Amniotic Fluid Stem Cells: a Promising Therapeutic Resource for Cell-Based Regenerative Therapy

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Abstract: Stem cells have been proposed as a powerful tool in the treatment of several human diseases, both for their ability to represent a source of new cells to replace those lost due to tissue injuries or degenerative diseases, and for the ability of produce trophic molecules able to minimize damage and promote recovery in the injured tissue. Different cell types, such as embryonic, fetal or adult stem cells, human fetal tissues and genetically engineered cell lines, have been tested for their ability to replace damaged cells and to restore the tissue function after transplantation. Amniotic fluid -derived Stem cells (AFS) are considered a novel resource for cell transplantation therapy, due to their high renewal capacity, the "*in vitro*" expression of embryonic cell lineage markers, and the ability to differentiate in tissues derived from all the three embryonic layers. Moreover, AFS do not produce teratomas when transplanted into animals and are characterized by a low antigenicity, which could represent an advantage for cell transplantation or cell replacement therapy. The present review focuses on the biological features of AFS, and on their potential use in the treatment of pathological conditions such as ischemic brain injury and bone damages.

Keywords: Cell-based therapy, amniotic fluid, amniotic fluid-derived mesenchymal stem cells.

INTRODUCTION

Regenerative medicine is a recently developed multidisciplinary discipline aimed to replace or restore normal function of cells, tissues, and organs that are damaged by disease [1-19]. Unlike conventional surgical therapies, which, although including some element of reconstruction, remain predominantly ablative for most diseases, regenerative medicine incorporates the fields of tissue engineering, cell biology, nuclear transfer and materials science to allow a truly regenerative approach, by reconstructing, repairing, or replacing missing or damaged tissue to a state as close as possible to its native architecture and function. In the last two decades, regenerative medicine has shown the potential for ''bench-to-bedside'' translational research in specific clinical settings [5]. A crucial point in regenerative medicine is represented by the cell type to be transplanted in the affected patients, alone or in conjunction with specific biomaterials i.e. scaffold. Based on their origin, whether from the individual to be treated or from a donor, these cells can be defined as autologous or allogenic [20-23]. Autologous cells would represent the ideal transplantation source, since they are not rejected by the immune system, avoiding the use of immunosuppressant drugs. Nevertheless, the use of these cells is limited by their low "ex vivo" expansion abilities, particularly in cases of patients with end-stage organ disease who do not produce enough cells for transplantation [1].

In these cases, the use of allogenic cells may represent an advantage, but this may require the creation of specific cell banks, able to provide a large number of samples from different donors immunologically matched with the potential patients.

For both purposes ("ex vivo" expansion of cells collected from affected patients for autologous transplantations vs banking of cells from different donors for allogeneic transplantation) the biological features of the selected cells represent a crucial point. In order to be used for regenerative medicine on a large scale, the cells to be transplanted must show the following features:

a *Accessibility*: the collection of cells should not require the use of invasive procedures,

b *Availability*: the cells should be readily available in large number for transplantation,

c *Plasticity*: the cells should be able to differentiate in different cell types,

d *Mitotic stability*: the cells should retain a stable karyotype along several cell divisions,

e *Low* risk: the use of the cells should not induce transplanted related diseases, such as immunoreaction and cancer

Table 1. Differentiation Abilities of c-kit+ vs Unselected AF Cells

Lineage	c-kit+	Unselected
Osteogenic	+	+
Adipogenic	+	+-
Chondrogenic	+	+
Hepatocytic	+	+
Myogenic	+	+
Cardiomyogenic	+-	+-
Endothelial	+	+
Blood	+ (only Lin- cells)	-
Neural	+	+

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1873-4286/12 $58.00+.00

- *Ethical acceptability*: the use of the cells should avoid ethical concerns.

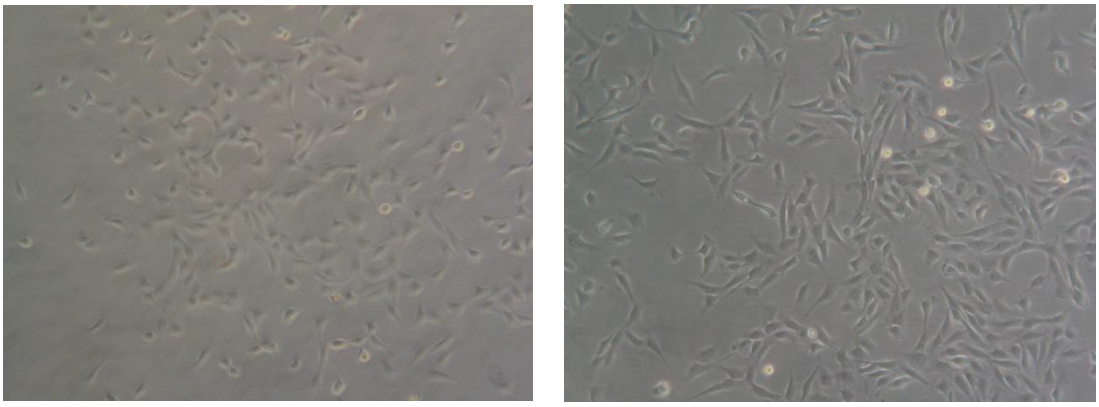
Another crucial point for cell based medicinal products is the limited availability of classical toxicology studies from the preclinical development. Human cells may be administered only to immunedeficient animals to avoid the host rejection of the donor cells. Homologous models using cells from the same species to simulate the behavior of the cell based medicinal product might be limited by the differences in cell physiology between species. Thus, clinical study should be performed with the highest attention to the safety and ethical issue involved, with an accurate analysis leading to perform the best clinical development possible with the lowest risk for the patients involved [6]. In the last years, several studies have explored the potentiality of different cell types for their use in regenerative medicine through "*in vitro*" and pre-clinical experiments on animal models, and great attention has been focused on the stem cell model. Stem cells are able to divide through asymmetric mitosis leading on one side, to the differentiation into diverse specialized cell types and on the other side, to their self renewing and to the production of more stem cells [24]. Stem cells are usually classified in two broad types: embryonic stem (ES) cells, isolated from the inner cell mass of blastocysts [25], and adult stem cells, found in various tissues in adult organisms and acting as a repair system for the body [2, 26-33]. ES cells are considered pluripotent stem cells, being able to to grow indefinitely and to differentiate in tissues deriving from all three germinal layers [34], while adult stem cells are considered to be multipotent cells, being able to differentiate in a limited number of specific tissues.

Although ES cells show the highest abilities in terms of pluripotency and proliferation, their use for cellular therapy in humans is hampered by their high risk of tumorigenesis [35-37]. In fact, ES cells present cellular and molecular features very similar to those showed by tumour cells and cancer cell lines, such as rapid proliferation rate, lack of contact inhibition, genomic instability, high telomerase activity, high expression of some oncogenes, and important similarities in their overall gene expression patterns and epigenetic status [38]. Not surprisingly ES cells form teratomas when injected into immunodeficient mice [36]. Furthermore, treatment protocols in animal models using ES were shown to be fatal, inducing the formation of teratoma-like tumors [39-40]. Due to this risk of cancer formation, and to the ethical controversies raised by the use and sacrifice of human embryos, ES cells are no longer proposed as a useful source of cells for regenerative medicine in human [41-43]. On the other hand, great interest has been devoted to the discovery of human induced pluripotent stem cells (iPSCs) [44-51]. This is due largely to the novelty of a much greater plasticity of somatic cells than previously thought [52-61] and to the ability to provide a robust model for autologous, patient-specific cell therapy, without any of the ethical concerns related to the use of ES cells [44, 62-71]. iPS cells have been originally derived from adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions, showing the morphology and growth properties of ES cells and express ES cell marker genes [44]. Thus, iPS cells and ES cells share the basic properties of self-renewal and pluripotency [51] . However, they also both exhibit cellular and molecular phenotypes resembling cancer cells and induce the formation of teratomas injected into immunodeficient mice [67, 72-74]. Thus, while the studies on IPs have been largely directed to their analysis in patients with different pathologies, due to their unique ability to modelling different human diseases [75-102], adult stem cells appear to remain so far the only available source of cells to be used in the field of regenerative medicine in the clinical practice, also considering their ability to be reprogrammed and contribute to a much wider spectrum of differentiated progeny than previously anticipated under appropriate microenvironmental cues. [103].

Different sources of adult stem cells have been identified, such as mesenchymal cells from bone marrow (BM) [104-113], umbilical cord [114-131] , placenta [132-135], amniotic fluid [136-139], adipose tissue [140-149] and other tissues [29, 150-157]. In this review, we will focus our attention to the Amniotic Fluid Stem cells (AFS), which represent an interesting model due to their unique features and the possible advantages of their use in regenerative medicine.

The amniotic fluid (AF), contained in the sac of membranes known as the amnion, surrounds the embryo and foetus, protecting it from outside injuries and acting as a vehicle for the exchange of body chemicals with the mother. AF samples can be easily collected from women undergoing amniocentesis for prenatal diagnosis. Cultures can be obtained starting from as little as 2 mL AF, the amount which is usually discarded after diagnosis. Since in western countries a large number of women require prenatal diagnosis during pregnancy, a huge amount of AF samples can be easily collected in the structures performing prenatal genetic investigations. In recent years, different reports have demonstrated the presence of stem cells in human AF able to differentiate into multiple lineages [137, 139, 158-169]. Human AF obtained during the process of amniocentesis (around the 16^{th} week of pregnancy) contains a heterogeneous population of cell types originating from embryonic and extra-embryonic tissues [137] . These cells can be classified into three types: epitheloid (E-type) cells, derived from fetal skin and urine; amniotic fluid specific (AF-type) cells, derived from fetal membranes and trophoblasts; fibroblastic (F-type) cells, derived from fibrous connective tissue and dermal fibroblasts [170-171]. The first two cell types are invariably found in the beginning of AF culture samples. Only the AF-type cells, however, persist during the cultivation process, while it has been observed that E-type cells rapidly show a significant decrease [172-173]. Conversely, F-type cells are not invariably found in AF, appearing after 3 days of culture in 85% of samples [173]. About 1% of the cells in AF cultures express the surface antigen c-Kit (CD117) [138], a receptor present on ES, primordial germ cells and many somatic stem cells. Isolated cells grow rapidly in culture and are capable of more than 250 population doublings [138]. Importantly, AFS cells display a normal karyotype and maintain telomere length during long-term culture [169]. AFS are broadly multipotent, and have been induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages, never showing signs of aging and tumorigenicity even after living for more than 2 years in culture [138, 174-178]. Moreover, it has been demonstrated that AFS cells can become nestin-positive neural stem cells, and then dopaminergic and glutamate-responsive neurons, although the ability to produce functional neurons is still debated. In appropriate medium, these cells also form functional osteoblasts producing mineralized calcium . Furthermore, hepatic lineage cells obtained by differentiation of AFS cells were able to secrete high levels of urea and express liver proteins such as albumin, α-fetoprotein, hepatocyte nuclear factor and growth factor [138 ,179].

This feature of clonal AFS led some authors to classify these cells as pluripotent stem cells [138, 179]. As a matter of fact, AFS cells appear to be different both from pluripotent ES cells and from multipotent adult stem cells, and may represent a new class of stem cells with properties of plasticity intermediate between embryonic and adult stem cell types. In fact, about 90% of AFS express the specific markers of embryonic carcinoma cells, embryonic germ cells, and ES cells, like Oct-4 and TERT [138, 159, 180-182]. However, unlike ES cells, AFS are not tumorigenic after transplantation in mice, thus representing an intermediate stage between embryonic and adult stem cells in terms of their versatility [138].



a) **b)**

Fig. (1). a) AFMSCs obtained after 8 days of culture. **b**) AFMSCs obtained after 10 days of culture

AF MESENCHYMAL STEM CELLS (AFMSCS)

AFS show the typical "fibroblast-like" morphology similar to those of mesenchymal stem cells (MSCs) derived from other sources (umbilical cord, blood and bone marrow) Fig. (**1a** and **1b**). This morphology is characterized by the presence of a small cell body with a few long and thin cell processes. The cell body contains a large, round nucleus with a prominent nucleolus, surrounded by finely dispersed chromatin particles, and a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria, and polyribosomes. These cells are positive for mesenchymal markers such as CD90 (Thy-1), CD105 (SH-2, Endoglin), CD73 (SH-3, SH-4), CD166, CD29 and CD49. On the other hand, AFS cells are negative for the hematopoietic markers such as CD45 (Leucocyte Common Antigen), CD34 (Siamolucin) and CD14 (LPS receptor) [138, 183-184]. Most adherent amniotic cells present in a typical AF sample (80-90%) represent mesenchymal cells [184]. In a recent study, the frequency of AFMSCs in AF was estimated to be between 0.9 and 1.5%, while approximately 2.7 x 10^5 cells can be isolated at starting culture from each sample [185]. The group of In't Anker [186] obtained 180x10^6 AFMSCS after 4 weeks of culture, while Kim *et al*. [187] obtained an estimated number of 7.7 x10^{23} cells after 27 passages and 66 cell doublings. AFMSCs have been successfully cultured over a period of 8 months, displaying a high proliferation rate and a stable karyotype [187].

About 90% of AFMSCs express Oct-4 mRNA and Oct-4 protein, a transcription factor playing a key role in the maintenance of pluripotency [158,161]. Oct-4 is active in the oocyte and after fertilization remains active during the first steps of embryo development throughout the preimplantation period. Oct-4 expression is associated with an undifferentiated phenotype and play a crucial role in human embryonic stem cell self-renewal. High expression levels of this gene have been noted in AFS only until the 19th passage [187]. Since AFMSCs reach senescence at the 27th passage, the disappearance of Oct-4 expression at the 20th passage may be related to the onset of aging at this time [187].

AFMSCS ISOLATION

Selected AFMSCs

Literature data show that different protocols have been used for the isolation of AFMSCs and their differentiation into several cell lineages. Some authors have reported the use of the immunoselection with c-Kit specific antibodies in order to isolate AFMSCs starting from confluent human amniocentesis cultures [164, 138]. C-Kit (CD117) is a protein that in humans is encoded by the KIT gene with multiple transcript variants encoding different isoforms. CD117 is a cytokine receptor expressed on the surface of hematopoietic stem cells as well as other cell types, and its activity is mediated by the binding to the cytokine stem cell factor. The selected c-Kit cells have been shown to express several embryonic stem cell markers such as Oct-4, Nanog, and SSEA-4, although other markers like SSEA-3 or Tra-1-81 have not been detected [138]. c-Kit+ cells are also positive for several mesenchymal markers (vimentin, CD105, and CD90) and negative for hematopoietic markers such as CD34, CD45, and CD133 [138].

Unselected AFMSCs

Although the majority of studies about AFMSCa are based on c-Kit selected cells, it has been reported that CD117 positive cells from human AF produce abnormal cell differentiation and host immune response after transplantation in rat myocardium [188]. In fact, although it was expected that AFS cells would have been protected from immune rejection, these cells were surprisingly rejected when transplanted in a xenogenic immuno-competent host. Authors suggested that the procedure of AFS cell isolation, i.e., a cell sorting based on expression of c-Kit, could be responsible for the powerful host immune response and concluded that the c-kit-sorted cell subpopulation of human AF is not endowed with a tolerogenic potential suitable for these cells to survive in the immunoincompatible rat heart [188]. As a consequence, it can be hypothesized that a protocol based on the direct culture of AF cells without any selection could provide a better chance for homing and differentiation after transplant. Several groups have directly cultured unselected amniotic fluid cells in media allowing the proliferation of AFMSCs, and subsequently induced their differentiation [137, 159-161, 165-166]. These studies have demonstrated that also unselected AFS cells are able to differentiate in several lineages. However, a crucial point is to evaluate if the stemness and differentiation ability of these unselected cells are identical or different to those of c-Kit+ AFS cells. In a recent review, Davydova [189] addressed this question by analyzing literature data concerning the specific properties of unselected AFS cells in terms of stemness and differentiation ability. Based on the reported data, there is good evidence that both cells types are able to differentiate in tissues deriving from the three embryonic germ layers, but with some specific differences. In fact, adipogenic differentiation appears to occur invariably in c-Kit+ AFS cells, while contradictory data have been reported about the adipogenic differentiation in unselected AFS cells. Tsai *et al*. [161-162] and Kim *et al*. [187] reported that unselected AFS cells are able to differentiate in adipocytes, as evidenced by red oil staining. On the other hand, Saulnier *et al*. [190] reported that AFMSCs were not able to undergo adipogenesis in their culture conditions. Blood forming stem cells are not present in AF at the time of amniocentesis (16-18th week of gestation), being present in human AF only early in pregnancy. As a consequence, neither un-

selected nor c-kit+ cells are able to differentiate in blood forming cells. However, it has been demonstrated that selected c-Kit+Lin- AFS cells are capable of forming erythroid, granulocyte-macrophage and mixed colonies [191]. In fact, when cultured under appropriate differentiation conditions, murine and human c-Kit+ Lin- cells were able to generate all the blood lineages, although human cells generated immature T-cell precursors only, suggesting that the used T-cell culture conditions were not fully appropriate for this population [191].

Taken together, these data seem to suggest that c-Kit+ and unselected AFS cells show similar but not identical properties. It must be stressed, however, that in many cases other factors such as different culture conditions can be invoked to explain such differences. In fact, it has been reported that a high serum content (20% FBS) together with the use of alpha MEM medium promotes the selection of a mesenchymal phenotype [161, 192]. Moreover, some culture strategies can improve the effectiveness of differentiation protocols. Such is the case of a recently reported study, which describes a novel single step culture protocol able to differentiate AFMSCs into osteogenic cells, allowing a 20 days reduction of the culture time, thus achieving a full differentiation within one month from withdrawal [165] Fig. (**2**).

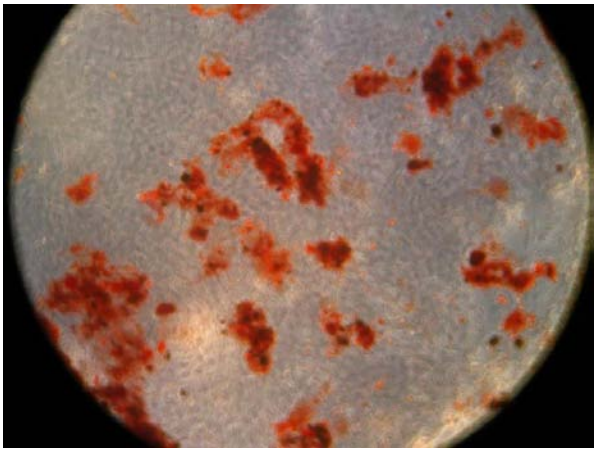


Fig. (2). Alizarin red staining of AFMSC after osteoblastic differentiation (22 days of culture in osteogenic medium)

AFMSCS DIFFERENTIATION

As above described, AFS cells have been induced to differentiate into cell types derived from each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. Here we report a more detailed analysis of literature data concerning some of the most investigated differentiation models, namely osteogenic, neurogenic and cardiomyogenic differentiations.

Osteogenic Differentiation

The ability of AFS cells to differentiate in osteogenic precursors have been demonstrated by several authors which demonstrated an excellent ability of AFS cells to differentiate in osteogenic precursors and to produce *in vivo* mineralized matrix and bone tissue [138, 165-166, 174, 193-197]. These results has been achieved using both c-kit selected [138] and unselected AFS [165-166, 174, 193-197] In particular, it has been demonstrated that osteogenic differentiation can be achieved in a very short time when using a single step culture protocol [165]. Interestingly, it has been evidenced that the treatment with ethanol during the first hours of the differentiation protocol alters this process, producing an enhancement in the osteogenic differentiation, demonstrated by an increase of "*in vitro*" calcium deposition alkaline phosphatase activity [194]. Moreover, the ability of differentiated AFS cells to growth on scaffolds and surfaces commonly used in orthopedic implantology have been clearly demonstrated [165-166, 197]. In particular, Antonucci *et al*. evidenced that AFS cells maintain a good ability to proliferate on titanium surfaces even after their osteogenic differentiation [165], and that this growth ability was showed also when these cells were cultured on titanium screws [166]. Taken together, all these studies strongly support the potential usefulness of AFS cells in the treatment of traumatic and degenerative bone disorders.

Neurogenic Differentiation

Many groups have investigated the potential of human AFS to differentiate into neurogenic cells. Purposefully, uncultivated or cultivated AFS, either in standard medium or in neurogenic differentiation medium, were analysed for the presence of morphologic features and specific markers of neurogenic differentiation.

In several studies, neurogenic differentiation has been investigated using unselected AFMSCs. Prusa *et al.* [170] described that native human AF contains a very little amount of neurogenic cells, and that differentiation only sporadically occurs in standard culture conditions, while it is strongly increased in neurogenic induction medium. These results were confirmed by the study of Tsai *et al* [162], confirming the existence of neural progenitor cells in second-trimester AF from normal pregnancies. On the other hand, other authors reported that AFS do not alter their morphology after exposure to neurogenic specific media, despite the presence of early and late neuronal antigens after 2 weeks culture [198]. Since the results of Prusa *et al*.[170] were obtained using a medium containing 2% serum and 1.25% dimethyl sulfoxide (DMSO), it can be hypothesized that the appearance of a neurogenic phenotype could be induced by the presence of DMSO in culture, as reported by other authors [199]. In fact, Lu *et al*. explored the potential of simple chemical methods to transdifferentiate different cell types (primary rat fibroblasts, primary human keratinocytes, HEK293 cells, rat PC-12 cells, and rat bone marrow stromal cells), evidencing that all cells except for keratinocytes adopted at least partial "neuron-like" morphology upon stimulation with different chemicals including DMSO. Moreover, apparent increases in immunolabeling for the neuronal markers were detected in the cell soma, but nor confirmed by RT-PCR- Authors concluded that the morphological changes and increases in immunolabeling for certain cellular markers upon chemical induction are likely the result of cellular toxicity, cell shrinkage, and changes in the cytoskeleton, not representing regulated steps in a complicated cellular differentiation process [199]. Several groups have investigated the gene expression of specific neuronal markers in AFCs uncultivated and cultivated in neurogenic differentiation medium. RT-PCR and Western blot analyses demonstrated that culture in neurogenic medium can trigger an induction/ up-regulation of some genes, such as CD133, nestin, CNPase, p75 and neurotrophin-3, representing specific markers of neurogenic differentiation [158-164, 170]. More recently, other studies have performed different experiments in order to address the question whether AFS cells are actually able to differentiate into mature neurons [200]. Although neurogenic differentiation of AFS cells has been demonstrated in mouse, pig and human [173, 201-203], and adenovirus and baculovirus transductions have been showed able to induce the appearance of neuronal characteristics in human and mouse AFS, respectively [204-205], so far a definitive proof that AFS cells can differentiate to mature fully functional neurons is still missing [200]. A crucial point could be represented by the evidence that the efficiency of neurogenic differentiation is related to the presence of specific extracellular growth factors in culture medium [178, 206]. Finally, inconsistent data have been reported about the ability of AFS to differentiate into dopaminergic neurons, since some authors reported data supporting this ability [162, 207], while other provide evidence that human AFS cells do not differentiate into dopaminergic neurons [208]. In addition to "*in*

vitro" studies, also the potential of AFS cells in peripheral nerve regeneration "*in vivo*" has been investigated. In particular, the group of Pan *et al*., using the model of the injured sciatic nerve rat model, reported in several studies that AFS cells can augment the growth of the injured nerve across the nerve gap, suggesting this to be due to neurotrophic factors secreted by the amniotic fluid cells or to interacting effects with Schwann cells [209]. Moreover, increased nerve myelination and improved motor function after AFS transplant has been observed [209-211]. However, the underlying molecular mechanism of the observed beneficial effects must be clarified, in particular as concerning the ability of transplanted AFS cells to become part of the organic structure or just release factors with supportive effects [200].

In addition to the above described studies, based on the use of unselected AFS cells, other reports have investigated the ability of neurogenic differentiations of c-kit selected AFS. The existence in c-kit + AFS cells of committed neuronal progenitor cells able to express transcriptional profiles highly similar to those of mesencephalic dopaminergic neurons has been demonstrated by McLaughlin *et al*. [163] who evidenced the expression of different dopaminergic markers (TGF-b3, FGF8, Shh and b-catenin), suggesting that cell lines can be derived from subcultures of amniocentesis, primarily composed of a population of progenitors with a phenotype similar to that of committed mesencephalic dopaminergic neurons.

Furthermore, after application of a different neurogenic induction protocol using Neuronal Growth Factor (NGF), c-Kit+ AFS cells acquired the ability to secrete the excitatory neurotransmitter L-glutamate in response to stimulation by potassium ions [138]. An interesting feature has been provided by the "*in vivo*" experiments of De Coppi *et al*. [138], which showed that human c-Kit+ AFS cells injected in the lateral cerebral ventricle of newborn mice after induction in neurogenic medium were successfully implanted into the lateral ventricles and survived efficiently for at least 2 months.

Cardiomyogenic Differentiation

The ability of AFS cells to differentiate into cardiomyocites has been very recently suggested by studies carried out on c-kit+ cells**.**

The group of Bollini *et al*. reported the results of the induction of cardiomyogenic differentiation in c-Kit-sorted, GFP-positive rat AFS by co-culture with neonatal rat cardiomyocytes, demonstrating their ability to acquire a cardiomyogenic phenotype and to preserve cardiac function after transplantation in the heart of animals with ischemia/reperfusion injury, even if their potential appears to be limited by poor survival in an allogeneic setting [212] . The group of Guan *et al*. investigated whether human AFS cells could be a potential source of cells for cardiac cell therapy, by testing their "*in vitro*" differentiation capabilities. This group reported that undifferentiated AFS cells express different cardiac genes, including MEF2, CX43, H- and N-cadherin. AFS cells were induced to differentiation along the cardiac lineage by incubation with 5-aza-2'-deoxycytidine, and morphological changes, upregulation of cardiac-specific genes and redistribution of CX43 were analyzed as markes of caryomiogenic differentiation. Also in this study, AFS cells were co-cultured with neonatal rat cardiomyocytes, showing the formation of mechanical and electrical connections. Authors concluded that hat AFS cells can be differentiated into a cardiomyocyte-like phenotype and can establish functional communication with neonatal rat cardiomyocytes [213].

The group of Yeh *et al*. investigated whether human AFS cells can be differentiated into cardiomyogenic cells and toward the maturation of endothelial cell lineage "*in vitro*" using mimicking differentiation milieu, and observed that these cells were differentiated into cardiomyocyte-like cells and cells of endothelial lineage, when cultured with rat neonatal cardiomyocytes or in endothelial growth medium enriched with vascular endothelial growth factor, respectively. These authors also evaluated human AFS cells for the therapeutic potential of cardiac repair using an immune-suppressed rat model with experimental myocardial infarction. After intramyocardial injection conducted with a needle directly into the peri-infarct areas, animals treated with AFS cells showed after 4 weeks a preservation of the infarcted thickness, an attenuation of left ventricle remodeling, a higher vascular density, and thus an improvement in cardiac function. Trasnplanted AFS cells expressed cardiac-specific markers such as Nkx2.5, alpha-actinin, and cardiac Troponin T. Moreover, Cx43 was clearly expressed at the borders of the transplanted/transplanted and host/transplanted cells, thus indicating an enhancement of cell connection. Authors concluded that human AFS cells can induce angiogenesis, have cardiomyogenic potential, and may be used as a new cell source for cellular cardiomyoplasty [214].

LOW IMMUNOGENIC CHARACTERISTIC OF AMNIOTIC FLUID-DERIVED MESENCHYMAL STEM CELLS

AFMSCs have been considered to be suitable cells for allogenic transplantation, based on their low immunogenicity. Several studies have reported that both c-kit+ and unselected AFMSCs are positive for antigens HLA-ABC (MHC class I), but only a small fraction of these cells are slightly positive for antigens HLA-DR (MHC class II) [138, 160, 184]. These cells appear resistant to rejection because they express immunosuppressive factors such as CD59 (protectin) and HLA-G [184]. CD59 inhibits the complement membrane attack complex by binding C5b678 and hampering C9 from binding and polymerizing, thus preventing complement from damaging cells. HLA-G plays a key role in immune tolerance in pregnancy, being expressed in the placenta, while HLA-A and HLA-B genes are not expressed. Other recent studies have shown immunomodulatory properties of AFMSCs, that can inhibit the proliferation of T lymphocytes. Sessarego *et al*., to verify the immunosuppressive activity of AF-MSC, performed standard proliferative assays on peripheral blood mononucleated cells, evidencing a statistically significant inhibition of T-cell proliferation at peripheral blood mononuclear cell/AF-MSC ratios of 1: 1 and 1: 4 [215]. These immunological properties indicate that AFMSCs could survive after allogeneic transplant without using immunosuppressive therapy, offering advantages over cells derived from other sources. Moreover, it has been suggested that, due to this specific features, after establishment of low cost protocols to isolate AFS cells it should be possible to create banks encompassing all MHC immunotypes, which could be used for allogenic clinical applications [216].

GENE EXPRESSION PROFILES OF AFMSCS

AFMSCs possess gene expression profiles largely characteristic of undifferentiated cells and showing modifications with passage number during the culture period. These profiles have been investigated both in unselected and c-kit+ AFMSCs.

Unselected AFMSCs

Expression studies carried out by RT-PCR analysis have demonstrated the presence in AFS of Rex-1, SCF, GATA-4, Vimentin, CK18, HLA ABC, and FGF-5 transcripts throughout the culture period [29]. All these genes play a crucial role in the differentiation and function of several tissues. On the other hand, BMP-4, AFP and nestin genes have been reported to be specifically expressed only from the 16th to the 20th passage [187]. Finally, other genes, such as Pax-6, NCAM, BMP-2 and HLA DR genes have not been found to be expressed.

Several other expression studies have demonstrated that the multilineage differentiation ability of AFMSCs is mediated by the expression of specific genes. In fact, during osteoblastic differentiation, AFMSCs after 30 days of culture show the expression of all markers typical of late stage osteoblasts (COL1, ONC, OPN, OCN, OPG, BSP, Runx2) [165].

During hepatocyte differentiation, AFMSCs express specific markers such as AFP, albumin, CK18, HNF1a, C/EBPa, CYP1A1 [217]. Endothelial AFMSCs differentiation, stimulated by growth factors, produces the expression of angiogenic factors such as VEGF, PGF and HGF [138, 217]. AFMSCs under conditions of myogenic differentiation are positive for MyoD, Mrf4 and Desmin transcripts [138]. Finally, both unselected and c-kit+ AFMSCs cultivated in neurogenic differentiation medium express neuronal marker genes (CD133, nestin, neurofilament, CNPase, p75, BDNF, and neurotophin-3) [138, 162-163, 170]. Using high-density oligonucleotide microarrays and functional network analyses, Tsai *et al*. [218] examined whether MSCs derived from four different origins (amniotic fluid, amniotic membrane, umbilical cord blood, and adult bone marrow) exhibited unique gene expression profiles. AFMSCs evidenced an up-regulation of genes involved in uterine maturation and contraction, such as OXTR (oxytocin receptor) and PLA2G10 (regulation of prostaglandin synthesis), as compared to the other cell types. Thus, AFMSCs probably may initiate the interaction with the uterus by up-regulating the oxytocin and thrombin receptors. Authors suggested that the most prominent functions of AFMSCs may be the regulation of uterine contraction and its related signaling transduction pathways, since other genes involved in these pathways were up-regulated, such as thrombin-triggered responses (F2R and F2RL), hedgehog (HHAT), and G-protein related (RHOF, RGS5, PLCB4, and RGS7). These results for the first time suggest a mechanistic role of fetal MSCs in regulating uterine contraction [218].

Selected AFMSCs

Multiplex RT-PCR analyses carried out on isolated c-Kit$^+$Lin$^-$ (KL) cells from both human and murine amniotic fluid during the hematopoietic differentiation demonstrated the expression of specific genes such as Gata2, Lmo2, Aml1, Mpo, Pu1, Cmyb, Fog1 [191]. Conversely, specific genes of the lymphoid differentiation (Il7ra and Ets1) were absent or very poorly expressed in these cells [191].

Phenotypic Characterization of AFMSCs

Tsai *et al* [161] characterized the phenotype of unselected AFMSCs at passages 4-8 by flow cytometry. This analysis revealed expression of surface antigens, such as SH2 (low positivity until passage 8), SH3, SH4, CD29, CD44 and HLA-ABC (MHC class I) and low positivity for CD90 and CD105. On the other hand, CD10, CD11b, CD14, CD34, CD117, HLA-DR, DP, DQ (MHC class II) and EMA were negative. Further characterization studies revealed the presence in both unselected and c-kit+ AFMSCs of antigens TRA-1-60, SSEA-3 and SSEA-4, typical markers of embryonal carcinoma cells and embryonal cells [138, 187] . These cells also exhibit markers such as collagen types I, II, III, IV and XII, fibronectin, CD44 (homing cell adhesion molecule, HCAM), CD54 (intercellular cell adhesion molecule-1, ICAM-1), CD31 (platelet/endothelialadhesion molecule-1, PECAM-1), CD106 (vascular cell adhesion molecule-1, VCAM-1), α-SMA (alpha-smooth muscle actin), CK18, desmin, vimentin, vWF, FSP [187]. Interestingly, it has been reported that antigen expression is not affected by gestational age or the type of culture medium used [219]. Flow cytometry analysis also demonstrated the presence of DAZL and c-Kit-expressing cells among AFMSCs population [220]. DAZL proteins are germ cell-specific, RNA-binding proteins essential for gametogenesis [221]. The expression of this gene is of specific interest since it demonstrates the presence in the AFMSCs of some key marker of the embryonic germ cells. To date, there is no evidence for the presence in AF of germ-like cells, but surely the expression of DAZL gene suggests that some additional feature about the plasticity and versatility of AFS should be investigated.

Proteomic Analysis of AFS

Proteomic analysis has identified 432 different gene products in unselected AFMSC cells, the majority of which is represented by enzymes and structural proteins mainly localized in cytoplasm, mitochondria, and nucleus [222]. Characterization of these proteins have suggested that the heterogeneous AF cell population is originated from a variety of fetal compartments (placenta, fetal tissues). In fact, many proteins are specific of the three cell populations present in the amniotic fluid. Some proteins are characteristic of epithelial cells (ATP synthase D chain, NADH-ubiquinone oxidoreductase 30 kDa subunit, annexin II, annexin IV, 40S ribosomal protein SA, glutathione S-transferase P, major vault protein and keratins type I cytoskeletal 19, and type II cytoskeletal 7). Other proteins have been reported to be expressed in fibroblasts (fibronectins, tropomyosins, transgelin, arp2/3 complex 34 kDa subunit, gelsolin, elongation factor 1-b and others), keratinocytes (keratins, ribonucleoproteins, annexin II, acetyl-CoA acetyltransferase and others), foreskin and epidermis (tropomyosins and keratins) and mesenchymal cells (vimentin 1). Since the afore mentioned proteins have already been reported to be expressed in the differentiated cell types, these results indicate that certain types of fetal cells, like epithelial cells, fibroblasts, keratinocytes and mesenchymal cells, are already differentiated at the time of amniocentesis [222].

The group Roubelakis *et al*, compared the human mesenchymal stem cells derived from AF with bone marrow mesenchymal stem cells in a study of molecular and proteomic characterization. Through two-dimensional gel electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) approach, these authors generated for the first time the protein map of cultured AF-MSCs and identified about 261 proteins. The functional pattern of the identified proteins from both sources was similar. However, the cultured AF-MSCs showed a number of unique proteins related to proliferation and primitive phenotype which are able to diversify the two cell types [223]. Recently, the same group conducted a proteomic analysis on two morphologically distinct adherent AF cell types, namely spindle-shaped (SS) and round-shaped (RS), showing the presence of 25 proteins differentially expressed between the two subpopulations, which could explain the different proliferative , migration and differentiation abilities of two cell types. The SS cells compared to RS cells showed upregulation of reticulocalbin-3 precursor, collagen alpha-1(I) chain precursor, FK506-binding protein 9 precursor, Rho GDP-dissociation inhibitor 1, chloride intracellular channel protein 4, tryptophanyl-tRNA synthetase and heat shock proteins 1 70 kDa and beta-1. On the other hand, proteins such as peroxiredoxin-2, 60 kDa heat shock protein, glutathione S-transferase P and annexin A4, were up regulated in RS cells, which in addition showed the presence of cytokeratin 8, 18 and 19, cathepsin B, coactosin-like protein and integrin alpha, not present in SS cells. These latter cells overexpressed alpha-1 collagen, a molecule important for directing the MSCs into osteogenic lineages, thus explaining their greatest ability to differentiate *in vitro* into osteoblast lineages. Moreover, SS cells showed an increased expression of proteins involved in cell migration, which could explain their best motility *in vitro*. On the other hand, the high rate of proliferation of SS cells could be linked to the presence of proteins such as intracellular chloride channel protein 4 [224].

EXPERIMENTAL APPLICATIONS OF AFS IN CELLULAR THERAPY

Successful cell based therapy needs the identification of an appropriate cell source that is easily accessible and that can provide a large cell number after expansion. AFS cells show a high proliferative capacity and a good ability to express markers of pluripo-

tency at high passage numbers, representing a promising source for the treatment of a large number of diseases. Although the identification of AF as an useful source of stem cells is quite recent, as compared to other cell types, several reports in recent years have shown the possibility of using these cells in the field of cell based therapies. Some of these reports have been based on the use of unselected AFMSCs cells, while others used c-kit+ cells.

Unselected AFMSCsOne of the first applications of AFS in cellular therapy was reported by Kunisaki *et al.* [225], who compared the efficacy of mesenchymal amniocytes and fetal myoblasts in the diaphragmatic

constructs in neonatal lambs. This application demonstrated that diaphragmatic repair with AFS leads to improved structural outcomes when compared with equivalent fetal myoblast. In particular, AFS cells based constructs showed increased cellularity and higher elastin content, as well as enhanced modular and ultimate tensile strengths. Authors concluded that, since amniocentesis is part of the normal prenatal diagnostic workup in patients with congenital diaphragmatic hernia, it is possible to obtain autologous amniotic fluid for the isolation and expansion of mesenchymal amniocytes from fetuses in which this condition has been prenatally diagnosed without any added morbidity, suggesting that AF is a preferred cell source for tissue-engineered diaphragmatic reconstruction [225]. The same group also investigated the usefulness of AFS cells in other models, such as fetal cartilage engineering [226] and fetal tracheal reconstruction [227]. In the first of these studies, authors investigated whether cartilage could be engineered from mesenchymal progenitor cells form ovine AF. The engineered constructs derived from mesenchymal AFS cells showed histological evidence of chondrogenic differentiation and maintained their original size and three-dimensional architecture. Quantitative assays of the engineered constructs revealed lower concentrations of collagen type II, but similar amounts of glycosaminoglycans, elastin, and DNA, when compared to native fetal hyaline cartilage. Authors concluded that AFMSCs could represent an useful source for the engineering of cartilaginous tissue *in vitro* and a promising tool for the surgical treatment of select congenital anomalies [226]. These results were confirmed in the second study, where authors demonstrated that AFS cells can also be used to engineer 3-dimensional cartilage suitable for tracheal repair, at least in the fetal environment. This engineered cartilage can provide sustained mechanical support to the trachea, maintaining a hyaline extracellular matrix profile, and becoming epithelialized over time *in vivo*, eventually allowing for spontaneous breathing of the implanted animals at birth [227]. AFS cells were also used in a study aimed to the evaluation of the effects of a combined therapy based on the treatment with AFS and fermented soybean extracts of peripheral nerve injury in rats [229]. This study demonstrated attenuation of inflammation and a rescue from apoptosis of both transplanted AFS and endogenous Schwann cells. Authors also evidenced the addition of fermented soybean extracts prevents the AFS from apoptosis by inhibiting the fibrin deposition, which paralleled the suppression of macrophage aggregation and pro-inflammatory cytokines expression. Authors suggested that the paracrine effect of AFS can be regarded as the most likely mechanism of nerve regeneration [229]. Hauser at al. [231] reported on the use of AFS in the treatment of glycerol-induced acute kidney injury, comparing their potential to the one of BM derived mesenchymal stem cells, and evidencing a rapid normalization of renal function compared with both cell types. Interestingly, BM cells showed an higher efficacy in inducing proliferation, while AFS cells were more antiapoptotic. Yeh *et al.* used human AFS cells as a source for the fabrication of cell sheet fragments which were transplanted into the peri-ischemic area of an immune-suppressed rat model at one week after myocardial infarction induction. Authors observed that treated animals showed a superior heart function as evidenced by echocardiography as compared to controls. AFS cell sheet fragments had a better ability of cell retention and proliferation than dissociated AFS cells upon transplantation to the host myocardium. Moreover, transplantation of AFS cell sheet fragments stimulated a significant increase in vascular density, thus contributing to an improvement in wall thickness and a reduction in the infarct size. Histological and molecular analyses demonstrated that the transplanted human AFS cells can be differentiated into cardiomyocyte-like cells and cells of endothelial lineages and modulate expression of multiple angiogenic cytokines and cardiac protective factor with the potential to promote neo-vascularization, likely contributing to the improvement of ventricular function [234].

C-kit+ AFMSCs

Carraro *et al*. [228] investigated the ability of AFS cells to integrate into murine lung and to differentiate into pulmonary lineages after injury, demonstrating that these cells can integrate into the epithelium. Moreover, these authors demonstrated the plasticity of AFS to respond in different ways to different types of lung damage by means of the expression of specific markers of alveolar or bronchiolar epithelial cell lineage, based on the different type of injury.

Ghionzoli *et al.* [230] evaluated the ability of AFS cells to diffuse systemically and to integrate into tissues of healthy newborn rats after intraperitoneal injection, demonstrating the absence of adverse effects as well as the homing and integration of AFS into various organs (intestine, liver, spleen, heart, lungs, and femur), but not in the brain.

In a similar experiment, Perin *et al*. [232] injected human AFS cells in the damaged kidney of a mouse model with glycerol induced rhabdomyolysis and Acute Tubular Necrosis. The results showed a protective effect and a significant immunomodulatory function over the course of the pathological condition. The therapeutic potential of human AFS cells in a rat model of acute myocardial infarction has been recently investigated by Bollini *et al*. [233] , who observed a cardioprotective effect, with improvement of myocardial cell survival and decrease of the infarct size. In this study, AFS cells were demonstrated to secrete Tβ4, previously shown to be both cardioprotective and pro-angiogenic.

The above described reports demonstrate that AFS cells have been widely used in different fields of cellular therapy. However, particular interest must be devoted to two other possible applications of these cells in regenerative medicine, namely the treatment of bone damages and ischemic brain injury, both representing pathologies with a large prevalence in the population.

EXPERIMENTAL APPLICATIONS OF AFS IN BONE REPAIR

Traumas and degenerative diseases are among the main causes of bone defects. The current cure for bone repair is autologous bone graft, but this approach is limited by non-structural integration of autologous fragments. Cell-based therapies may be particularly effective for the treatment in patients with reduced presence of endogenous stem or progenitor cells because of advanced age [235]. A major challenge in this field is to identify a rich source of cells capable of synthesizing a robust mineralized matrix and generating functional osteoblasts [236]. Both c-kit+ and unselected AFMSCs have been tested for their ability to provide these functions.

c-kit + AFMSCs

AFS human cells cultured in the presence of osteogenic-inducing medium are able to form bone after subcutaneous transplantation in mice [138], Peister *et al*. [235] demonstrated the ability of AFS cells to produce 3D mineralized bioengineered constructs *in vitro* and *in vivo*. In addition, the *in vitro* pre-differentiated AFS cells continued to produce minerals for 4 weeks after subcutaneous transplantation in immunodeficient rats. However, authors suggested that AFS cells may not require pre-

differentiation when placed in a site of bone injury, since they will likely receive differentiation cues from the fracture hematoma and adjacent bone. Based on these results, authors suggested that AFS cells can be successfully used to produce 3D mineralized bioengineered constructs *in vitro* and *in vivo* and that these cells may be an effective source for functional repair of large bone defects [235]. The group of Sun [237] investigated the levels of response to bone morphogenetic proteins (BMPs) in the AFS cells, compared to MSCs derived from human BM. The BMPs (BMP-2, 4, 6, 7 and 9) are involved in osteogenic activity and are probably the most important growth factors in bone formation and healing [238]. Compared to BM-MSCs, the AFS had a stronger response to BMP 7 and a more substantial mineralization [237].

Unselected AFMSCs

A recent report showed that the choice of cell source for bone tissue engineering may influence the rate of osteogenic differentiation *in vitro* [239]. This study revealed a difference of mineralization potential between the two sources of mesenchymal stem cells, namely AF and BM. In fact, AFS cells produce a large mineralized matrix for long periods of culture while the mineralization capacity of BM-MSCs is limited to the first few weeks in culture. Taken together, these data confirm the potential of AFS to differentiate into osteoblastic cell line and their ability to produce mineralized matrix, suggesting that these cells could represent in the future the gold standard for orthopedic implantology in the treatment of traumatic bone diseases. In this view, it must be stressed that unselected AFS human cells have the ability to grow on titanium scaffold and screws commonly used in oral and orthopedic implantology [165-166].

EXPERIMENTAL APPLICATIONS OF AFS IN THE TREATMENT OF STROKE

Another promising field of clinical application of stem cell based cell therapy is represented by stroke. Stroke is one of the major causes of death and disability across the world [240-241]. Approximately 750,000 people in US suffer a stroke annually [242], and approximately one-half of the survivors remaining with permanent disabilities [243-244]. Some interventions during the acute phase of stroke such as the use of thrombolytic agents have been recognized to improve the outcome including survival and residual disability [245]. Although the current treatments are aimed to prevent and reduce the damage, the lost tissue (infarct) cannot be salvaged. Due to this loss of tissue, the majority of patients who suffered stroke experience lifelong disability [240].

Several studies have recently been published investigating the therapeutic potential of either endogenous [241] or transplanted stem cells in laboratory models of stroke, providing evidence that regenerative cell-based therapies can lead to functional recovery in stroke patients [246-251]. Kondziolka *et al.* investigated the safety and feasibility of human neuronal cellular transplantation in patients with basal ganglia stroke and fixed motor deficits, showing no adverse cell-related serologic or imaging-defined effects after 12-18 months follow up, with improvement in the total European Stroke Scale score [246]. Nelson *et al*. reported the first postmortem brain findings of a phase I clinical stroke trial patient implanted with human hNT neurons (derived from the NTera2 teratocarcinoma cell line) adjacent to a lacunar infarct 27 months after surgery. These authors identified neurofilament immunoreactive neurons in the graft site, showing by fluorescent in situ hybridization (FISH) analysis the presence of polyploidy in groups of cells at this site similarly to polyploid hNT neurons *in vitro*. No evidence for neoplastic degeneration was observed. Based on these results, authors suggested that that implanted hNT neurons survive for >2 years in the human brain without deleterious effects [247]. The usefulness of mesenchymal stem cells in the treatment of stroke was investigated by Bang *et al*., who examined the feasibility, efficacy, and safety of cell therapy using culture-expanded autologous MSCs from BM in a group of patients with cerebral infarcts within the middle cerebral arterial territory and with severe neurological deficits [248]. MSC-treated patients showed a significant improvement during the follow-up period as compared with the control patients, and serial evaluations showed no adverse cell-related, serological, or imaging-defined effects. Authors concluded that in patients with severe cerebral infarcts the intravenous infusion of autologous MSCs represents a feasible and safe therapy able to improve functional recovery, also considering that the use of patients' own bone marrow cells should circumvent the problems of host immunity and graft-versus- host disease [248]. In a recent review, Bersano *et al*. summarized clinical studies on stem cell transplantation in stroke patients to evaluate the safety, feasibility of administration and tolerability of this experimental treatment, analyzing the criticisms related to this kind of therapeutical approach. In fact, the potential success of transplantation in stroke appears to be influenced by some critical issues including anatomy, localization and size of infarct area, time of transplantation, vascular supplies, route and site of implantation and patient selection. Authors suggested that it would be necessary that cell sources do not have restricted fates, being able to differentiate into appropriate cell types in relation to ectopic site other than they should be able to produce functional connections. Anyway, authors concluded that the results of the initial clinical studies analyzed indicate that stem cell therapy may be safe and technically feasible in stroke patients [249]. As a matter of fact, stem cell therapy appears to be an interesting model for stroke therapy for different reasons. In fact, these cells have the capacity to respond actively to their environment, migrate to the areas of injury, and secrete neuroprotective compounds, in addition to their potential for generating a variety of new functional cell types. As observed by Burns *et al*., exogenous stem cells from multiple sources can generate neural cells that survive and form synaptic connections after transplantation in the stroke-injured brain. Moreover, stem cells also exhibit neuroprotective properties that may ameliorate stroke deficits. Although the exact mechanisms underlying functional benefits remain poorly understood, in many cases the observed improvement is likely independent of neural [250]. Currently, there are two main theories behind the therapeutic effect of stem cell transplantation in the treatment of stroke. The first concept is the cell replacement theory, in which transplanted stem cells differentiate into progenitor and specialized somatic cells to replace dying cells. The other hypothesis is based on the immuno-modulatory, neuro-protective and neuro-trophic abilities of stem cells inducing a reduction of stroke size and increasing the recovery of behavioral functions [251]. In both cases, the therapeutic effects of the implanted stem cells or their precursors would be dependent upon their functional and structural integration into the brain tissue. Thus, cell-based therapeutic strategies can be classified into three categories, namely i) transplantation of stem or progenitor cells into the injured site to replace the nonfunctional cells, ii) enhancement of proliferation or differentiation of endogenous stem or progenitor cells, and iii) immunomodulatory and anti-inflammatory effects. As a matter of fact, evidence strongly suggests that all effects are obtained by means of stem cells transplantation, and that all strategies can be pursued by a single approach. Various cell types can serve as potential sources for transplantation, such as neural stem cells (NSCs), NT2 neurons (NT2N), umbilical cord blood cells (UCBCs), ES cells, MSCs, and some immortalized cell lines [252]. Experimental studies showed that all of these cell sources have been successful to some extent in attenuating the ischemic damage and improving functional recovery after brain injury. BM derived MSCs appear to be the most widely used and well characterized cells [250, 253-254] . These cells can be easily collected from the bone marrow of affected patients and employed for autologous transplantation. However, many questions concerning the use of BM derived stem cells in the therapy of stroke must be answered. It has been reported that only about

0.02% BM stem cells injected into the carotid artery stained for neuralmarkers in the ischemic hemisphere, suggesting that the mechanisms of recovery are more likely due to the release of trophic factors, possibly promoting endogenous repair mechanisms, reducing cell death, and stimulating neurogenesis and angiogenesis, rather than neuronal differentiation and implant integration to the injured ischemic site [253]. Bliss *et al*. claimed that the use of BM stem cells shows several advantages as compared to other models, since these cells lack the ethical issues associated with embryonic-derived cells, are easily obtained offering the potential of autologous transplants, obviating the need for immunosuppression regimes, and even with xenogenic transplants are thought to be hypoimmunogenic, as they do not initiate T cell priming or humoral antibody production. Moreover, another advantage of these cells is that they are already in clinical use for malignant and non-malignant disorders. However, these authors also observed that BM stem cells show poor survival when injected, likely due to the lack of trophic support or through triggering the innate immune system, and that such poor survival could represent a disadvantage of these cells, although functional recovery is sustained out to one year [254]. An important point concerning the cellular model to be used in the cell therapy of stroke is that it has been demonstrated that in allogenic transplantation the age of the cells donor plays a crucial role in the determination of the therapeutic efficiency of the transplant [255-258]. Stolzing investigated human MSC from donors of various ages and determined their ''fitness'' by measuring various age and senescence markers used routinely to characterise the aging of somatic cells in relation to their differentiation capacity and functionality. These authors observed an age related reduction in specific cell subtypes and a reduced capacity for proliferation and differentiation. Moreover, an increasing in the markers of cellular aging, including oxidative damage, ROS levels p21 and p53 expression was observed, suggesting a progressive loss of fitness with age [255]. Kretlow *et al.* observed differences in the adipogenic, chondrogenic, and osteogenic differentiation capacity of murine BM stem cells harvested from donor animals of different age and number of passages. In particular, cells from younger donors adhered to tissue culture polystyrene better and proliferated in greater number than those from older animals. Moreover, chondrogenic and osteogenic potential decreased with age for each group, while adipogenic differentiation decreased only in cells from the oldest donors. Authors concluded that consideration of age and passage in combination will prove to be critical to the success of any strategy that seeks to regenerate tissue through the use of implanted progenitor cells [256]. Zhou *et al*. tested the effect of age on senescence-associated beta-galactosidase, proliferation, apoptosis, p53 pathway genes, and osteoblast differentiation of human BM stem cells, evidencing the presence of fourfold cells positive for senescence-associated beta-galactosidase and 1.7-fold longer doubling time in samples from older than younger subjects. Moreover, authors observed that with age more cells were apoptotic and showed an increased expression of p53 and its pathway genes, p21 and BAX, which could play a critical role in mediating the reduction of cell proliferation and differentiation [257]. Kastara *et al*. examined the effect of gender, age, and *in vitro* culture on the basic properties (proliferation, differentiation, and immunosuppressive potential) of BM-MSCs, evidencing a decline in the progenitor frequencies from the BM of adult mice [258]. All these data suggest the usefulness of alternative models in which "young" stem cells can be used. In this view, AFS cells represent a very interesting model, representing extremely "young" cells. Some studies have already highlighted the enormous potential of unselected AFMSCs transplantation therapy for stroke. Cipriani and colleagues [164] demonstrated that AFMSCs can survive and migrate after transplantation into a rat ischemic brain. In this study AFMSCs have been transplanted into rat brains in basal condition and in a model of cerebral ischemia, respectively, and obtained results demonstrated that the transplanted cells were able to migrate from the injection site, both at short and long distances along the corpus callosum, and to gain access to multiple brain regions. The AFMSCs injected into the striatum were seen to migrate towards multiple regions in control animals and mostly towards the injured region in the ischemic rats. Rehni *et al* [259] investigated the possible ameliorative effect of mouse AFMSC transplant on the behavioural deficits experimentally induced in mice by cerebral ischemia-reperfusion. After the induction of stroke by the Middle Cerebral Artery Occlusion (MCAO), the authors injected AFMSC or Embryonic neuronal stem cells (ENSC) into cerebral ventricles of ischemic mice. This study produced a significant result, since AFS cells exert a protective effect on the ischemic brain comparable to ENSC. Preliminary results of our group, based on the transplantation of fresh and cultivated rat AF cells in the jugular of rats submitted to MCAO, suggest the usefulness of this cellular model in the therapy of stroke, since treated rats have showed an improvement both in neurologic and behavioral scores at 48 h from the transplant, and this positive effect was still evident 28 days after transplantation (unpublished results). Although these results could encourage the use of AFS in ischemic cerebral vascular disease, further studies are required to evaluate the histological changes in the ischaemic brain after AFS transplantation and to elucidate the molecular mechanism of neuroprotective events provided by these cells.

ADVANTAGES OF AFS COMPARED TO OTHER RESOURCES OF STEM CELLS

Much of the recent interest surrounding human progenitor cells and stem cells is related to their potential use for replacing dysfunctional cells within a tissue. Since the use of ES cells is limited by ethical and logistic issues, special attention has been devoted to the use of alternative sources of stem cells such as mesenchymal cells from bone marrow, umbilical cord, placenta and amniotic fluid. Adult BM is the most common source of MSCs used in clinical settings. However, the use of adult BM shows some limitations [260]. First, the frequency of MSCs in adult BM is low (about 0.001-0.01% of nucleated cells) [261]. Moreover, harvesting BM from a patient is an invasive procedure. Therefore, other alternative sources of MSCs useful for clinical application have been investigated [262-263]. Umbilical cord blood represents another potential source of MSCs; however, low yield and interindividual variation have been reported as factors limiting their use in cell therapy [264-269].

Several groups, in recent years, have turned their attention to the human term placenta as a possible source of stem cells. The placenta is discarded after child birth, the isolation of cells from this tissue does not involve any invasive procedures and the use of placenta-derived cells raises no ethical issues. The placental cells are readily isolated, are able to differentiate into multiple lineages and also have immunomodulatory effects both *in vivo* and *in vitro* [262, 270-271]. Several reports have described the ability to isolate stem cells from the four regions of the fetal placenta: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic. From these regions the following cell populations have been isolated: human amniotic epithelial cells (hAEC), human amniotic mesenchymal stromal cells (hAMSC), human chorionic mesenchymal stromal cells (hCMSC), and human chorionic trophoblastic cells (hCTC). The cells from different regions have a variable plasticity; in fact, the properties of self renewal and "hierarchy," belonging to the stem cells, have not yet been clearly demonstrated in different cell types of fetal placenta [272] . Finally, great interest has been raised by the development of the induced Pluripotent Stem cells (iPS) obtained from terminally differentiated somatic cells *via* nuclear reprogramming [44, 273]. However, a number of scientific problems appear to hamper the use of iPS in cell therapy, such as their tumorigenic potential, the risk of insertional mutagenesis caused by viral integration into the genome, the presence of copy number variation (CNV) and somatic coding mutations [274-275] , and the existence of aberrant epigenomic repro-

gramming [276]. Moreover, generation of iPSCs still suffers from low efficiency and high cost. In addition to the above described models, the recent discovery of a stem cell population in the AF offers a promising alternative source of stem cells for cellular therapy. The ability of AFSs to differentiate into cells of all three embryonic germ layers and their high proliferation rate are two advantages of this model. AFS cells represent a new class of stem cells with properties somewhere between embryonic and adult stem cell types. Compared to embryonic stem cells, amniotic stem cells can be obtained without destroying human embryos, thus solving much of the ethical controversy. Furthermore, they are easily retrieved during routine prenatal testing, and they can be isolated and grown in laboratory dishes. The expansion potential of amniotic fluid-derived MSCs exceeded the one of BM-derived MSCs, since these cells are able to expand extensively without feeder cells [159] . The AFS cells are duplicated faster than stem cells from other sources; in fact, AFS cells take about 20 to 24 h to double the number of cells, while umbilical cord stem cells take 28 to 30 h, BM stem cells more than 30 h and placenta-derived cells approximately double every 36 h [277-279]. The presence of certain markers of undifferentiated cells shows that AFMSCs cells may be less differentiated than most BM-MSCs, and may more closely resemble pluripotent ES cells. Finally, while transplantation studies using human ES are also hampered by possible immunological rejection and tumorigenicity, AFS have proved to be not tumorigenic after transplantation in mice [138] and to retain stable karyotype along several culture passages [138] and after differentiation [165].

SUMMARY

Stem cell transplantation offers a new therapeutic avenue for the treatment of several human diseases [280-285]. However, the cells to be used therapeutically must meet strict criteria regarding their large expansion capacity in culture, their efficacy in the treatment of the disease and their stability and safety after transplantation. The use of adult stem cells may alleviate ethical and availability concerns, with the additional advantages, in some cases, to allow autologous grafts to be performed. The recently demonstrated presence of stem cells within AF have raised great interest due to: a) the large accessibility of these cells by means of routine amniocentesis; b) their ability to differentiate in several cell lineages; c) the absence of tumorigenicity after transplantation and d) the lack of ethical problems related to their use. Of all the many sources of MSCs described to date, the AF has been increasingly accepted as the ideal one for cellular therapy of different human diseases. Many groups have shown that AF-derived MSCs can be isolated relatively easily, proliferate quickly under standard culture conditions, have a remarkable multilineage potential, display negligible immunogenicity, demonstrate no evidence for teratoma formation, while presenting no ethical concerns. In addition, and perhaps more importantly, in several countries a diagnostic amniocentesis is routinely offered to any mother with advanced age or with a fetus in which a congenital anomaly has been suggested by prenatal imaging or sierological tests on peripheral blood. Since a quantity of 2 mL amniotic fluid is sufficient to culture these cells, and given the high percentage of pregnancies undergoing prenatal diagnosis by amniocentesis, collection and preservation of AF of fetuses in the future could represent the basis for autologous cell therapy approaches for treating various diseases, as well as for the creation of stem cells banks which could be used for allogeneic transplantation. Another important point is that in all industrialized countries prenatal diagnosis at present is widely performed in structures scattered throughout the entire territory. This will provide a unique opportunity to create several centres for the isolation and banking of these cells, covering a large portion of the territory. As a consequence, in the case of acute diseases (such as stroke) in which a cell treatment should be carried out within a few hours from the event, the presence of a well integrated network for the collection and storing of AFS cells will provide a prompt and ready-to -use source of cells to be used for the treatment of these diseases, also considering the low immunogenicity of these cells.

Another interesting feature is represented by the possibility of cultivate AF cells from foetuses affected by genetic diseases. In fact, since amniocentesis is mostly performed in mothers at risk of generating a child affected by a genetic disease (due to the presence of advanced maternal age, familiarity for a mendelian disease, presence of abnormalities at ultrasound examination or altered hormone values in sierological tests), a large prevalence of affected foetuses should be expected. While AF cells from these foetuses are likely not suitable for a therapeutic use in the treatment of human pathologies, they could represent interesting models for the study of genetic diseases. In fact, due to their ability of differentiating in tissues derived from all the three embryonic layers, AF cells could be used for studying the processes of tissue differentiation in pathological conditions, shedding light on the molecular mechanism underlying the development of congenital malformations. In some cases, prenatal diagnosis allows the detection of very severe diseases not allowing the survive of the affect child at birth. In this case, the use of AFS could provide a unique opportunity for the modelling of such diseases. In this aspect, AF cells could represent an interesting alternative to iPS cells for the study of genetic diseases. However, another interesting approach could be represented by the use of AFS as a source for the production of iPS. In fact, it has been recently demonstrated that iPS can be rapidly and efficiently obtained starting from the sixth day after infection with four key factors (OCT4/SOX2/KLF4/C-MYC), which represents a very rapid time as compared to the several weeks required when starting from human fibroblasts [286-287].

Taken together, all these data provide evidence that AF represents a new and very promising source for stem cell research, and that in a next future AFS cells will play a key role in regenerative medicine. Certainly stem cells from AF could be useful both for a personalized cells supply for newly born children and for banking cells to be used for therapeutic cell transplantation in immunogically matched recipients.

CONCLUSIONS AND OPEN QUESTIONS

Despite the large number of studies which have investigated AFS cells, several questions about the biological features of these cells as well as about their efficiency as a therapeutic tool must be ,answered. Are AFS cells pluripotenti or multipotent stem cells? Which are the molecular basis of the ameliorative effects evidenced in different pre-clinical applications? Can we actually consider these cells as "younger" cells as compared to other stem cells sources, such as BM, and would this represent an advantage in their use in a clinical setting? Do these cells actually present a low immunogenity, so that their banking could also allow transplantation from non-matched donors or by using pools of cells obtained by different donors? Some of these answers will likely come from further "*in vitro*" investigations, others from pre-clinical studies. Several very recent reports, published while this review was in progress, have further investigate the biological properties of AFS cells. Lange *et al.* have pointed their attention to the AFS cells model for preparing autologous tissue-engineered organ constructs prenatally, evidencing that, although this approach is still in its experimental stages, further preclinical and clinical studies could define its exact role in the pediatric laryngological setting [288]. Park *et al.* evaluated the ability of several types of human MSCs, including those derived from AF, to differentiate " *in vitro*" and "*in vivo*" when this cells are encapsulated in a fibrin hydrogel mixed with TGF-β3, showing an high expression of genes and proteins specific to cartilage forming tissues. Authors concluded that cultured or transplanted hMSCs mixed with TGF-β3 in a fibrin hydrogel differentiated into chondrocytes, suggesting that these cells would be suitable for reconstruction of hyaline articular cartilage [289]. Liu *et al*. demonstrated that that CD44+ human AFS cells

can be induced to become functional dopaminergic neuronal-like cells *in vitro*, and that when these cells are transplanted into 6-hydroxydopamine (6-OHDA)-treated rats with Parkinson disease they express multiple dopaminergic neuron cell markers and are ameliorative to behavioral recovery after induction both "*in vitro*" and "*in vivo*", akthough a full differentiation into dopaminergic neuronal-like cells was not observed. Authors suggest that CD44+ human AFS cells could be a source of dopaminergic neuronal-like cells with a potential use in cell-replacement therapy for Parkinson disease [290]. Buckley *et al*. examined the cytokine profile of alveolar epithelial type 2 cells (AEC2) damage milieu, hypothesizing that it would promote endogenous epithelial repair whilst also recruiting cells from other locations and instructing their engraftment and differentiation. Authors demonstrated that the AEC2 damage milieu was chemotactic for exogenous uncommitted human AFS cells, increasing migration >20 fold. AFS cells attached within an *in vitro* AEC2 wound, and expedited wound repair by contributing cytokines MIF and PAI-1 to the AEC2 damage milieu, which also promoted differentiation of a sub-population of human AFS cells to express phenotypic markers of distal alveolar epithelium sich as SPC, TTF-1 and ABCA3 [291]. Dupont *et al*. investigated the ability of a self-complementary adeno-associated viral vector encoding bone morphogenetic protein 2 (scAAV2.5-BMP2) to enhance human stem cell osteogenic differentiation *in vitro*, evidencing that human AFS cells seeded on scAAV2.5-BMP2-coated three-dimensional porous polymer Poly(ε-caprolactone) scaffolds displayed significant increases in BMP2 production compared with controls during 12 weeks of culture, suggesting that the analyzed model could represent a novel acellular bone-graft-free endogenous repair therapy for orthotopic tissue-engineered bone regeneration [292]. A very interesting report has been recently published by Lu *et al*. These authors co-expressed through lenti-viral delivery the four transcription factors commonly used for the generation of iPS (Oct4, Sox2, Klf4 and c-Myc) in AF cells, obtaining ES-like colonies which were picked onto a traditional feeder layer. An high percentage AF-iPS with partial to no AP activity was found, while an overwhelming majority of fully stained AP positive (AP+) AF-iPS colonies was observed when colonies were first seeded on a feeder-free culture system, and then transferred to a feeder layer for expansion. This screening step decreased the variation seen between morphology and AP assay. The feeder-free screened AP+ AF-iPS colonies were also positive for pluripotent markers, OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81, SSEA-3 and SSEA-4 as well as having differentiation abilities into three germ layers *in vitro* and *in vivo*. With this protocol, authors provided a simple, one-step method for selection of AP+ AF-iPS cells *via* feeder-free screening [293]. The study of the properties of AFS cells obtained from species different from human and rat is in progress. Investigations have been carried out about the properties of ovine AFS cells and their efficiency as a therapeutic approach in the treatment of experimentally induced Achille's tendon defect (Colosimo *et al*., personal communication). Chen *et al*. carried out a study aimed to isolate and characterize porcine AFS cells from the amniotic cavity of pregnant gilts in the early stages of gestation. The primary culture of AF showed multiple cell types, including the epithelial-like cells and fibroblast-like cells, while after 6 to 8 days of culture the epithelial-like cells disappeared and the remaining cells presented the fibroblastoid morphology. Authors observed a cell doubling time of about 34.6 h, and wereable to continually culture the cells over 60 passages *in vitro*. The flow cytometry results showed that porcine AFS cells were positive for CD44, CD117 and CD166, but negative for CD34, CD45 and CD54. Moreover, pAF-MSCs expressed ES cell markers, such as Oct4, Nanog, SSEA4, Tra-1-60 and Tra-1-81. Very importantly, three germ layer markers, including FGF5 (ectodermal marker), AFP (endodermal marker) and Bra (mesodermal marker), were detected in embryoid bodies derived from porcine AFS cells, which were capable of differentiating into neurocytes, adipocytes and beating cardiomyocytes, without forming teratomas when injected into immunodeficiency mice. Authors concluded that these optimal features of porcine AFS cells can provide an excellent alternative stem cell resource for potential cell therapy in regenerative medicine and transgenic animal [294]. Zheng *et al*. isolated stem cells from AF of goat at terminal gestational age and transferred enhanced green fluorescence protein (EGFP) gene into the stem cells to evaluate the capability of multipotent differentiation (neurogenic, adipogenic, osteogenic and endothelial) of the transgenic stem cells. Authors demonstrated that the transgenic AFS cell were capable of self-renewal and were positive for the undifferentiated cell markers, Oct4, Nanog, Sox2 and Hes1, while following differentiation cells expressed markers for neurogenic cells such as astrocyte (GFAP) and neuron (NSE), adipogenic cells (LPL+), osteogenic cells (Osteocalcin+ and Osteonectin+) and endothelium (CD34+ and eNOS+). Authors concluded that the EGFP gene transgenic AFS cells have the capability of multipotent differentiation, suggesting their usefulness in cell transplantation studies in future [295]. The same group performed a similar study also on AFS cells isolated from transgenic porcine foetuses, showing also in this case thew ability of chondrogenic, osteogenic and neurogenic differentiation, as evidenced by the expression od specific markers [296]. Filioli *et al*. isolated and characterized canine MSCs from foetal adnexa, such as AF, amniotic membrane, and umbilical cord matrix. These authors evidenced that for AFS and amniotic membrane cells the viability did not change with passages. AFS cells expressed Oct-4 and CD44 but not ematopoietic markers CD34 and CD45. Dog leucocyte antigens (DLA-DRA1 and DLA-79) were expressed only in AFS cells at passage 1. Isolated cells at passage 3 showed multipotent capacity, and differentiated *in vitro* into neurocyte, adipocyte, osteocyte, and chondrocyte Cells at passage 4 showed normal chromosomal number, structure, and telomerase activity. Based on these results, authors concluded that in dog MSCs can be successfully isolated from foetal adnexa and grown *in vitro*, and, due to their proven stemness and chromosomal stability, they could be used as a model to study stem cell biology and have an application in therapeutic programs [297]. Finally, some recent review have provided novel and stimulating pre-clinical data about the use of AFS cells in cellular therapy. Lee *et al*. cultivated human AFS cells on a multiwelled methylcellulose hydrogel system to form spherically symmetric cell bodies for cellular cardiomyoplasty. The grown AFS cells bodies enriched with extracellular matrices were xenogenically transplanted in the peri-infarct area of an immune-suppressed rat, *via* direct intramyocardial injection, showing an enhancement of cell retention and engraftment in short-term and long-term observations, when compared with dissociated AFS cells. Moreover, authors observed an attenuation in the progression of heart failure, an improvement of the global function, and an increase in the regional wall motion. An upregulation of HGF, bFGF and VEGF was evidenced, suggesting a significantly increased vessel densities in the hearts treated with AFS cell bodies. Authors suggested that the injected AFS cell bodies could undergo differentiation into angiogenic and cardiomyogenic lineages, contribute to functional benefits by direct regeneration and attenuate cell loss by providing an adequate physical size thus improving heart function [298]. Mirabella *et al*. reported that human AFS cells, seeded onto hydroxyapatite scaffolds and subcutaneously implanted in nude mice, were able to mount a response resulting in the recruitment of host's progenitor cells forming tissues of mesodermal origin such as fat, muscle, fibrous tissue and immature bone. Moreover, implanted AFS cells migrated from the scaffold to the skin overlying implant site but not to other organs. Based on their results, also these authors concluded that AF is a very appealing reserve of stem cells potentially useful for clinical application in regenerative medicine, mostly due to their ability of recruitment of host progenitor cells, homing towards injured sites and multipotentiality in tissue repair [299].

PERSPECTIVES

One of the most promising field of study related to the stem cells is the one involving the use of novel tools able to provide wide information about the biological features of a specific cell type, such as functional genomics, transcriptomics, proteomics and interactomics. The application of such technologies, capable of analyze thousands of molecules in a single experiment, will likely provide an increase in our knowledge of the biology of AFS cells. In fact, despite the large number of data obtained about the stem cells' ability to differentiate into multiple lineages, very little is known about the genes that govern this special property [300]. Studies aimed to the identification of the global molecular signature of stem cells, rather than to the investigation of the expression of single genes, would provide information able to enable the control and direction of differentiation into particular phenotypes [300]. An example came from the attempt to identify a set of genes that are commonly expressed in multiple stem cell types in order to obtain a genetic signature of "stemness" and to understand the molecular basis of the main features of stem cells, such as self-renewal and the ability to differentiate into multiple lineages [301]. The improvement in our knowledge about the genetic signature of the different stem cell types could have important effects also on their use in clinical trials. In fact, it has been suggested that the analysis of lineage-specific gene expression and cell surface markers, commonly used to describe a differentiated phenotype, could be not appropriate for the determination of the quality of the cells, the specificity of differentiation, and the assessment of mixed phenotypes. Since these informations will likely be necessary in order to ensure the quality of the cells to be used in any any type of clinical therapy, global gene expression profiling will probably represent the gold standard for a non-biased evaluation of the quality of cells [300]. Thus, the therapeutic potential of stem cells largely relies on understanding the molecular signature of these cells and their derivatives [300].

In concusion, the study of AFS cells and of their application in cellular therapy represents a very promising resource in the field of the regenerative medicine. If the properties of pluripotency and safety of these cells will be definitively demonstrated, this model will likely representing the definitive answer in the "embryonic stem cells vd adult stem cells" debate.

ACKNOWLEDGEMENTS

This study was partially supported by the Cari-Chieti Foundation (Italy)

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Received: November 11, 2011 **Accepted: December 8, 2011**

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