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Amniotic Fluid Progenitor Cells and Their Use in Regenerative Medicine

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1. Introduction

In recent past, the potential use of stem cells and the advancement in stem cell research for Regenerative Medicine is considered as an alternative therapeutic strategy for a broad range of genetic and acquired diseases.

The interest in stem cells has been increasing over the past years, since their discovery in the early '90s, and they might represent a promising tool for regenerative purposes because of their capability to become almost any cell of an adult organism.

Despite the discoveries and the promising results, many are the controversies raised by stem cells. Feasibility of their use for human therapeutic purposes is regulated by many requirements such as safety, accessibility to a source that can provide an adequate amount of cells for in vitro expansion, absence of ethical issues and repeatability of the results.

Different lines of stem cells are investigated for understanding the basic mechanism of cellular differentiation and the potential for regenerative medicine purposes. However, to overcome safety and ethical issues, scientists are still looking for alternative sources that may provide easy and safe access to a cell population that may be used for cellular therapy.

Amniotic fluid, due to its contact with the fetus, has been considered an interesting source for undifferentiated or partially differentiated cells.

More recently, interest has been rising on more committed cell lines that may possibly provide new, more specific, tools for tissue regeneration. In particular, the isolation of cells already committed to a specific fate has been performed for kidney, pancreas and other organs and the study of these novel cell populations may give us an insight on cellular development and provide a more precise way of driving cell differentiation into a mature cell type.

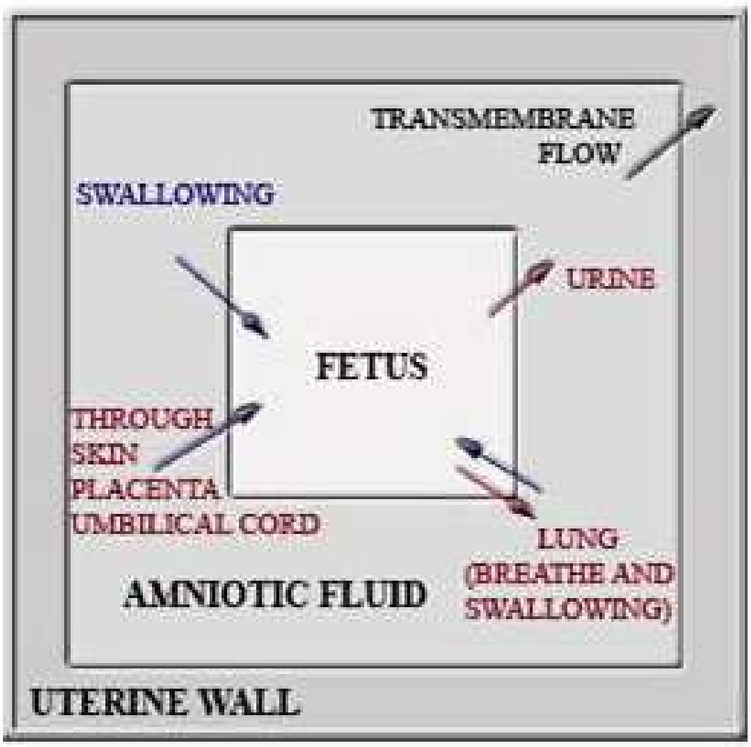
Nevertheless, our knowledge of amniotic fluid cellular composition is still incomplete and only in the last few years some studies have been published describing the different cell types that can be retrieved. As long as new discoveries are shared and new insights are given on amniotic fluid cellular composition and cell differentiation we can gain a better understanding of the mechanism underlying development. The main goal of this chapter is to provide the readers with a broad knowledge regarding the work that has been done until now to undisclose the heterogeneous amniotic fluid cellular composition and their use for regenerative medicine purposes.

2. Amniotic fluid

Amniotic fluid is a clear, fluid that fills the amniotic cavity. It provides an ideal and protective environment in which floats the developing embryo and later on the fetus. It also helps to regulate the temperature of the fetus during the pregnancy.

2.1 Origin and molecular composition

During embryogenesis, maternal plasma is the main protagonist of amniotic fluid volume increase and water flows osmotically through fetal membranes, and, later on, through the placental membrane. The volume and the composition change during pregnancy following the physiological variations of the developing fetus (Fig. 1).



Amniotic fluid composition and volume are the result of exchanges and interaction with many different sources, either fetal or maternal. the above figure shows a schematic representation of the most important overall contributions to amniotic fluid.

Fig. 1. Amniotic fluid origin and composition

During the first weeks of gestation, the composition is comparable to the fetal plasma and its volume increases from 25 ml at 10 weeks to about 400 ml at 20 weeks (Underwood et al., 2005). By 8 weeks of gestation the fetal kidney begins fluid production that rapidly increases in volume during the second trimester. The exchange of fluids through the skin is present until keratinisation that occurs between 20 and 24 weeks of gestation. The molecular composition and the presence of nutritive substances have been shown to play a key role, in animals, in the proliferation and differentiation of various intestinal cell types such as epithelial and mucosa cells (Underwood et al., 2005).

2.2 Amniotic fluid in diagnostic

The use of amniotic fluid to determine the status of health of the fetus has been an important diagnostic tool for many years. Back in the 60s, it was considered an invaluable source of information for the diagnosis of fetal distress, haemolytic disease and fetal maturity (Horger et al., 1969), neural tube defects and lung maturity (Underwood et al., 2005). Over the years, the diagnostic techniques have been greatly improved and new fields of investigation have tried to tie various conditions with preterm labour, infective processes and embryo diseases. In particular, it has been used as a safe and reliable screening tool for genetic and congenital diseases in the fetus.

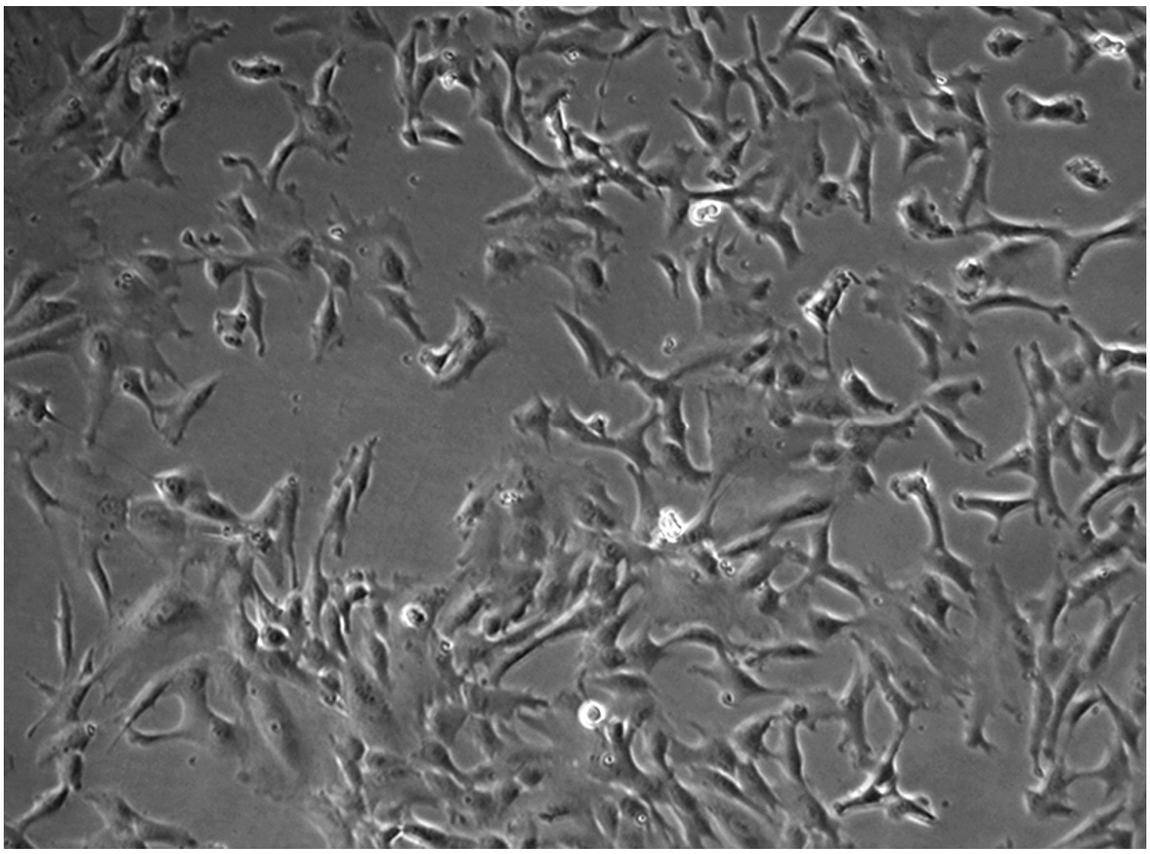
2.3 Isolation, expansion and characterization of amniotic fluid cells

The possibility of using amniotic fluid derived pluripotent and multipotent stem cells has been found appealing due to the relative easiness and safe procedure required to retrieve the cells from its source. Furthermore, the use of multipotent progenitors has been considered an attractive alternative to the use of pluripotent cells due to their already committed phenotype. Cells can be isolated from the liquid collected by amniocentesis. Briefly, prior to amniocentesis an ultrasound is performed to confirm fetal viability, gestational age, number of fetuses, placental location, volume, fetal anatomical survey, uterine cavity abnormalities and to evaluate the best needle insertion site.

A 20 cc syringe is used to aspirate the liquid. The first 2 cc collected should be discharged and then using another syringe and then, using another syringe, additional 15 to 20cc are aspirated.. Removal of the fluid generally takes less than 1 minute. After collection the cells are seeded with specific culture media and the adherent fraction is expanded.

Contact between amniotic fluid and compartments of the developing fetus, such as lung and gastrointestinal tract can explain the presence of different types of cells (Fig. 2). Moreover, cells detaching from the forming kidney or exfoliating from the fetal skin may contribute significantly to cellular composition. In particular, the presence of mature cell lines derived from all three germ layers has been identified (Hoehn et al., 1982; Gosden et al., 1983). Mesenchymal and hematopoetic progenitor cells have also been shown to exist before the 12th week of gestation in humans (Torricelli et al., 1993) together with cells expressing proteins and various genetic markers from specific tissue types including brain, heart, and pancreas have all been discovered (Tsangaris et al., 2004, Bossolasco et al., 2006; McLaughlin et al., 2006; Da Sacco et al., 2010).

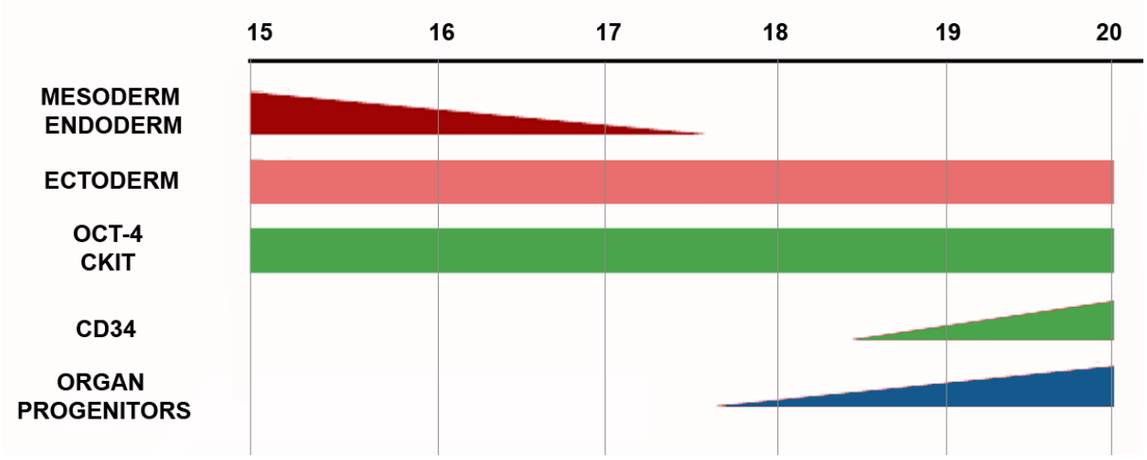
Fauza et al. reported the successful isolation and expansion of unfractioned mesenchymal stem cells (AFMC) from human samples between 20 and 37 weeks of gestation, confirming the presence of a multipotent mesenchymal cell types over the progression of gestation (Kunisaki et al., 2007). A fully characterization of amniotic fluid pluripotent cell population has first been reported by Atala in 2007 (De Coppi et al., 2007). This newly isolated stem cell population (AFSC) is characterized by expression of c-kit, a surface marker expressed by stem cells of mesenchymal origin. AFSC express some surface markers and transcription factors distinctive of ESC such us OCT-4 and SSEA-4 indicating they can actually posses some important characteristics that also ESC have, showing their pluripotential capability. They stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, including CD29, CD44 (hyaluronan receptor), CD73, CD90 and CD105 (De Coppi et al., 2007).



Amniotic fluid cells can be easily collected and expanded in vitro and exhibit a heterogeneous morphology with a preponderance of fibroblastoid, mesenchymal like cell shape (Unpublished Picture, Da Sacco et al.)

Fig. 2. Amniotic fluid cells morphology

However, a comprehensive analysis of amniotic fluid cellular composition has been missing and only in 2010 Da Sacco et al. for the first time demonstrated that the cellular composition varies in a timely fashion (Fig.3). Expression of markers for cells belonging to early endodermal and mesodermal germ layer differentiation pathway is predominant in the earlier weeks of gestation and constantly decreases over time to disappear at 17-18 weeks of gestation. Ectodermal markers, probably because of the exfoliating fetal skin, maintain a stable expression in all the samples analyzed. Interesting, it is shown that concurrent with the decrease of germ layers markers there is a noticeable increase of organ specific progenitor cell marker expression. Proteins expressed during lung, liver, heart and kidney differentiation are highly expressed starting form 17-18 weeks while expression of pluripotent markers such as OCT-4 and c-kit was found stable over time in all the samples analyzed, suggesting that, at least in the time range analyzed, the pluripotent cells are undergoing self renewal. Furthermore, mesenchymal marker CD90 is present in all the samples analyzed while hematopoietic marker CD34 decreased its expression over time.



Mesodermal and endodermal cellular markers decrease over time and are not detectable after 17-18 weeks of gestation while various organ progenitor cell markers concentration rise after 17-18 weeks. Pluripotent markers OCT-4 and c-kit remain unchanged, as well as ectodermal markers, probably because of skin exfoliation. CD34 cellular marker rises after 18 weeks of gestation.

Fig. 3. Schematic representation of changes in composition within amniotic fluid between 15 and 20 weeks of gestation. (Da Sacco et al., 2010)

3. Amniotic fluid cells and organ specific regenerative medicine

Due to an easy and safe collection procedure, amniotic fluid has quickly gained interest as a potential source of pluripotent/multipotent cells for regenerative medicine purposes. Amniotic fluid stem cells have been shown to be easily cultured and expanded upon collection and isolation De Coppi et al., 2007, Perin et al., 2007, Da Sacco et al., 2010, Kunisaki et al., 2007). De Coppi et al. (De Coppi et al., 2007) and Arnhold et al. (Arnhold et al. 2011) proved that after c-kit selection these cells are still exhibiting optimal growth rate. Their potential for differentiation has been proved in many published works and cells can be retrieved from different species like humansDe Coppi et al. 2007, Perin et al., 2007, Da Sacco et al., 2010), pigs (Zheng et al., 2010), goat (He et al., 2011; Zheng et al., 2011), mouse (De Coppi et al., 2007) and buffalo (Yadav et al., 2010).

A recent report following a comparative analysis of AFSC and BM-MSC cells on proliferative potential and immunogenicity analysis showed the AFSC are less immunogenic and harbor a higher proliferation rate than BM-MSC (Mirebella et al., 2011).

3.1 Amniotic fluid cells and kidney

The complexity of the kidney and the multiple functions of the renal compartment are a great challenge to a successful therapeutic approach for its recovery and the regeneration. Beside the use of endogenous stem cells and other traditional and advanced therapies, the administration of exogenous stem cells, including AFSC has been proposed (Perin et al., 2011).

In the recent past, Perin et al. showed the capability of AFSC to participate in vitro to the development of embryonic kidneys. In particular, cells labeled with the surface marker CM-Dil were shown able to integrate within the structures of the developing kidney. Integration into the metanephric structures was additionally confirmed by the migration of the injected

cells to the periphery of the embryonic kidney. This data strongly correlates to the centrifugal pattern of induction, morphogenesis and differentiation of the metanephros, proceeding from the center to the periphery of the embryonic organ (Perin et al., 2007).
Moving into an in vivo model, the same group for the first time proved the potential of human AFSC to participate to the regeneration of kidneys undergoing acute tubular necrosis (Perin et al., 2010). After intra renal injection, cells were showed to survive, integrate into renal structures, and differentiate into tubular cells expressing proximal as well as distal epithelial tubular markers and persist over the long term.
However, as the Authors highlight in their study, the main mechanism of action seems to lie into the ability of AFSC to modulate the immune response by lowering pro-inflammatory cytokines while stimulating the expression of anti inflammatory molecules, and by lowering apoptosis and increasing endogenous proliferation.
On a different model of acute renal injury, Camussi's research group confirmed the positive results and the comparable efficacy between BM-MSC and AFSC (Hauser et al., 2010).
Beside the use of pluripotent cells, in 2010, we reported the isolation and characterization of more committed Amniotic Fluid derived Kidney Progenitor Cells (AFKPC) (Da Sacco et al., 2010). Cells expressing both CD24 and OB-Cadherin were sorted and characterized for a wide range of kidney markers such as PAX-2, LIM-1, GDNF, ZO-1. Additional selections were performed on the CD24+OB-cadherin+ cells to isolate cells committed to mesangial differentiation, podocyte differentiation, mesenchymal to epithelial transition cells and vascular progenitors. Characterization of marker expression for these subpopulations showed significant differences in gene expression, confirming their different commitment to renal fate.

3.2 Amniotic fluid cells and lung

In uterus, the developing lungs of the fetus are filled with fetal lung liquid which is actively secreted into the amniotic fluid. In the late gestational period, surfactant produced by the fetal lungs contributes to the composition of amniotic fluid and can be measured to determine the developmental stage of the surfactant system within the organ. Contact between the developing lung and the fluid make it a possible important reservoir for cells to be used in lung regenerative medicine. In fact, AFSC were shown able to integrate and proliferate into mouse embryonic lung and express human lung epithelial cell markers (Carraro et al., 2008).
Following hyperoxia injury, a tail vein injection of cells into nude mice showed localization in the distal lung with expression of both TTF1 and type II pneumocyte marker surfactant protein C. In the same work, specific Clara cells damage through naphthalene injury was followed by integration and differentiation of AFSC at the bronchioalveolar and bronchial positions with expression of specific Clara cell 10-kDa protein (Carraro et al., 2008). The positive results obtained by Warburton's research group were the first to prove the use of AFSC for in vivo organ regeneration. However, as underlined by the author, the number of cells homing and integrating within the lung was considerably low and the effects on tissue regeneration may be due on mechanisms different from integration and proliferation. However, our knowledge on this field is still lacking and more studies should be performed to clarify molecular pathways and suggest a plausible mechanism of action.

3.3 Amniotic fluid cells and heart

Heart failure remains one of the major causes of mortality in the United States (Honold et al., 2004). Stem cells have been proposed as an alternative, innovative approach for the

treatment of heart disease and cardiac differentiation. AFSC have been tested in the past years for their potential of becoming functional cardiomyocytes.
Hoerstrup's research group used amniotic fluid derived cells to successfully repopulate heart valves. After isolation, CD133- and CD133+ cells were isolated, characterized and subsequently seeded onto tissue engineered scaffolds. Feasible heart valve leaflets were obtained in vitro with the use of both fibroblast-like and endothelial like cells (Schmidt et al., 2007). However, Chiavegato et al., in 2007 showed that injections of human AFSC into a rat normal or ischemic myocardium was ineffective and cells were targeted by the immune response with consequent rejection of the xenotransplanted cells. On the other hand, the use of a xenotransplantation model, even when cells were injected in immunodeficient animals, may not be ideal for immunogenicity studies.
New insights on the cardiomyogenic potential of amniotic fluid cells have been published in 2010 by Soker et al. showing the in vitro capability of AFSC to be differentiated into cardiac cells when co-cultured with rat cardiomyocytes (Guan et al., 2010). Along with this work, Sung's research group reported the differentiation of AFMC into cardiomyocytes and endothelial cells (Yeh et al., 2010). Bollini et al. in two different works demonstrated the potential of AFSC to differentiate into cardiomyocytes both in vitro (Bollini et al., June 2011) and in vivo showing their cardioprotective effect following acute myocardial infarction (Bollini et al., May 2011).
In summary, the results obtained with amniotic fluid derived stem cells for cardiomyocyte differentiation are contrasting, mostly due to the lack of a specific model and the use of different species and differentiation protocols. More studies should be performed in order to truly confirm their capability to provide an effective tool for cardiovascular regenerative medicine.

3.4 Amniotic fluid cells and hematopoietic system

C-kit positive/ Lin – cells derived from both human and mouse, have been shown to have hematopoietic potential (Ditadi et al., 2009). These cells were capable of differentiating into erythroid, myeloid, and lymphoid lineages in vitro as well as in vivo, in the peripheral blood of irradiated mice. Furthermore, single cells analysis was able to assess the expression of several genes important during different stages of hematopoietic differentiation.

3.5 Amniotic fluid cells and pancreas

The occurrence of pancreatic damage and diabetes has dramatically increased in the last years. The rise of this emergency has strongly encouraged physicians and scientist to search for alternative therapeutic approaches. In 2009 was suggested that stem cells derived from amniotic fluid could be of use for pancreatic regeneration (Furth et al., 2009).
However, the first attempts to differentiate amniotic fluid cells into functional pancreatic cells were unsuccessful. In fact, the use of obestatin, a molecule proven to efficiently increase expression of pancreatic beta cell genes, was unable to stimulate pdx-1 expression these cells (Trovato et al., 2009).
A better knowledge of developmental pathways and gene cascades involved in pancreatic specification brought, a year later, to a growing number of successes. In fact, differentiation into pancreatic cells was proven using a variety of different procedures. In particular, transfection with the PDX-1 gene was able to induce pancreatic features on cells from AFMC (Gage et al., 2010).

With an interesting approach, Li et al. were able to prove differentiation into insulin producing cells by silencing several neuronal genes by use of small interference NRSF RNA. This was shown as crucial for pancreatic differentiation and for the expression of pancreatic markers including Pdx1, Hnf4α, Isl-1, Nkx6.1, Insulin, and Glut (Li et al., 2010). A different approach was taken by Zou et al. Knowing that the expression of particular surface markers can identify cell populations with specific traits and defined commitment, a CD44+/CD105+ population was isolated and successfully differentiated into pancreatic cells expressing PDX-1 (Zou et al., 2011). The increasing number of studies reported in the last two years suggests that the interest for amniotic fluid cells for beta cell differentiation is a growing research subject. Moreover, differentiation into pancreatic beta cells is been proven as possible in vitro settings. However, no in vivo studies have been published reporting their potential in acute and chronic pancreatic diseases.

3.6 Amniotic fluid cells and brain

The differentiation of pluripotent and multipotent cells into neural cells has been considered fundamental for understanding brain differentiation and for the establishment of innovative approaches for the healing of brain injuries. Many different studies have been performed on amniotic fluid cells and their expression of neuronal markers. However, their ability to differentiate into functional brain cells has being highly debated.

First reports on amniotic fluid progenitor cells commitment to neuronal cell lineage were published in 2006. In fact, McLaughlin's research group reported the ability to isolate and expand them in culture. Their studies showed that these novel progenitors are committed to mesencephalic dopaminergic neurons (McLaughlin et al., 2006). AFMC were shown to be able to differentiate into brain cells both in vitro (Prusa et al., 2004, Tsai et al., 2004; Tsai et al., 2007; Jiang et al., 2010, Mareschi et al., 2009) and in vivo (Cheng et al., 2010). Selection of specific cell population based on specific surface marker expression didn't show to really improve the neuronal potential of amniotic fluid cells. Cells isolated by use of different surface markers like c-kit, (De Coppi et al., 2007), sox-2 (Jezierski et al., 2010) were shown able to differentiate into neuronal like cells. However, in 2009 was reported the inability of AFSC to differentiate into dopamine neurons both in vitro and in vivo assays (Donaldson et al., 2009). An interesting recent study, investigated the impact of extracellular signals on neural differentiation, where it was confirmed that extracellular matrix has an essential role on neurogenic differentiation and therefore regulates its efficiency (Orciani et al., 2011).

While many studies seems to prove that differentiation of amniotic fluid stem cells, either AFSC or AFMC, into neural cell types, there are still too many open questions about functionality of the differentiation, ideal cell population and best differentiation cocktail. While the current status of the research gives great hope for the future, to confirm of deny the possible use of amniotic fluid cells for brain regeneration more in vitro and in vivo data are certainly required.

3.7 Amniotic fluid cells and liver

Only a few studies have been reported that investigate the potential of amniotic fluid derived cells for hepatocyte differentiation. Zheng et al. in their work, claimed that AFSC had a better response to the differentiation when compared with BM-MSC under the same conditions (Zheng et al., 2008). Later on, differentiation into the hepatic lineage was

successfully obtained by Gasbarrini research group (Saulnier et al., 2009) that showed the equal potential of adult and fetal derived cells, including AFSC, for liver regeneration.
However, beside these encouraging results, more studies are required prior to confirm the suitability of amniotic fluid stem cells for liver therapy.

3.8 Amniotic fluid cells and bone

In 2010, it was reported a positive effect of transient ethanol exposure during early differentiation of AFSC into osteoblasts (Hipp et al., 2010).
Papaccio's research group showed the ability of AFMC to differentiate into bone cells when co-cultured with dental pulp cells proving potential for bone engineering (De Rosa et al., 2010).
Osteogenic progenitors have been found within amniotic fluid (Antonucci et al., February 2009). In this work, they were able to obtain calcium mineralization and osteogenic differentiation of AFMC. Expression of various osteogenic markers after 30 days in culture was demonstrated. Similar results were obtained by two other research groups (Antonucci et al., October 2009, Steigman et al., 2009 and Sun et al., 2010).
Peister in 2011 showed that AFSC were capable of a greater differentiation potential compared to mesenchymal stem cells although the latter response to the differentiative cocktail was occurring at earlier times (Peister et al., 2011). However, in vivo data are still lacking and the osteogenic potential of amniotic fluid cells in a complex environment should be undisclosed.

3.9 Amniotic fluid cells and chondrocytes, adipose tissue and skeletal muscular cellular differentiation

3.9.1 Chondrocytes

The regenerative capacity of the cartilage is limited. The ability to differentiate stem cells into cartilage may provide a better alternative to primary culture of chondrocytes that in vitro dedifferentiate losing their characteristics (Kramer et al., 2008).
Fauza's research group demonstrated the ability of ovine AFMC to successfully differentiate into chondrocytes on 3D scaffolds expressing several markers of cartilage (Kunisaki et al., 2006). Atala's group showed the ability of AFSC to differentiate into chondrocytes (De Coppi et al., 2007). However, no functional studies were performed to confirm the possible use of these amniotic fluid derived cartilage cells.

3.9.2 Adipocytes

Adipocyte differentiation was proven in 2007 for AFSC when these cells were first characterized and tested for their pluripotentiality (De Coppi et al., 2007).
In addition, adipogenic differentiation for goat derived AFMC was shown in 2011 (He et al., 2011) proving their differentiative potential.

3.9.3 Myocytes

Muscular tissue is well known to harbour endogenous stem cells that help recovering the tissue after an injury. However, the differentiation potential of these pluripotent stem cells and when the extent of the injury, due to an acute or chronic insult, is too heavy, muscular degeneration occurs with loss of motility and impaired function. The study of cells feasible

for muscular differentiation and regeneration has been considered essential for a successful therapeutic approach.
Amniotic fluid cells have been studied for their capability to differentiate into functional muscular cells. In particular, Streubel reported using non-hematopoetic AFMC for the conversion of amniocytes into myocytes. (Streubel et al., 1996). De Coppi showed the ability of AFSC to differentiate into myocites in vitro by expression of markers expressed by the differentiating and mature muscle fibers (De Coppi et al., 2007) and the results were later confirmed by studies both in vitro and in vivo on scid mice (Gekas et al., 2010)

4. Amniotic fluid derived cells and their role as cytokine modulators

In the last years new evidences have been found that correlates the administration of stem cells with the modulation of inflammatory and fibrotic processes through cytokine mediated cross-talk between the pluripotent cells and the surrounding environment. New studies have highlighted the possibility that the same mechanism of action can be used to explain the effect of amniotic fluid stem cells in many diseases. In particular, Perin showed that in a murine model of acute tubular necrosis, the expression of inflammatory cytokines is strongly regulated after injection of AFSC (Perin et al., 2010). Down regulation of pro-inflammatory molecules and up-regulation of pro-regenerative and anti-flogistic cytokines resulted in a faster regeneration of the damaged tissue with higher proliferation rate, lower apoptosis and an overall better physiological profile of different renal parameters. A broad study performed by Yoon (Yoon et al., 2010) investigated the in vitro production of cytokines by AFMC in the cultured media. Presence of several inflammatory molecules was reported such as IL-8, IL-6, TGF-β, TNFRI, VEGF, and EGF and other molecules involved in the TGFB/SMAD2 pathway. The conditioned culture media proved to be useful for enhancing wound healing in an in vivo murine model. While studying the angiogenic potential of AFSC, Teodolinda et al. (Teodolinda et al., 2011) reported the ability of the cells to produce and release several cytokines and chemoattractant molecules that are able to modulate not only the vessel growth but also the activity of macrophages/monocytes and other cells involved in inflammation and immunoresponse.

5. Conclusions

In the last few years, an increasing number of studies have been performed on amniotic fluid derived stem cells and progenitor cells. Exciting results have been reported on amniotic fluid cell population characterization of composition, growth kinetics and potential for specific organ regeneration. However, further investigation is still required to completely categorize cells according to origin and function. Improving the efficiency and specificity of differentiation into various mature and functional cell types to prevent their attrition towards unrelated cell types would be an important factor to control in regenerative medicine applications. In this very same direction, the establishment of protocols and differentiative media will better allow us to compare the different populations and understand their mechanism of action. In addition, knowledge about how the different compartments of the developing fetus are contributing to the cellular composition may undisclose important information about the development and the amniotic fluid composition.

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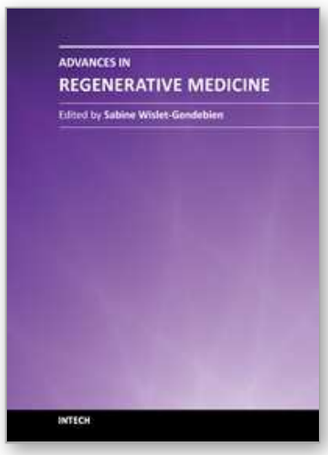

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Even if the origins of regenerative medicine can be found in Greek mythology, as attested by the story of Prometheus, the Greek god whose immortal liver was feasted on day after day by Zeus' eagle; many challenges persist in order to successfully regenerate lost cells, tissues or organs and rebuild all connections and functions. In this book, we will cover a few aspects of regenerative medicine highlighting major advances and remaining challenges in cellular therapy and tissue/organ engineering.

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