded fetal tissues might be better than BM-MSCs for GVHD therapy. This idea is based upon the fact that BM-MSCs have aforementioned limitations that may be overcome using an alternative MSC source. Second, we review literature that suggests that *in vitro* conditioning by cytokine exposure, called "licensing", of MSCs during expansion might improve their clinical effect in GVHD. This idea is based upon the relative plasticity of MSCs to culture conditions such as hypoxia, cytokine exposure, etc, that change the physiology of MSCs and may improve their clinical effect.

It is well-understood that BM-MSCs have limitations that may affect their clinical potency and impact. For example, BM-MSCs have a limited expansion potential and grow relatively slowly *in vitro*, and they require a painful and invasive collection procedure, and BM-MSCs from older individuals may not expand to clinically relevant number and may be unsuitable for therapy. To address these limitations, individuals at the EMBO workshop: "From fetomaternal tolerance to immunomodulatory properties of placenta-derived cells in cell therapy" First Bi-annual Meeting of the International Placenta Stem Cell Society (IPLASS) held in Brescia, Italy focused upon the deciduous tissues associated with fetal life, e.g., amnion, placenta and umbilical cord. Our thesis is that MSC derived from umbilical cord may be a effective, safely and painlessly collected alternative source of MSC, and replace BM-MSC, for GVHD prevention or treatment.

Our group works with MSCs that are derived from umbilical cord stroma, also known as Wharton's jelly (WJ, or WJCs below). WJ is a primitive, loose connective tissue that is rich in hyaluronan, and supports and cushions the umbilical vessels. WJ's contains an MSC population that is easily isolated following birth from the discarded umbilical cord after umbilical cord blood has been collected. WJCs grow more quickly and produce more cells during expansion *in vitro* compared with BM-MSCs [54,55] and, they have immune properties similar to adult-derived MSCs from bone marrow and adipose tissue [51,54,56–58]. It is their immune properties that make MSCs attractive for immunological disorders. These immune properties are: 1) low immunogenicity and naïve MSCs do not strongly stimulate allogeneic T-lymphocyte proliferation; 2) MSCs suppress the proliferation of activated T lymphocytes, 3) increased production of regulatory T-cells, and 4) a shift in the immune response towards tolerance or anergy since MSCs do not stimulate B cells and prevent B cells from becoming stimulated.

The mechanisms of MSC immune suppression have been reviewed elsewhere (see [29,59,60]). GVHD may be modeled *in vitro* since treatments which impact on the inflammatory response are reflected by assays of the suppression of mitogen-activated or allo-antigen activated T-cell proliferation, and the expansion of regulatory T-cells, which would reflect a critical component of tolerance induction. The mechanisms used by MSCs are under debate. Evidence exists to support both a direct, contact-dependent mechanism that is mediated at least in part by MSC expression of the cell death ligand, B7-H1 [61], and an indirect, contact-independent mechanism mediated by various cytokines and growth factors such as prostaglandin E2 (PGE2), cyclo-oxygenase (COX) 1 and 2, hepatocyte growth factor (HGF), transforming growth factor-β, interleukin 10, human leukocyte antigens G5 and E, leukemia inhibitor factor, indoleamine 2,3-dioxygenase (IDO), and others [28,29,56,62–64]. As seen below, the mechanisms are not fully determined and the literature is filled with example and counterexample.

Several studies compared the immune properties of BM-MSCs, WJCs and MSCs derived from adipose tissues [51,54,56,57,65]. Najar et al. reported that adipose-derived MSCs and WJCs had similar *in vitro* immunosuppressive effects for lymphocyte proliferation, compared to BM-MSCs; that MSCs target CD4+ and CD8+ T-cells for immune suppression equally; adipose-derived and WJCs inhibit T-cell activation, and that MSCs were immunosuppressive regardless of the type of stimuli used to activate the lymphocytes [57]. In their hands, MSC immune suppression was mediated by COX 1 and 2 enzymes and by the production of PGE2 and did not involve HGF. In agreement with Najar et al.'s findings, Chen et al. found that PGE2 synthesis, mediated by COX2, produces the majority of WJCs' suppressive effects on T-cell proliferation and on IFN-γ secretion [65]. PGE2 expression by WJCs was stimulated by inflammatory signals IFN-γ or interleukin-1β produced by peripheral blood mononuclear cells following mitogen or allogeneic stimulation. Critically, they found that WJCs cultured with unstimulated (naïve) T-cells do not secrete much PGE2, however, following co-culture with stimulated T-cells, WJCs excreted more PGE2. This finding fits with our own [58], and other labs' findings [23,51,54,56,61,66,67]: MSCs have little effect on unstimulated T-cells and exposure to activated T-cells or inflammatory cytokines changes MSCs so they display immunosuppressive behavior.This has been termed as licensing or priming of MSCs. Chen et al. found that IDO and TGF-β played little role in MSC's suppression of the T-cell proliferation. As a counterexample to Chen et al.'s finding that IDO had little role, Yoo et al. had diametrically different findings when they compared the immunoregulatory properties of adipose-derived, umbilical cord blood-derived MSCs, WJCs and BM-MSC [51]. They found that MSCs from all four tissue sources responded to either IFN-γ or tumor necrosis factor-α (TNF-α) secreted from activated T-cells by inducing IDO secretion, and the released IDO from MSCs suppressed T-cell proliferation, and led to decreases in TNF-α and IFN-γ. Yoo et al. reported that, while MSCs responded to IFN-γ or TNF-α exposure to upregulate IDO expression, they did not increase expression of HGF, Cox 1 and 2, interleukin-10, and transforming growth factor-β. Prasanna et al. also examined the immune properties of MSCs from BM and from WJ, and the effect of IFN-γ and TNF-α exposure on these properties [56]. They found that IFN-γ or TNF-α stimulation produced subtly different responses between BM-MSCs and WJCs. For example, IFN-γ or TNF-α exposure increased the expression of the immune-adhesive ligand, CD54 in both BM-MSCs and WJCs. However, IFN-γ increased expression of HLA class 2 in BM-MSCs and not in WJCs. Prasanna et al. also reported that IFN-γ exposure did not strongly affect the immunogenicity of MSCs in their *in vitro* proliferation assays [56]. In contrast to these findings, Cho et al. reported that IFN-γ exposure induced expression of MHC class II in swine and human WJCs, and that IFN-γ stimulate WJCS produced an antibody response following subcutaneous or intravenous injection of allogeneic WJCs faster than when unlicensed WJCs were used in a swine model [66]. One unexpected finding in the Prasanna et al. report was the importance of MSC proliferation (possibly) on immune suppressive properties: both BM-MSCs and WJCs that had been mitotically inactivated lost their immune suppressive effect. To our knowledge, this was the first report to correlate MSC proliferation with suppression of lymphocyte proliferation. If MSC proliferation is critical for immune modulation, this would significantly impact upon how MSCs are derived for therapeutic use. The differences between Cho et al. and Prasanna et al. on changes in HLA expression are explained by work from Deuse et al., who compared the immunogenicity of allogeneic BM-MSCs and WJCs both *in vitro* and *in vivo* following exposure to different doses of IFN-γ [54]. At doses of IFN-γ below 50 ng/ml, IFN-γ upregulated HLA-DR and doses from 100 to 500 ng/ml of IFN-γ down-regulated HLA-DR. Interestingly, in all cases, WJCs had lower expression of HLA-I and HLA-DR compared

to BM-MSCs, WJCs had weaker allogeneic T-cell stimulation compared to BM-MSCs, and WJCs had longer survival following allogeneic transplantation in immunocompetent Balb/c mice. To summarize these studies, MSCs from Wharton's jelly, adipose tissue and bone marrow can potently suppress T-cell activation, and suppress both CD4+ and CD8+ T-cell proliferation induced by mitogen or allogeneic stimulation. Both soluble factors and direct contact are important for full effect of MSC_s on immune cells. These studies did not consistently identify soluble factors involved; rather indicate a role for PGE2, IDO, COX 1 and 2, and other factors. The reason for these differences is unknown. Several studies indicate that differences exist between MSC sources, but the physiology that accounts for these differences is not understood, currently. For example, does MSC proliferation, or some other attribute, limit MSC immune suppression [56]? MSCs from adipose, BM and WJ have similar *in vitro* and *in vivo* immune properties. The advantages of WJCs, e.g., their lower immunogenicity, less immune activation and slower rejection compared to BM-MSCs, would not be apparent without direct comparisons (as was conducted in these studies). While some studies found that WJCs and adipose MSCs have equal or superior suppression of activated T-cells proliferation compared to BM-MSCs, in other studies the differences were less apparent. Finally, consistently, adipose and Wharton's jelly MSCs have superior *in vitro* expansion properties compared to BM-MSCs.

As mentioned above, MSCs' immune properties, specifically their immunogenicity, their ability to suppress T-cell activation and their immune suppression of activated T-cell and B cell proliferation, can be modified by manipulating their environment. This has been called licensing or priming. Thus, the therapeutic effect of MSCs may be "tuned" to improve performance for a particular therapeutic application by appropriate priming. Several studies that address this hypothesis are discussed briefly below [23,56,61,66,67]. Cho et al. showed that exposure of WJCs to IFN-γ increases the expression of MHC class I and induces the expression of MHC class II [66]. This was accompanied by increases in the immunogenicity of WJCs in an allogeneic swine model. Similar and different findings were reported by Prasanna et al., as was discussed above [56]. Again the theme is that IFN-γ exposure modifies MSC effect on immune properties including expression of IDO, HLA class I and class II surface marker expression, etc. Tipnis et al. reported that IFN-γ caused WJCs to upregulate the expression of cell death ligand B7-H1, in addition to confirming that IFN-γ stimulates increased expression of IDO, and induces HLA class II expression [61]. Valencic et al. evaluated two variables: the priming effect of IFN-γ exposure on WJCs and the timing of lymphocyte exposure to WJCs [67]. They found that the timing of WJC priming was critical to reveal their immune suppressive effects on lymphocytes and priming WJCs increased their immune suppressive action in both contact and non-contact settings. In contrast, if pre-stimulated lymphocytes were added to non-primed WJCs, the lymphocytes showed normal or enhanced proliferation. Deuse et al. examined the dose-dependent effects of IFN-γ on BM-MSCs and WJCs and found that higher levels of IFN-γ stimulation produce a stronger effect of WJCs on immune suppression [54]. The *in vitro* work suggests that primed MSCs would be more effective at treating chronic GVHD, where they are placed into an environment which will rapidly license them to begin immune suppression, which fits with animal model and human clinical observations [23,40]. It also suggests that unprimed MSCs given together with hematopoietic stem cells during allo HCT would be ineffective at preventing GVHD, which again is supported by animal GVHD model work by Polchert et al. [23], and such speculation might be retrospectively confirmed from clinical data. Additionally, the *in vitro* work suggests that IFN-γ-priming would improve MSCs' therapeutic effect when given together with hematopoietic stem cells before GVHD has developed; which has been confirmed in a GVHD mouse model [23]. While Polchert's work fits with *in vitro* work that indicates that IFN-γ priming will have beneficial effects in GVHD, primed MSCs have not yet been tested in clinical use. In that regard, the clinical findings reported by Dander et al. [30] fit with what we might predict MSCs might do based upon our basic understanding of their immunophysiology. Currently, there is no reason to believe that primed MSCs would not be safe and effective for clinical use. In fact, the *in vitro* and animal model data suggest the primed MSCs would have more potent therapeutic effect than naïve MSCs. We further speculate that hindsight will clarify the target tissue effects reported for MSCs in GVHD [47] once the interactions of MSCs with Tregs, Th1, Th17 and Th2-cell subsets are resolved. Unfortunately, there is not space to discuss this critically important topic.

5. Summary and conclusions

In summary, MSCs appear to be safe and well-tolerated, and they offer a hope for treatment of steroid-refractory GVHD patients. The clinical outcomes to date are good, and there is room for improvement. Most clinical trials have used BM-MSCs; adipose-derived MSCs were used in a few trials for GVHD, and WJCs have not yet been tested clinically for GVHD. As discussed above, *in vitro* testing of MSCs suggests that off-the-shelf, unmatched cryopreserved MSCs derived from either adipose or WJ may be a second-generation of MSC-based cell therapy for GVHD. Finally, we must expand our understanding of the concept of priming MSCs since it improves effectiveness in an animal GVHD model and in pertinent *in vitro* assays. In conclusion, new information about MSC biology should be translated rapidly to clinical evaluation for safety and efficacy for therapy in steroid-resistant GVHD.

Contributors

MLW presented the abstract at the EMBO workshop: "From fetomaternal tolerance to immunomodulatory properties of placenta-derived cells in cell therapy" First Bi-annual Meeting of the International Placenta Stem Cell Society (IPLASS) 3rd–6th October, 2010 held in Brescia, Italy; drafted the Ms and wrote the MSC section. JPM prepared the clinical review.

Conflict of interest

The authors declare no conflicts of interest. This paper is based upon an abstract presented at the EMBO workshop: "From fetomaternal tolerance to immunomodulatory properties of placenta-derived cells in cell therapy" First Bi-annual Meeting of the International Placenta Stem Cell Society (IPLASS) 3rd–6th October, 2010 held in Brescia, Italy.

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paper is dedicated to MLW's wife, Betti G Weiss and to MLW's mother, Sharon P Weiss.

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Wharton's Jelly stem cells: Future clinical applications

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ABSTRACT

This review focuses on the therapeutic potential of stem cells harvested from the Wharton's Jelly of the human umbilical cord. Recently, investigators have found that a potent stem cell population exists within the Wharton's Jelly. In this review, the authors define a new subset of stem cells, termed perinatal stem cells, and compare them to other sources of stem cells. Furthermore, cryopreservation of Wharton's Jelly stem cells is described for potential use in future cell based therapies and/or regenerative medicine applications. Current evidence of the application of mesenchymal stem cells from various sources in both pre-clinical and clinical trials is reviewed in the context of potential indications of use for Wharton's Jelly derived mesenchymal stem cells.

1. Introduction

The term perinatal encompasses the time from the 20th week of gestation to the neonatal period (the first 28 days of life). The tissue that sustains natal development is typically discarded as medical waste post-delivery. As such, harvesting stem cells from these tissues represent a safe, non-invasive means for attaining therapeutically beneficial stem cells. These include amnion/amniotic fluid, umbilical cord blood, placental tissue, umbilical cord vein, and the Wharton's Jelly contained within the umbilical cord sometimes referred to as umbilical cord tissue [1–9].

Perinatal stem cells are not embryonic stem cells (ESCs), nor are they somatic (adult) stem cells (ASCs); they represent a bridge between embryonic and adult stem cells. Human embryonic stem cells are derived from the inner cell mass of the developing blastocyst in the initial post-fertilization cell divisions [10,11]. They possess the ability to produce all the cells from all germ layers, including adult tissue-specific (somatic) stem cells and differentiated cells. Adult stem cells, on the other hand, are multipotent tissue-specific stem cells that maintain cell turnover units within the tissue [8,12]. Furthermore, adult stem cells may possess the ability to trans-differentiate to other cell types from other tissues. Perinatal stem cells possess characteristics of both embryonic stem cells and adult stem cells as they possess pluripotency properties, as well as multipotent tissue maintenance [13].

Despite these inherent characteristics, both embryonic and adult stem cells have significant drawbacks. For one, human embryonic stem cells will form teratomas when transplanted [14]. As a result, embryonic stem cells cannot directly be transplanted into human patients and, therefore, must be manipulated *in vitro* and differentiated along tissue-specific lineages to form, for instance, adult stem cells or differentiated progeny, prior to transplantation. Furthermore, the derivation of human embryonic stem cells inherently destroys the development and potential of a human life. Not surprisingly, as a result, the use of human embryonic stem cells, in both research and clinical settings, possess a tremendous ethical cloud [15]. Adult stem cells, on the other hand, do not encompass similar ethical challenges (when proper IRB approval and/or patient consent are obtained). However, adult stem cells have relative limited proliferative potential (i.e. multipotent), are extremely rare *in vivo* and, generally speaking, are difficult to expand *ex vivo* [8]. Furthermore, the procurement of adult stem cells from patients is invasive and represents significant risk and discomfort to the patient [8].

These described limitations can be overcome with the utilization of various sources of an additional stem cell paradigm, termed here as perinatal stem cells. For one, perinatal stem cells, unlike embryonic stem cells, do not form tumors when transplanted. Additionally, perinatal stem cells may possess greater pluripotency capability than adult stem cells, as there is evidence that these cells can produce cells from all three germ layers [13,16–19]. This property will ultimately allow for greater tissue differentiation capacity. Since stem cells from perinatal tissue are procured from tissue that would otherwise be discarded as medical waste, there is little risk to the mother or newborn. Of the perinatal stem cell sources, Wharton's Jelly has great potential to emerge as a useful stem cell source to treat various diseases in the clinics. The

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potential clinical applications and indications for Wharton's Jelly stem cells are numerous [1,16–18,20]. This review will focus on the stem cells derived from the Wharton's Jelly of the human umbilical cord and their potential use in therapeutic applications.

2. What is Wharton's Jelly?

Wharton's Jelly is the primitive mucous, connective tissue of the umbilical cord lying between the amniotic epithelium and the umbilical vessels. First observed by Thomas Wharton in 1656, this gelatinous substance is comprised of proteoglycans and various isoforms of collagen. The main role of the Wharton's Jelly is to prevent the compression, torsion, and bending of the umbilical vessels which provide the bi-directions flow of oxygen, glucose and amino acids to the developing fetus, while also depleting the fetus and placenta of carbon dioxide and other waste products [16,18,21]. Cells found in Wharton's Jelly are a primitive mesenchymal stem cell (MSC), likely trapped in the connective tissue matrix as they migrated to the AGM (aorta-gonad-mesonephros) region through the developing cord, during embryogenesis (prior to E10.5) [21].

During early embryogenesis, hematopoiesis takes place in the yolk sac and later in the AGM region. Colonies of early hematopoietic cells and mesenchymal cells migrate through the early umbilical cord to the placenta between embryonic day 4 and 12 of embryogenesis [21]. There is a second migration from the placenta again through the early umbilical cord to the fetal liver and then finally to the fetal bone marrow where hematopoietic stem cells (HSCs) and mesenchymal stem cells engraft and predominantly reside for the duration of life. Included in these migrating hematopoietic colonies are early precursors of HSCs, as well as primitive mesenchymal stromal (stem) cells. Researchers have postulated that during this migration to and from the placenta through the umbilical cord, mesenchymal stromal cells become embedded in the Wharton's Jelly early in embryogenesis and remain there for the duration of gestation [21].

The formation of these perinatal stem cells at such an early embryonic state allows them to retain a resemblance to embryonic stem cells (ESCs), while still maintaining the properties of somatic mesenchymal stem cells found in bone marrow, as defined by the International Society for Cellular Therapy (ISCT) [22]. For the purposes of this article, Wharton's Jelly Stem Cells (WJSCs) are defined as native stem cell populations residing within the *in situ* umbilical cord extracellular matrices and umbilical cord mesenchymal stem cells (UC-MSCs) are defined as *in vitro* cell populations derived from Wharton's Jelly Stem Cells (WJSCs).

The problem of how to define UC-MSCs is further exacerbated because 'within the scientific literature, the acronym MSC has been used to represent (bone) marrow stromal cells, mesenchymal stem cells, and multipotent mesenchymal stromal cells' [22]. Further complicating the matter, researchers have used a variety of methods for isolation, culture, and characterization. The use of various methods leads to considerable ambiguity when study comparisons are attempted. In 2006, in order to pursue standardization, the Mesenchymal and Tissue Stem Cell Committee of the ISCT proposed minimal criteria for defining MSCs. To begin with, they proposed that these cells be designated as multipotent mesenchymal stromal cells (MSC). They further proposed three criteria for defining these cells: adherence to plastic culture ware, specific surface antigen expression, and multipotent differentiation potential. In the case of adherence to plastic, the adherence must be maintained under standard cell culture conditions using tissue culture flasks. Flow cytometric analysis of MSCs should demonstrate surface antigen expression of CD105, CD73, and CD90. Furthermore, these cells should show minimal expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II proteins. Finally, these cells must demonstrate the ability to differentiate along osteogenic, adipogenic and chondrogenic lineages under standard *in vitro* differentiating conditions [22]. UC-MSC possess plastic adherence, can be differentiated along chondrogenic, adipogenic, and osteogenic lineages and possess expression of the following cell markers: CD105+ (endoglin, SH2), CD73+ (SH3), CD90+ (Thy-1), HLA-A,B,C+ (MHC class I), CD34, CD45-, HLA-DR- (MHC class II) [16–18].

Interestingly, UC-MSCs not only possess MSC properties but they exhibit properties to those attributed to ESCs. Specifically, UC-MSCs express human ESC markers Tra-1-60, Tra-1-81, SSEA-1 (stage-specific embryonic antigen-1), SSEA-4, alkaline phosphatase and even form embryoid bodies *in vitro* [23]. Additionally, UC-MSCs express the pluripotency markers Oct-4, Sox-2, and Nanog [13,16,24,25], albeit at relatively lower levels than ESCs [19,23]. Both qRT-PCR and microarray data have confirmed the relative lower expression of pluripotency markers in UC-MSCs, compared to human ESCs, irrespective of early- or late-passaged cells [19,23]. These markers are up-regulated in undifferentiated human embryonic stem cells and have been shown to maintain pluripotency in self-renewing human ESC populations [26]. Although lower relative expression of pluripotent markers would suggest that UC-MSCs are not as pluripotent as ESCs, it would suggest, however, that UC-MSCs are highly multipotent. In fact, microarray studies confirm that UC-MSCs express markers of all three primordial germ layers independent of passage length [19]. Furthermore, the regulation of these pluripotent genes has been shown to induce pluripotency in somatic cells (otherwise known as iPS; induced pluripotent stem cell) [26,27]. Not surprising, Oct-4, Sox-2 and Nanog are not expressed by adult stem cell sources, including bone marrow-derived MSCs [13], although forced expression of Oct-4 and Nanog in human BM-MSCs has been reported to improve their stemness [28].

Since WJSCs are trapped within the Wharton's Jelly between day 4 and 12 of embryonic development and reside there for the duration of gestation, they can be procured after birth of the newborn. The ability to procure these cells, in ethically unchallenged ways, is quite significant. Furthermore, these cells that formed during the earliest ontogenic time period result in significant differences in expansion potential compared to (adult) bone marrow mesenchymal stem cells (BM-MSCs). As previously reported, the number and potency of BM-MSCs specifically decreases with age, as indicated by lower *in vitro* CFU-F and proliferative potential, lower telomerase activity, longer population doubling times, and shorter times to senescence [29–32]. UC-MSCs maintain the same multipotent differentiation potential with relatively higher CFU-F and proliferative potential, higher telomerase activity, shorter population doubling times, and longer times to senescence, without loss of stem cell potency. Thus, UC-MSCs appear to be more primitive mesenchymal stem cells than those found in bone marrow and represent an earlier stage mesenchymal-like stem cell than those derived from adult fat or bone marrow [13,18,20].

In approximately 99% of all deliveries, these potentially therapeutic cells are discarded as medical waste. In the United States alone, every one of the approximately 4,000,000 annual births represents an opportunity to collect these cells. The same WJSCs which are trapped in early embryonic development during the migration to and from the placenta through the early umbilical cord can be easily collected and harvested from the Wharton's Jelly of the umbilical cord at the time of delivery. This ease of collection has obvious advantages over the collection of adult stem cells from fat and bone marrow, for which the donor has to undergo an invasive surgical procedure. This factor, coupled with the great expansion capabilities of UC-MSCs, enables this cell source to represent a virtually inexhaustible source of stem cells for both autologous and allogeneic cellular therapies and regenerative medicine products [24,33].

3. Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs) as a universal source

In the field of cellular therapy, there are two models typically pursued. One is an autologous model which utilizes a patient's own cells for the therapy; the other is an allogeneic model which utilizes cells from another donor for the therapies. Most often these models are determined based on the capabilities of the cell source. In order for an allogeneic model to be considered, the cell source must be immunologically privileged, suggesting that the cells do not immunologically cross-react and, therefore, do not have to be human leukocyte antigens (HLA) matched for transplantation or they are HLA-matched to the recipient.

UC-MSCs, like bone marrow mesenchymal stem cells, are immunologically privileged. MSCs invoke only minimal immune reactivity, and, furthermore, may possess anti-inflammatory and immuno-modulatory effects [30,34–37]. UC-MSCs express MHC class I antigens and express low levels of MHC class II antigens, relatively less than BM-MSCs. As several studies currently suggest, UC-MSCs, like BM-MSCs, do not require tissue matching, thus, allowing for an allogeneic cell therapy source, as any donor can give cells to any other person without rejection or need of immuno-suppressant drugs [37]. This characteristic suggests that UC-MSCs can be used as a 'universal' or 'off-the-shelf' stem cell product.

WJSCs also work as a cell source for an autologous model. Presently, collection and cryogenic preservation of WJSCs along side matching umbilical cord blood (UCBs) units in private banking storage are being conducted. In this model, both the cord blood unit and the WJSC unit are processed using minimally manipulated procedures. Studies in mice have shown that a co-transplant of a single cord blood unit and a WJSC unit from either a related or an unrelated donor increases the engraftment efficiency of the infused cord blood HSCs, although the mechanism of action is currently unclear [20,38]. As such, preservation of matching WJSCs along side private umbilical cord blood units can significantly increase the chances of having a successful transplant if that cord blood unit is needed for transplantation. WJSCs are currently being banked along side public and private cord blood units. AuxoCell Laboratories, Inc. and the New Jersey Public Cord Blood Bank are working together to bank WJSCs units that match public cord blood units listed on the National Marrow Donor Program's (NMDP) registry, as well as private units. Currently, however, WJSC units are not listed on the NMDP's registry.

By currently building an inventory of WJSC units, this partnership anticipates a future where WJSCs will be used to potentially enhance every cord blood transplantation. Currently, investigative trials in animals are being conducted and will need to be translated into humans to further prove the safety and efficacy of these cells for this indication. Moreover, this publicly banked inventory will greatly increase the therapeutic potential of the existing worldwide public cord blood inventory as low potential therapeutic units (based on total nucleated cell count) can now be enhanced and administered as a clinically sufficient therapeutic dose for transplantation. As interest in banking of WJSCs continues to increase, various methods to process umbilical cord have been developed and implemented to bank WJSCs, in conjunction with umbilical cord blood stem cells. These advances have allowed the procurement, processing and cryopreservation of WJSCs to be conducted in existing infrastructure and, importantly, in a similar timeframe as current cord blood banking (unpublished data).

At the time of delivery, after the donor's umbilical cord blood has been collected, the umbilical cord, in its entirety, is clipped, and placed in the included collection jar. The collected cord blood and cord are placed in the kit and shipped to the processing center within 48 h, where the umbilical cord blood is processed using a FDA-certified automated method; the umbilical cord is processed using a separate technique, which involves a series of processing and separation steps. The final homogenous cell product is cryopreserved in a 25 mL cryopreservation bag, similar to the one used for the associated cord blood unit. WJSCs are then cryopreserved at a controlled rate, and then transferred to liquid nitrogen for long-term storage, once the units have passed all quality controls and are found to be free of pathogens and contaminants.

Samples are taken from each unit and characterized for expression of cell surface proteins using flow cytometry. WJSCs express CD105 (Endoglin Receptor), CD73 glycoprotein, CD90 (Thy-1), CD44 (homing-associated cell adhesion molecule; H-CAM), CD29 (Integrin β1), HLA-ABC, HLA-DR and lack expression for CD34 and CD45. Further characterizations may include CFU-F (colony forming unit–fibroblast), expansion potential and multipotential differentiation along osteogenic, chondrogenic, and adipogenic lineages [16,18]. Unit sterility (i.e. lack of bacterial contaminants) and the indicated characterization is sufficient for release of the unit for transplantation.

In the United States, AuxoCell Laboratories, Inc.'s proprietary processing technology is licensed by ViaCord for private banking. Additionally, AuxoCell has partnered with select private cord blood banks internationally, and continues to develop partnerships with both private and public cord blood banks worldwide.

4. Regenerative medicine applications of MSCs

Additional properties of MSCs make them useful stem cell candidates for use in various cell based therapies, beyond umbilical cord blood hematopoietic engraftment. For instance, UC-MSCs share the natural homing capabilities of BM-MSCs. For MSCs, an injury serves as a homing beacon, as they home to sites of inflammation and to locally effect the inflammatory/immune mediated tissue damage with subsequent ability to support tissue healing. They shift the spectrum of local cytokines from pro-inflammatory to anti-inflammatory [39,40]. Studies are currently ongoing to take full advantage of these unique properties for specific indications. The immunosuppressive ability of these cells has the potential to treat many disorders including graft-versus-host disease (GvHD) [41–44], diabetes [45,46], Crohn's disease [44], heart disease [39,47,48], and solid tumor cancers [40,49].

5. MSCs for the treatment of heart disease

There are many different heart conditions for which stem cell treatments are potentially valuable. The rationale to use MSCs to treat heart conditions is based on the ability of MSCs to home to areas of injury/inflammation and/or on their ability to down regulate the immune response and support the tissue repair process. Stem cells may be shown to reduce the amount of scar tissue and increase the pumping strength of the heart in myocardial infarcted patients.

According to the National Heart, Lung and Blood Institute, 1.1 million people suffer heart attacks in United States annually. Coronary heart disease, which causes heart attacks and angina, is a leading cause of death in the United States with nearly 450,000 related fatalities in 2005, according to the American Heart Association. As such, cellular therapies to treat cardiac disease are aggressively being pursued, and cardiac stem cell therapies could be commonplace within several years.

BM-MSCs have been shown to benefit patients early after myocardial infarction by exhibiting lower incidence of arrhythmias [47,48,50]. A recent study using a mesenchymal stem cell therapy presented by Osiris Therapeutics, Inc. showed that an intravenous injection of bone marrow-derived mesenchymal stem cells

repaired heart damage in patients who had experienced heart attacks within 10 days [47,48]. The trial now has moved to a phase II study in 50 hospitals in the United States. However, one of the limitations of BM-MSCs cell therapies is the difficulty to expand early-passage BM-MSCs to sufficient numbers and doses required to have a therapeutic benefit in the patient [24,33]. This is due primarily to increased senescence of BM-MSCs when expanded *in vitro* (unpublished data).

6. MSCs for the treatment of cancer

One of the other exciting research areas that utilize MSCs for cellular therapies is in the field of cancer. Two recent studies examine ability of MSCs to home to tumors to treat metastatic cancer [40,49]. Progress of this kind would be a major breakthrough in the treatment of solid tumors. The first study from the London Research Institute reported that MSCs engineered to produce and deliver tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) home to and kill cancer cells in a lung metastatic cancer model. This study was the first to show a significant reduction in metastatic tumor burden with frequent eradication of metastases using induced TRAIL expressing MSCs. The researchers concluded that this method 'would have a wide potential therapeutic role, including the treatment of both primary tumors and their metastases, possibly as an adjuvant therapy in clearing micro-metastatic disease following primary tumor resection' [49]. TRAIL has also been used with umbilical cord blood [51].

A second study takes advantage of MSC homing capacities by combinatorial treatment with intraperitoneal (IP) injections of 5-fluorouracil (5-FU) and targeted interferon beta (IFN-β) gene therapy in UC-MSCs to treat metastatic human breast cancer in SCID mouse lung cancer [40]. UC-MSCs were found in the lung and not in other observed tissue, although this is not surprising as there is overwhelming evidence that intravenous injected cells initially home to the lung [52]. Although both treatments alone, significantly resulted in reductions in lung tumor area, the combined treatment of IFN-β transduced UC-MSCs and 5-FU resulted in greater lung tumor reduction, compared to each treatment alone [40]. Although only two cancer studies utilizing MSCs are highlighted here, the use of MSCs to combat tumors continues to progress toward therapeutic utilization.

7. Conclusion

Although there are no current clinical trials ongoing with WJSCs or UC-MSCs, several pre-clinical trials have been conducted to suggest the possible clinical benefits of this cell source. Several indications have been investigated in animals including hematopoietic reconstitution [20,38], Parkinson's [24], diabetes [45,46], Macular Degeneration [53] and spinal cord injuries [54]. Before WJSCs can be safely translated into human trials, further investigation and characterization in animals must be completed to ensure safety and efficacy. WJSCs are immuno-privileged, immunosuppressive, have a multipotent/pluripotent differentiation capacity and are readily available as a cell source; WJSCs may be an important cell therapy source for specific indications in the near future to treat several diseases and improve the quality of life in many patients.

Conflict of interest

RRT and KJC are employees of AuxoCell Laboratories, Inc. CLC is on the Board of AuxoCell Laboratories, Inc. and has worked as a consultant for AuxoCell Laboratories, Inc.

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Prenatally harvested cells for cardiovascular tissue engineering: Fabrication of autologous implants prior to birth

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ABSTRACT

Using the principal of tissue engineering, several groups have demonstrated the feasibility of creating heart valves, blood vessels, and myocardial structures using autologous cells and biodegradable scaffold materials. In the current cardiovascular clinical scenario, the main medical need for a tissue engineering solution is in the field of pediatric applications treating congenital heart disease. In these young patients, the introduction of autologous viable and growing replacement structures, such as tissue engineered heart valves and vessels, would substantially reduce today's severe therapeutic limitations, which are mainly due to the need for repeat reoperations to adapt the current artificial prostheses to somatic growth. Based on high resolution imaging techniques, an increasing number of defects are diagnosed already prior to birth around week 20. For interventions, cells should be obtained already during pregnancy to provide tissue engineered implants either at birth or even prenatally.

In our recent studies human fetal mesenchymal stem cells were isolated from routinely sampled prenatal amniotic fluid or chorionic villus specimens and expanded *in vitro*. Fresh and cryopreserved samples were used. After phenotyping and genotyping, cells were seeded onto synthetic biodegradable scaffolds and conditioned in a bioreactor. Leaflets were endothelialized with either amniotic fluid- or umbilical cord blood-derived endothelial progenitor cells and conditioned. Resulting tissues were analyzed by histology, immunohistochemistry, biochemistry (amounts of extracellular matrix, DNA), mechanical testing, and scanning electron microscopy (SEM) and were compared with native neonatal heart valve leaflets.

Genotyping confirmed their fetal origin, and fresh *versus* cryopreserved cells showed comparable myofibroblast-like phenotypes. Neo-tissues exhibited organization, cell phenotypes, extracellular matrix production, and DNA content comparable to their native counterparts. Leaflet surfaces were covered with functional endothelia. SEM showed morphologically cellular distribution throughout the polymer and smooth surfaces. Mechanical profiles approximated those of native heart valves.

These *in vitro* studies demonstrated the principal feasibility of using various human cell types isolated from fetal sources for cardiovascular tissue engineering. Umbilical cord blood-, amniotic fluid- and chorionic villi-derived cells have shown promising potential for the clinical realization of this congenital tissue engineering approach. Based on these results, future research must aim at further investigation as well as preclinical evaluation of prenatally harvested stem- or progenitor cells with regard to their potential for clinical use.

1. Introduction

In the field of tissue engineering adult stem- and progenitor cells have proven to be a useful tool for maintaining or restoring the function of damaged or diseased tissues and have recently attracted increasing attention as a possible highly clinically relevant source [1]. Although their differentiation potential is much more restricted than that of embryonic or induced pluripotent stem cells, adult stem- and progenitor cells lack the major risk of tumor induction after transplantation which makes them ideal sources for therapeutical applications [2]. With regard to the realization of a minimally invasive approach, several progenitor cell types have been assessed for the generation of tissue engineered heart valves or

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vascular grafts, including bone-marrow or blood-derived sources [3]. Ideally, these autologous stem- or progenitor cells should be harvested during pregnancy as soon as the cardiovascular defect is detected enabling the generation of tissue engineered autologous implants with the potential to grow, to remodel, and to regenerate, ready to use at or shortly after birth. Furthermore, the ideal cell source should be easily accessible and allow for cell harvest without substantial risks for both, the mother and the child and without the sacrifice of intact donor tissue [4]. This approach of pediatric cardiovascular tissue engineering displays a promising strategy to overcome the lack of autologous replacement materials for the early repair of congenital malformations in order to prevent secondary damage to the immature heart. Different studies have proven the principal feasibility of using prenatally harvested cells isolated from different sources. The present work summarizes relevant cell types and discusses possible future applications of the pediatric tissue engineering approach.

2. Heart valve tissue engineering: *In vitro* fabrication of viable constructs

Langer and Vacanti defined the term 'tissue engineering' as an interdisciplinary field, applying the principles and methods of engineering to the development of biological substitutes that can restore, maintain or improve tissue formation [5]. According to this pre-definition the successful *in vitro* fabrication of autologous viable heart valve replacements similar to the native prototype is supported by three main elements: First, autologous cells that resemble their native counterparts in phenotype and functionality are isolated and expanded using standard cell culture techniques. Second, these cells are seeded onto a biodegradable matrix, fabricated in the shape of a trileaflet heart valve, termed the *scaffold*, which promotes tissue strength until the cell derived ECM (extracellular matrix) guarantees functionality on its own. Third, in order to promote tissue formation and maturation, the seeded scaffolds are exposed to stimulation transmitted via a culture medium (biological stimuli) or via 'conditioning' of the tissue in a bioreactor (mechanical stimuli). This aims at adequate cellular differentiation, proliferation, and ECM production to form viable tissue. This construct is subsequently implanted orthotopically as a valve replacement, and further *in vivo* remodeling is intended to recapitulate physiological valvular architecture and function (Fig. 1) [4,6,7].

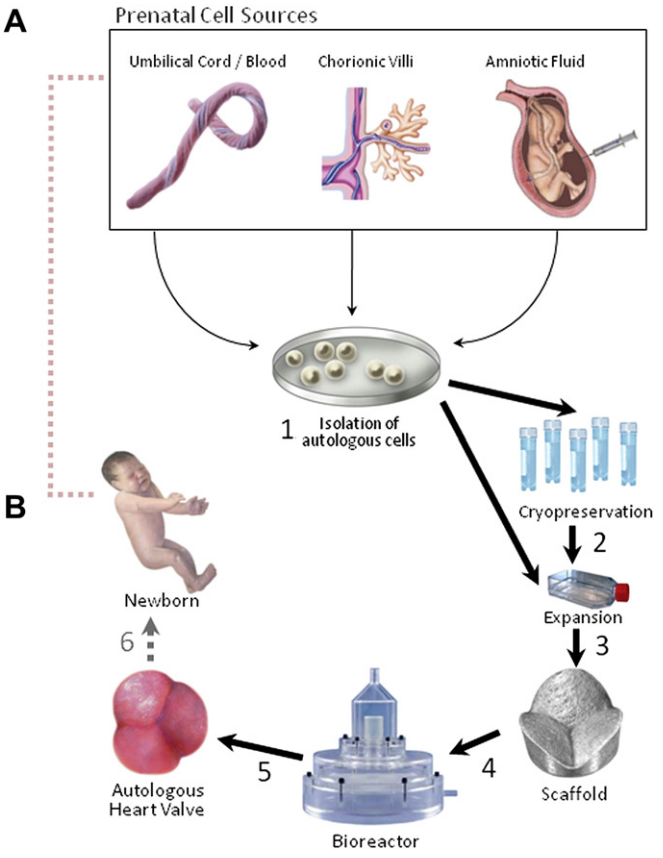

Fig. 1. Concept of prenatal accessible cell sources for cardiovascular tissue engineering. (A) Prenatal cell sources for progenitor and/or stem cells e.g. umbilical cord- and/or cord blood, chorionic villi, and amniotic fluid. (B) After autologous fetal cells have been prenatally harvested (1), they can either be cryo-preserved (2) or directly further expanded (3) depending on the optimal time point for the surgical intervention. When sufficient cell numbers are reached, cells are seeded onto a biodegradable scaffold. After a short static phase, the constructs are positioned in a bioreactor (4) and conditioned. When optimal tissue formation is achieved, tissue engineered constructs (5) are available for implantation (6).

2.1. Chorionic villi-derived multipotent stem cells

The human placenta, particularly its chorionic villi (Fig. 2A), provides extra-embryonically situated fetal mesenchymal cells, including progenitor cells (Fig. 2B) that are routinely obtained for prenatal genetic diagnostics by biopsy (*reviewed by* Pappa and Anagnou [8],). These cells might present a further attractive cell source for pediatric tissue engineering applications as indicated by recent investigations [9]. Schmidt et al. first demonstrated the feasibility of this approach in heart valve tissue engineering using chorionic villi-derived cells for the *in vitro* fabrication of viable heart valves [10]. However, in these initial studies fetal origin of the cells has only been shown after harvest, whereas several studies have shown that maternal cell overgrowth in advanced passages is not uncommon [11], which has to be addressed by future approaches.

2.2. Amniotic fluid-derived stem cells

Amniotic fluid has been demonstrated to be a unique pool of fetal cells that bear resemblance to multipotent stem cells with potential for therapy (Fig. 2C) [12,13]. It particular, the easy of prenatal harvest makes them a highly attractive fetal cell source for this concept as they provide access to progenitor cells from all three germ layers in a relatively low-risk procedure [13–15]. Several studies have shown promising results based on human amniotic fluid-derived cells (AFDCs) with regard to tissue engineering [16,17]. In 2007, Schmidt et al. demonstrated the feasibility to tissue engineer *ex vivo* viable autologous heart valve leaflets, using human amniotic fluid as a single cell source (Fig. 2D). Cell populations required for the fabrication of heart valves, namely mesenchymal- and endothelial-like cells have been successfully differentiated and expanded [4]. In order to expand the versatility of these cells also for adult application, cryopreserved AFDCs were investigated as a potential life-long available cell source, once again showing successful fabrication of viable heart valve leaflets *in vitro* [18]. Despite the futuristic strategy of this novel class of replacements potentially leading to innovative therapies for both pediatric and elderly patients in the future, several concerns possibly related to this cell source, such as cancerogenicity, long-term durability and adequate growth behavior have to be addressed in further *in vivo* investigations.

2.3. Umbilical cord blood derived-endothelial progenitor cells

As a fetal cell source, umbilical cord blood (UCB) contains hematopoietic cells, multipotent stem cells, unrestricted somatic

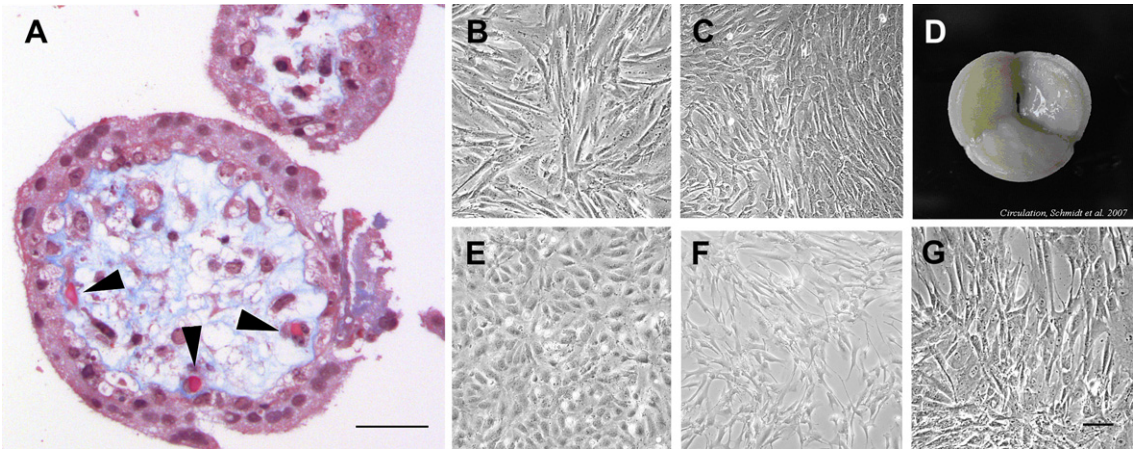

Fig. 2. Prenatally fabricated autologous human heart valve based on amniotic fluid derived-progenitor cells as single cell source. (A) Cross section through chorionic villus as example for a prenatally available fetal cell source (Kindly provided by Dr. J. Achermann). Blood vessels are highlighted by arrows. Scale bar represents 100 μm. Morphology of different primary cell isolates is given for chorionic villi (B), amniotic fluid (C), Wharton's jelly (G), derived-multipotent stem cells, and umbilical cord blood derived-endothelial progenitor cells (E) and multipotent stem cells (F). Scale bar represents 100 μm. (D) Macroscopic image of tissue engineered heart valve. Leaflets with intact and densely covered amniotic fluid-derived cells [4]. (Reprinted with permission from Schmidt D. et al: Circulation 2007:11,116:I64–70).

stem cells and endothelial colony-forming cells (ECFCs) and has been suggested as source for tissue regeneration [19–29]. ECFCs can give rise to true progenitors of endothelial cells (Fig. 2E). ECFCs exhibit extraordinary proliferative capability *in vitro* and structurally contribute to *de novo* blood vessel formation by the process of vasculogenesis when implanted *in vivo* [30,31]. Because of such favorable properties of ECFCs and millions of UCB units already cryobanked worldwide, term UCB is considered to be a valuable candidate source to provide the high endothelial cell number required in regenerative medicine approaches. Examples are prevascularization of *in vitro* engineered tissue constructs or *in vitro* endothelialization of synthetic blood vessel replacements prior to transplantation [32].

Schmidt et al. developed tissue engineered blood vessels generated from human myofibroblasts seeded on biodegradable vascular scaffolds, followed by endothelialization with differentiated UCB-derived endothelial cells. These results indicate that tissue engineered vascular grafts (TEVGs) with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human UCB-derived progenitor cells. Thus, UCB-derived progenitor cells obtained pre- or postnatally may enable the clinical realization of tissue engineering constructs for pediatric applications [33,34]. While UCB appears to be a source for large-scale amounts of ECFCs, concerns are existing regarding their safety for clinical application after expansion [35].

2.4. Umbilical cord- or cord blood derived- multipotent stem cells

Sodian et al. recently demonstrated tissue engineered autologous heart valves with the potential to grow and to remodel fabricated from cryopreserved UCB cells as a single cell source [36]. Erices et al. described in 2000, UCB-derived adherently growing, fibroblast-like cells which exposed similar to BM-derived MSCs an immunophenotype of $CD45^-$, $CD13^+$, $CD29^+$, $CD73^+$, $CD105^+$ [25]. The potential of cord blood-derived MSCs to differentiate along the mesodermal lineage has also been demonstrated by multiple groups (Fig. 2F) [26,27,37].

However, the transplantation of UCB-derived MSCs is in contrast to established treatment in a variety of haematoblastoses in the stage of infancy. But further research with UCB-derived MSCs for transplantation could improve the prognosis in diseases, which are related to degeneration and/or injuries of body cells and organs as indicated by ongoing clinical trials in phase I–III [38,39]. Schmidt et al. described in 2006 the fabrication of tissue engineered living blood vessels generated from human umbilical cord-derived cells (Fig. 2G) seeded on biodegradable vascular scaffolds, followed by endothelialization with differentiated cord blood-derived endothelial progenitor cells. These results indicate that tissue engineered vascular grafts with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord- or cord blood progenitor cells. Thus, blood-derived progenitor cells obtained before or at birth may enable the clinical realization of tissue engineering for pediatric applications [34]. Because of such favourable properties of MSCs and millions of UCB units already cryobanked worldwide, term UCB is considered to be a valuable candidate source to provide the high MSC number required in regenerative medicine approaches. However, Kögler et al. demonstrated that MNC outgrowth after isolation is very low and that cells do not adhere to tissue culture flasks when using unseparated frozen CB units. Only volume-reduced CB units, in which the majority of erythrocytes were already depleted at the time of cryopreservation, revealed successful generation of unrestricted somatic stem cells (USSCs) although in low frequency [20,40].

3. Future

Prenatally harvested stem- and progenitor cells hold great potential for advancing therapeutical options in patients with severe congential cardiovascular disorders. The proof of concept using several different cell types has been made, however up to date it seems unclear which one is the most favorable cell source for clinical use. As a source, amniotic fluid distinguishes itself through the relative ease of cell harvest and isolation, in chorionic villus-sampling the early time point of harvest is outstanding. Therefore, before ultimate clinical implementation of the pediatric tissue engineering principle, the identification of the best available cell source with regard to harvest and tissue formation capacities seems indispensable.

Conflict of interest statement

Benedikt Weber and Steffen M. Zeisberger have no conflict of interest. Simon P. Hoerstrup is scientific advisor of Xeltis Inc.

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Amniotic membrane and amniotic cells: Potential therapeutic tools to combat tissue inflammation and fibrosis?

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ABSTRACT

In addition to the placenta, umbilical cord and amniotic fluid, the amniotic membrane is emerging as an immensely valuable and easily accessible source of stem and progenitor cells. This concise review will focus on the stem/progenitor cell properties of human amniotic epithelial and mesenchymal stromal cells and evaluate the effects exerted by these cells and the amniotic membrane on tissue inflammation and fibrosis.

1. Introduction

1.1. Human amniotic epithelial and mesenchymal stromal cells

The human amniotic membrane (AM) contains two distinct cell populations. Human amniotic epithelial cells (hAEC) are cuboidal to columnar cells that form a monolayer lining the membrane and are in direct contact with the amniotic fluid. hAEC arise from the embryonic epiblast and are amongst the first cells to differentiate from the conceptus [1,2]. In contrast, human amniotic mesenchymal stromal cells (hAMSC) are dispersed in an extra-cellular matrix largely composed of collagen and laminin, and are derived from the extraembryonic mesoderm [1]. Both cell types originate during the pre-gastrulation stages of embryogenesis before the delineation of the three primary germ layers [1,2]. Embryonal carcinoma cells that are formed prior to gastrulation have been shown to retain stem cell-like properties. Thus, the early origin of the AM cells was a major reason that led to investigations into the plasticity and stemness of these cells.

Efficient protocols have been established for hAEC and hAMSC isolation from term placenta and are generally based on the separation of the AM from the chorionic membrane and subsequent enzymatic digestion [2–5]. A typical term AM yields between $150–200 \times 10^6$ hAEC and $20–50 \times 10^6$ hAMSC [6]. In culture, hAMSC exhibit plastic adherence and fibroblast-like morphology, while hAEC display a typical cobblestone epithelial phenotype. Many of the surface and intracellular stem/progenitor markers expressed by AM cells are listed in Tables 1 and 2. However, there is considerable variation in the percentages of AM cells expressing these markers reported by different investigators. The levels and pattern of marker expression appear to depend on the isolation protocol used and vary with expansion [7]. Gestational age dependant changes in marker expression have also been found. Surface markers such as CD44, CD49e and CD13 were significantly lower in hAEC derived from third trimester compared to first trimester [8] and hAEC expressing the pluripotency associated Nanog, Sox-2, Tra-1-60 and Tra-1-80 genes were higher in second trimester AM compared to term [9]. Further, cells with pluripotency associated markers have been found to be randomly distributed in the epithelial layer of term delivered AM [10]. This heterogeneity in distribution and gestational age dependant changes is also likely to contribute to different sub-populations being analyzed [2,5,8,11–14] and impact on investigations into stem cell properties and possibly pregnancy related studies using AM cells.

Interestingly, hAEC and hAMSC also express a repertoire of lineage associated genes (Tables 1 and 2), suggesting that they could act as progenitors and differentiate into various cell types. Indeed, hAEC and hAMSC have the ability to differentiate *in vitro* into cells from each of the three germ layers (Tables 1 and 2). After stimulating cells in media supplemented with growth factors, hormones and/or other additives, differentiation was monitored by evaluating the

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Table 1
Phenotype of undifferentiated hAEC and hAEC induced to differentiate *in vitro*.

Undifferentiated hAEC			
Marker groups	Markers	Detection methods	References
Mesenchymal and hematopoietic markers	CD10+, CD13+, CD29+, CD44+, CD49e+, CD73+, CD90+, CD105+, CD117+, CD166+, STRO-1+, CD14−, CD34−, CD45−, CD49d−, HLA-ABC +, HLA-DQ+/−, HLA-DR−	FACS	[2,7,11–15]
Stem cell markers	POU5F1 (OCT-4)+, Sox-2+	RT-PCR, immunocytochemistry	[2,11,14,15]
	FGF-4+, Rex-1+, CFC1+, Nanog+, DPPA3+, PROM1+, PAX6+, FOXD3−, GDF3−, TERT−	RT-PCR	[2,11,14,15]
	SSEA-3+, SSEA-4+, Tra 1-60+, Tra 1-81+, SSEA-1-	FACS, immunocytochemistry	[2,7,13–15].
	GCTM2+	immunocytochemistry	[11,15]
Neural lineage associated markers	Nestin, GAD, MBP, NF-M, NSE, CNPase, PLP, DM-20	RT-PCR	[2,14]
	Nestin, MAP2, GFAP	immunocytochemistry	[15]
	Neurofilament proteins, MAP2, MAP2 kinase	Immunocytochemistry	[39]
Lung associated markers	Nkx2.1, mucin, occludin, aquaporin-5, caveolin-1	RT-PCR, FACS	[11]
Hepatic lineage associated markers	Albumin, α-FP	RT-PCR, western blot, immunocytochemistry	[40]
	Albumin, α-1AT, CK18, GS, CPS-1, PEPCK, CYP2D6, CYP3A4, α-FP, TTR, TAT, CYP2C9, HNF3-γ, C/EBP-α	RT-PCR	[41]
Cardiomyogenic lineage associated markers	GATA-4, Nkx 2.5, MLC-2A, MLC-2V, MYL-7, ANP, CACNA1C, KCND3	RT-PCR	[14,15]
Pancreatic lineage associated markers	PDX-1	RT-PCR	[14]
Others	CD31-, CD324+ (E-cadherin), ABCG2/BCRP+, vimentin+, PanCK+,	FACS, immunocytochemistry	[2,11]

Differentiated hAEC			
Lineages	Characterization of differentiation: markers/cell morphology/tissue specific functions	Detection methods	References
Adipogenic	PPARγ; LPL; cells enlarge three/four times and are multinucleated; cells contain lipid deposits	RT-PCR, Oil Red O stain	[7,15]
Osteogenic	OSC, OSN; cells enlarge two/three times ad are binucleated; cells contain calcium deposits	RT-PCR, Von Kossa stain, Alzarin Red stain	[7,15]
Chondrogenic	Expression of collagen I and II; synthesis of proteoglycans	immunohistochemistry, Toluidine Blue Stain	[7]
Myogenic	ATCA2; MyoD1, skeletal muscle myosin heavy chain; elongated cells, multinucleated cells	immunocytochemistry	[8,15]
Neural	Increased expression of nestin, GAD, MBP, NF-M, NSE; expression of GFAP, CNP; elongated cells with neuronal- or astrocyte-like morphology	RT-PCR, immunocytochemistry	[14]
	Few nestin+ and MAP-2+ cells with neuronal morphology; numerous GFA + cells with astrocyte-like morphology	immunocytochemistry	[15]
Lung	Production of SPs A–D, SP-D secretion; epithelial phenotype with lamellar body formation	electron microscopy, immunohistochemistry, ELISA	[11]
Hepatic	Albumin; α-1AT, HNF-4α; CYP1A activity	RT-PCR, immunohystochemistry, EROD assay	[14]
	Albumin, HGF; features of hepatocytes	immunocytochemistry, FACS, transmission electron microscopy	[15]
Cardiomyogenic	GATA-4, MYL-7, ANP, CACNA1C, KCND3, TTNT; features of relatively mature cardiomyocytes	RT-PCR, FACS, transmission electron microscopy	[15]
	Increased expression of GATA-4, Nkx 2.5, MLC-2A, MLC-2V; expression ofα-actinin	RT-PCR, immunocytochemistry	[14]
Pancreatic	Increased expression of Pdx-1; expression of Pax-6, Insulin, NKx 2.2, glucagon	RT-PCR, immunocytochemistry	[14]
	AMY2B, glucagon; features of exocrine acinar beta cells	RT-PCR, immunocitochemistry, FACS, transmission electron microscopy	[15]

morphological changes (e.g. changes into cells with neuron, hepatocyte and cardiomyocyte-like features), expression of various lineage-specific genes, as well as assessing acquired abilities to exert tissue-specific functions (Tables 1 and 2). However, the level of maturation achieved *in vitro* may be variable [2,15], and could be due to the inability to express genes present in the terminally differentiated cells, shortcomings in the induction media, extra-cellular matrices, oxygen tensions and culture conditions used.

Clonal colony formation is an important feature of adult tissue derived stem cells. There are conflicting reports on the clonogenicity of AM cells. hAEC and hAMSC were found to be clonogenic with hAMSC forming colonies that could be expanded for at least 15 passages [5,15], whereas others report the absence of clonal colony formation by hAEC and hAMSC [13]. Generally, at much higher seeding densities, hAEC and hAMSC can be kept in culture for 5-10 passages [5,12,13]. Interestingly, after a few passages hAEC change from the cuboidal epithelial shape into elongated stromal-like cells that express markers associated with mesenchymal and fibroblast cells and show reduced differentiation potential [12,13]. The reason/s for these changes remains uncertain, but may be due to senescence, epigenetic modifications and to the autocrine/paracrine effects of growth factors such as EGF and TGFβ that are known to induce an epithelial to mesenchymal transition. Although the phenotypic changes are not as marked compared to hAEC, the morphology of hAMSC and differentiation potential also declines with expansion [5,12].

There is some evidence that AM cells can differentiate into cardiomyocytes, neural, alveolar epithelium and pancreatic β-islet cells following xenotransplantation and secrete proteins produced by hepatocytes [11,16]. Further, trophic factors secreted by hAEC and hAMSC could exert angiogenic, growth promoting, anti-inflammatory and anti-fibrotic effects following transplantation [16]. Thus, with a view to potential therapeutic applications, researchers are also developing isolation protocols in accordance with current guidelines for clinical use [16,17]. Culture of hAEC in

Table 2
Phenotype of undifferentiated hAMSC and of hAMSC induced to differentiated *in vitro*.

Undifferentiated hAMSC			
Marker groups	Markers	Detection methods	References
Mesenchymal and hematopoietic markers	CD3-, CD13+, CD14-, CD29+, CD34-, CD44+, CD45-, CD49e+, CD54+, CD73+, CD90+, CD105+, CD117 (weak), CD166+, $CD27^{low}$+, STRO-1+, HLA-A-B-C+, HLA-DR−	FACS	[1,2,7,8,13,42]
Stem cell markers	SSEA-3+, SSEA-4+	FACS, immunocytochemistry	[1,13]
	POU5F1 (OCT-4)+, Rex-1+, BMP-4+	RT-PCR	[1,13]
Endothelial marker	CD31-, VEGF receptor 1 and 2: FLT-1+ and KDR+	FACS	[1]
Hepatic lineage associated markers	Albumin, CK18, α-FP, α1-AT, HNF4α	RT-PCR	[43]
Pancreatic lineage associated markers	PDX-1	RT-PCR	[44]
Cardiomyogenic lineage associated markers	GATA-4, MLC-2A, MLC-2V, MLC-2v, cTnI, and cTnT, α-subunits of the cardiac-specific L-type calcium channel, Kv4.3	RT-PCR	[45]
Neural lineage associated markers	Nestin and musashi1, Tuj1 and NF-M, GFAP	RT-PCR, immunocytochemistry	[46]
Others	CD349+, CD140b+, CD324 (E-cadherin)-, vimentin+	immunocytochemistry	[2]

Differentiated hAMSC			
Lineages	Characterization of differentiation: markers/cell morphology/tissue specific functions	Detection methods	References
Adipogenic	LPL; accumulation of lipid deposits	RT-PCR, Oil-red O stain	[5]
Chondrogenic	Collagen-II; cartilage-specific metachromasia	RT-PCR, Toluidine Blue stain	[5]
Osteogenic	OPN; induction of calcium deposition	RT-PCR, Alizarin red stain	[5]
Myogenic	myoD, myogenin,desmin	RT-PCR, immunocytochemistry	[42]
	myoD1, skeletal muscle myosin heavy chain; features of myotubes	immunocytochemistry	[8]
Hepatic	Increased expression of albumin, CK18, α-FP, α1-AT, HNF4α; storage of glycogen	RT-PCR, immunocytochemistry; PAS staining	[43]
Pancreatic	Increased expression of Pdx-1, Isl-1, Pax-4, Pax-6, expression of insulin, glucagon, somatostatin; appearance of islet-like cell clusters	RT-PCR, immunocytochemistry	[44]
Cardiomyogenic	GATA-4, MLC-2A, MLC-2V, cTnI, and cTnT, α-subunits of the cardiac-specific L-type calcium channel, Kv4.3, induction of Nkx2.5, ANP and cardiac-specific gene -myosin heavy chain; integrate in cardiac tissues in co-culture experiments	RT-PCR, immunocytochemistry	[45]
Angiogenic	Increased expression of FLT-1, KDR, ICAM-1, appearance of CD34 positive cells, expression of vWF; features of endothelial cells	FACS	[42]
Neurogenic	Increased expression of nestin, musashi1, Tuj1 and NF-M, GFAP	RT-PCR, immunocytochemistry	[46]

serum free medium appears to lead to the expression of hematopoietic and monocytic markers, high telomerase activity and long telomere lengths [17], whereas hAEC that are routinely cultured in fetal calf serum (FCS) supplemented media lack these markers and telomerase activity [12–14]. Comparisons of cells grown in FCS with serum free media or media containing acceptable alternatives for clinical use such as platelet lysate and human serum may be warranted, as for example, a high level of telomerase activity is linked to teratoma and tumor formation. Injection of primary or passaged hAEC and hAMSC that have been cultured in FCS into mice, rodents and swine has so far not led to tumour formation [14,15,18–20], but the fusion of amniotic cells with host cells to form dysplastic precursors cannot be ruled out. Further, under serum free conditions a selection of surface markers were differentially expressed by primary and passage 5 hAEC [17]. This reinforces the possibility that culture conditions may select different cell populations, thereby altering the phenotype of the naïve population. Thus, further investigation would be beneficial as results reported on cell replacement, inflammation and fibrosis following xenotransplantion have been reported using primary hAEC and hAMSC prepared using FCS.

1.2. Pre-clinical studies investigating amniotic cells

Lung and liver fibrosis, myocardial infarct and stroke are leading causes of mortality and together with their long term morbidity places major burdens on health care systems worldwide. In pre-clinical animal disease models, human amniotic cells were found to make a modest contribution toward replacing damaged alveolar epithelium, endothelium and heart muscle [11,21,22], whereas a more significant contribution is likely to be their anti-inflammatory and anti-scarring effects.

Pooled MSC from amniotic and chorionic membranes and hAMSC alone have been injected directly into infarcted rat hearts following arterial ligation. Treated rats showed increased capillary density, improved left ventricular function and fractional shortening and a reduction in fibrotic scar tissue [21,22].

hAEC and hAMSC have also been evaluated as a treatment for liver and lung fibrosis. Hereditary, pathogenic, environmental and lifestyle factors can induce inflammation in liver and lungs and lead to collagen deposition in response to wound healing. The repeated insults lead to apoptosis and necrosis of cells, immune cell infiltration, release of pro-inflammatory cytokines, activation of resident cells into collagen depositing myofibroblast cells, altered tissue architecture and compromised organ function. Administration of Bleomycin, is widely used to mimic the phases of lung inflammation and fibrosis observed in patients with generic pulmonary fibrosis and acute respiratory distress syndrome. A 1:1 mixture of hAEC and hAMSC/human chorionic MSC was administered intra-tracheally or intra-peritoneally into Bleomycin-treated, immunocompetent C57/Bl6 mice [19]. Irrespective of the route of administration, human DNA and cytokeratin-19 positive cells were localized over the two week test period in lungs of mice receiving xenotransplants. Importantly, treated mice showed reduced neutrophil infiltration and fibrosis area whereas macrophage and lymphocyte numbers did not show significant changes [19]. Another study using Bleomycin injured SCID mice tested hAEC [11]. The data showed that following intravenous delivery, some hAEC persisted in the lungs over the four week test period, reduced IL-1, IL-6, TNFα protein levels and collagen in lungs and augmented regeneration leading to improved lung architecture [11].

The toxin carbon tetra chloride (CCl_4) is used to provoke liver fibrosis in mice and rodents. A study investigating the effects of hAEC in CCl_4 injured C57/Bl6 mice, found that following intravenous delivery, cells engrafted and persisted for several weeks in the liver [18]. Similar to effects observed in Bleomycin injured lungs, IL-6, TNFα protein and collagen content declined in the liver. Furthermore, the number of hepatocytes undergoing apoptosis and

number of activated collagen depositing hepatic stellate cells declined significantly in hAEC treated mice [18].

The therapeutic potential of AM derived cells has also been examined in neurological disorders. In particular, stroke has been a major target disease for testing the efficacy of transplantation of AM derived cells. Stroke remains a serious unmet medical condition worldwide and in the US stroke is the primary cause of disability and the third leading cause of mortality. Following the initial stroke episode, inflammation is a major cause of secondary cell death. Although the anti-inflammatory effects of AM derived cells could be beneficial in reducing stroke progression the optimal time and mode of cell delivery need careful assessment. That inflammation may represent a double-edged sword is exemplified in stroke, in that a dynamic modulation of the many inflammatory components in response to ischemic injury is necessary in order to facilitate the functional benefits of cell therapy. For example, the chemokine stromal cell-derived factor-1 or SDF-1, an early pathological inflammatory factor secreted soon after the stroke facilitates the migration of transplanted cells and therefore the early mitigation of SDF-1 may be detrimental. In parallel, stroke may lend a non-conducive brain microenvironment, requiring control of inflammation to some extent to enhance graft survival. To this end, following middle artery occlusion in rats, hAEC and hAMSC were transplanted into the presumed ischemic penumbra (instead of the necrotic core) 2 days after stroke and found to significantly improve motor and neurological deficits by 7 and 14 days and increase the number of healthy host cells within the penumbra [16]. hAEC injection into the penumbra can also lead to reduced infarct size [23]. While direct cell transplantation into the ischemic penumbra is feasible, non-invasive peripheral cell injection may allow a larger patient population to benefit from cell therapy in view of stroke's abrupt onset and rapid progression of debilitating disease symptoms.

In addition to the amniotic cells, the AM membrane itself can exert ameliorative effects and are summarized below.

1.3. Ongoing clinical applications using amniotic membranes

Human AM have a long history in clinical utility. The first application was reported a century ago where the membranes were used as biological dressings to heal skin wounds; a practice that continues to the present day. Currently, AM are also used for treating dermal burns and for open non-healing ulcers and surgical, infected and traumatic wounds [6,24,25]. Since the 1940s, AM have been used in the management of ocular surface disorders. The membrane is used as a graft (with the amniotic epithelium facing outwards) or as a patch (epithelium facing inwards) to cover and repair corneal, conjunctival and limbal defects and surgical incisions made during corrective surgery [26–28]. Further, hAEC and frozen stored AM intact or denuded of the epithelium are being used as feeder layers for the expansion of limbal and corneal stem cells for subsequent transplantation [29]. These ongoing applications led to pre-clinical studies testing the effects of AM on inflammation and fibrosis in lungs and liver.

1.4. Potential innovative applications of amniotic membranes

Recently, small pieces of the entire AM were found to be effective against cardiac ischemia [30] and liver fibrosis [31]. Cardiac ischemia was induced in rats by coronary artery ligation and fragments of AM from human term placenta were applied as patches onto the infarcted myocardium. During the two month follow-up period, treated rats showed improved cardiac dimensions and contractile functions including higher left ventricular ejection fraction, fractional shortening and wall thickening compared to non-treated rats [30].

Liver fibrosis was induced in rats through bile duct ligation (BDL) and AM fragments patched onto the surface of the injured liver [31]. While fibrosis progressed rapidly in controls leading to cirrhosis within 6 weeks of BDL, fibrosis was confined to the portal/periportal regions of the liver in AM-treated rats, without any evidence of cirrhosis and a nearly 50% reduction in collagen deposition [31]. Furthermore, the application of AM significantly delayed the gradual progression of the ductular reaction and reduced the area occupied by activated hepatic myofibroblast cells that deposit collagen [31].

While these initial pre-clinical findings suggests that AM and its' cells may have potential uses in these disease settings, stringent evaluation in larger animal models and comparisons against other cells such as adult bone marrow MSC and hematopoietic stem cells that have also shown to exert beneficial effects and currently being evaluated in clinical trials would be useful. Furthermore, very little is known about the factors that enable survival following xenotransplantation and mechanisms that trigger and lead to the ameliorative effects; some of the potential mechanisms are described below.

1.5. Potential mechanisms involved

A notable feature in the pre-clinical studies outlined above is amniotic cell survival in the absence of overt host responses after xenotransplantation into immunocompetent animals that had not been previously treated with immunosuppressants. Cells that could be transplanted across MHC barriers, without immunosuppression, offer immense scope for wide allogeneic therapeutic applications. hAMSC and hAEC express low levels of HLA Class IA and lack HLA-DR, co-stimulatory molecules CD40, CD80 and CD86 that engage T-cell receptors or are presented indirectly via antigen-presenting cells (APC), to fuel T-cell expansion [1,2,8,13]. One-way lymphocyte reactions have also demonstrated that hAEC and hAMSC fail to induce human T-cell proliferation [32]. Indeed, hAEC have been transplanted into allogeneic volunteers and during trials for lysosomal storage diseases without adverse sequelae attributed to the hAEC [1]. While these studies support the notion that amniotic cells can be transplanted across MHC barriers, generation of antibodies, effects of repeated cell injection as opposed to the possibility of tolerance induction need careful evaluation.

Further, hAEC and hAMSC can modulate immune cell activities. Amniotic cells suppress T-cell proliferation [32,33] with cell–cell contact and trophic factors being likely contributors. Although little is known about the effector molecules responsible, PGE2, TNF-Ra, IL-10, TGFβ and soluble HLA-G from hAEC and/or hAMSC are likely to play a role. hAMSC also inhibit the generation and maturation of APC. In transwell experiments, that only allow passage of soluble factors, hAMSC blocked differentiation and maturation of peripheral blood monocytes into dendritic cells (DC) preventing expression of the DC marker CD1a and reducing HLA-DR, CD80 and CD83 expression [34]. Furthermore, the blockade of monocyte maturation impaired their stimulatory activities on allogeneic T-cells [34]. Investigation into possible mechanism/s showed that hAMSC arrest monocytes in the G0 phase of the cell cycle, abolish TNFα and chemokines CXCL10, CXCL9 and CCL5 whilst greatly elevating the secretion of Th2-related cytokines CCL2, CXCL8 and IL-6 [34]. Effect of hAEC on APC is not known, but hAEC may restrain monocyte migration and activation via MIF-1.

The beneficial effects exerted by AM in the pre-clinical models of myocardial infarction and liver fibrosis, cannot be attributed to cell replacement in the injured tissue. Indeed, no cells derived from transplanted AM were found to have migrated and engrafted into the myocardium or liver. Most likely, the effects observed were associated with the release of soluble factors by cells and molecules

bound to the collagenous stromal matrix of the AM patch that exert paracrine mechanisms to support survival, differentiation and proliferation of host cells. The mechanisms are still undefined, however it has been reported that AM release potent immunomodulatory and anti-inflammatory cytokines (IL-10, IL-6) [35], growth factors associated with wound healing, including angiogenic factors (VEGF, PDGF angiogenin) [36], inducing proliferation (epidermal-, keratinocyte-, hepatocyte- and basic fibroblast growth factors) [37] and differentiation (TGFβ) [36].

The AM was also found to have reduced scarring in the myocardial infarct and livers of animals receiving membrane patches. In ophthalmic investigations it has been shown that hyaluronic acid present in the matrix of the AM can suppress TGFβ and inhibit the differentiation of conjunctival and limbal fibroblasts into myofibroblasts [38]. As TGFβ is a potent pro-fibrogenic cytokine its' reduction can inhibit collagen synthesis. Potentially, similar mechanisms may partly account for the reduction in scarring following the patching of AM. hAEC and hAMSC transplantation was also shown to elicit potent anti-fibrotic effects. Lowering of TGFβ protein was noted in Bleomycin and CCl_4 injured lungs and livers respectively, of mice receiving hAEC, coupled with an induction of collagen degrading matrix metalloproteinases and a reduction of their inhibitors, the TIMP proteins [11,18]. Again paracrine mechanisms induced by factors secreted by the hAEC may be involved. However, while studies show that hAEC are retained for several weeks, cell numbers engrafting are low and decline over time. Further, there is mounting evidence of transdifferentiation of hAEC *in vivo*. Early studies reported differentiation into neural cells, while recent studies report the presence of surfactant protein producing cells in lungs and albumin and α-antitrypsin secreting cells characteristic of hepatocytes in the liver. Whether the growth factor and cytokine milieu of the differentiated cells contribute to inflammation and fibrosis reduction is unknown. A recent study by Tsuji provides insights suggesting that hAMSC differentiating into cardiomyocytes may indeed play such a role [22].

In summary, hAEC and hAMSC have the capacity to differentiate into multiple cell lineages. In addition, the anti-inflammatory and anti-fibrotic effects of these cells and the AM have been demonstrated following transplantation into animal disease models. Ongoing studies relating to safety and efficacy of the transplanted hAEC, hAMSC and AM and mechanisms leading to reparative effects in diseased organs would make a valuable contribution in assessing the true potential of these cells for clinical applications.

Conflict of Interest

The authors state they have no conflict of interest.

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