

A Paediatric Perspective on Stem Cells: Expression, Function and Clinical Relevance

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Abstract

In this paper, we provide an overview of the potential advantages and disadvantages of different stem and progenitor cell populations identified to date in amniotic fluid, along with their properties and potential clinical applications. In the last ten years, placenta, fetal membranes (i.e. amnion and chorion), and amniotic fluid have been extensively investigated as a potential non-controversial source of stem cells. They are usually discarded after delivery and are accessible during pregnancy through amniocentesis and chorionic villus sampling. Several populations of cells with multi-lineage differentiation potential and immune-modulatory properties have been isolated from the human placenta and fetal membranes; they have been classified by an international workshop as human amniotic epithelial cells (hAECs) [3-8] human amniotic mesenchymal stromal cells (hAMSCs) [9,10] human chorionic mesenchymal stromal cells (hCMSCs) by Igura et al. and Anker et al., and human chorionic trophoblastic cells (hCTCs). Although only recently described, these cells may, given the easier accessibility of the AF in comparison to other extra-embryonic tissues, hold much promise in regenerative medicine.

Keywords: Stem cells; Multi-lineage; Amniotic fluid; Gastro-intestine

Introduction

In the last ten years, placenta, fetal membranes (i.e. amnion and chorion), and amniotic fluid have been extensively investigated as a potential non-controversial source of stem cells. They are usually discarded after delivery and are accessible during pregnancy through amniocentesis and chorionic villus sampling [1]. Several populations of cells with multi-lineage differentiation potential and immune-modulatory properties have been isolated from the human placenta and fetal membranes; they have been classified by an international workshop [2] as human amniotic epithelial cells (hAECs) [3-8] human amniotic mesenchymal stromal cells (hAMSCs) [9,10] human chorionic mesenchymal stromal cells (hCMSCs) by Igura et al. and Anker et al., and human chorionic trophoblastic cells (hCTCs). In the amniotic fluid (AF), two main populations of stem cells have been isolated so far:

1. Amniotic Fluid Mesenchymal Stem Cells (AFMSCs) and
2. Amniotic Fluid Stems (AFS) cells.

Although only recently described, these cells may, given the easier accessibility of the AF in comparison to other extra-embryonic tissues, hold much promise in regenerative medicine.

Amniotic Fluid (AF): Function, Origin and Composition

The AF is the clear, watery liquid that surrounds the growing fetus within the amniotic cavity. It allows the fetus to freely grow and move inside the uterus, protects it from outside injuries by cushioning sudden blows or movements by maintaining consistent pressure and temperature, and acts as a vehicle for the exchange of body chemicals with the mother [11,12].

In humans, the AF starts to appear at the beginning of the second week of gestation as a small film of liquid between the cells of the epiblast. Between days 8 and 10 after fertilization, this fluid gradually expands and separates the epiblast (i.e. the future embryo) from the amnioblasts (i.e. the future amnion), thus forming the amniotic cavity [3-8]. Thereafter, it progressively increases in volume, completely surrounding the embryo after the 4th week of pregnancy. Over the course of gestation, AF volume markedly changes from 20 ml in the

7th week to 600 ml in the 25th week, 1000 ml in the 34th week and 800 ml at birth.

During the first half of gestation, the AF results from active sodium and chloride transport across the amniotic membrane and the non-keratinized fetal skin, with concomitant passive movement of water [13]. In the second half of gestation, the AF is constituted by fetal urine, gastrointestinal excretions, respiratory secretions, and substances exchanged through the sac membranes [14-16].

The AF is primarily composed of water and electrolytes 98% to 99%, but also contains chemical substances (e.g. glucose, lipids, proteins, hormones, and enzymes), suspended materials (e.g. vernix caseosa, lanugo hair, and meconium), and cells. AF cells derive both from extra-embryonic structures (i.e. placenta and fetal membranes) and from embryonic and fetal tissues [17,18]. Although AF cells are known to express markers of all three germ layers by Cremer et al. in 1981, their exact origin still represents a matter of discussion; the consensus is that they mainly consist of cells shed in the amniotic cavity from the developing skin, respiratory apparatus, and urinary and gastrointestinal tracts [19-21]. AF cells display a broad range of morphologies and behaviors varying with gestational age and fetal development [22]. In normal conditions, the number of AF cells increases with advancing gestation; if a fetal disease is present, AF cell counts can be either dramatically reduced (e.g. intrauterine death, urogenital atresia) or abnormally elevated (e.g. anencephaly, spina bifida, exomphalos) [23,24]. Based on their morphological and growth characteristics, viable adherent cells from the AF are classified into three main groups: epithelioid (33.7%), amniotic fluid (60.8%), and fibroblastic type (5.5%)

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[25]. In the event of fetal abnormalities, other types of cells can be found in the AF, e.g. neural cells in the presence of neural tube defects and peritoneal cells in the case of abdominal wall malformations [26-28]. The majority of cells present in the AF are terminally differentiated and have limited proliferative capabilities [29,30].

In the 1990s, however, two groups demonstrated the presence in the AF of small subsets of cells harboring a proliferation and differentiation potential. First, Torricelli reported the presence of hematopoietic progenitors in the AF collected before the 12th week of gestation [31]. Then Streubel was able to differentiate AF cells into myocytes, thus suggesting the presence in the AF of non-hematopoietic precursors [32]. These results initiated a new interest in the AF as an alternative source of cells for therapeutic applications.

Amniotic Fluid Mesenchymal Stem Cells (AFMSCs)

Mesenchymal stem cells (MSCs) represent a population of multipotent stem cells able to differentiate towards mesoderm-derived lineages (i.e. adipogenic, chondrogenic, myogenic, and osteogenic) [32,33]. Initially they are identified in adult bone marrow, where they represent 0.01% of total nucleated cells [34], MSCs have since been isolated from several adult (e.g. adipose tissue, skeletal muscle, liver, brain), fetal (i.e. bone marrow, liver, blood), and extra-embryonic tissues (i.e. placenta, amnion) [35].

The presence of a subpopulation of AF cells with mesenchymal features, able to proliferate *in vitro* more rapidly than comparable fetal and adult cells, was described for the first time in 2001 [36]. In 2003, In't Anker demonstrated that the AF can be an abundant source of fetal cells that exhibit a phenotype and a multilineage differentiation potential similar to that of bone marrow-derived MSCs; these cells were named AF mesenchymal stem cells (AFMSCs). Soon after this paper, other groups independently confirmed similar results.

Isolation and culture

AFMSCs can be easily obtained: in humans, from small volumes (2-5 ml) of second and third trimester AF [37,38], where their percentage is estimated to be 0.9% to 1.5% of the total AF cells [39], and in rodents, from the AF collected during the second or third week of pregnancy [40,41]. Various protocols have been proposed for their isolation; all are based on the expansion of unselected populations of AF cells in serum-rich conditions without feeder layers, allowing cell selection by culture conditions. The success rate of the isolation of AFMSCs is reported by different authors to be 100% [42,43]. AFMSCs grow in basic medium containing fetal bovine serum (20%) and fibroblast growth factor (5 ng/ml). Importantly, it has been very recently shown that human AFMSCs can be also cultured in the absence of animal serum without losing their properties [44]; this finding is a fundamental prerequisite for the beginning of clinical trials in humans.

Characterization

The fetal versus maternal origin of AFMSCs has been investigated by different authors. Molecular HLA typing and amplification of the SRY gene in AF samples collected from male fetuses [45] demonstrated the exclusive fetal derivation of these cells. However, whether AFMSCs originate from the fetus or from the fetal portion of extra-embryonic tissues is still a matter of debate [46].

AFMSCs display a uniform spindle-shaped fibroblast-like morphology similar to that of other MSC populations and expand rapidly in culture [47]. Human cells derived from a single 2 ml AF sample can increase up to 180×10^6 cells within four weeks (three

passages) and, as demonstrated by growth kinetics assays, possess a greater proliferative potential (average doubling time of 25-38 hours) in comparison to that of bone marrow-derived MSCs (average doubling time of 30-90 hours) [48-50]. Moreover, AFMSCs' clonogenic potential has been proved to exceed that of MSCs isolated from bone marrow (86×4.3 vs. 70×5.1 colonies) [51]. Despite their high proliferation rate, AFMSCs retain a normal karyotype and do not display tumorigenic potential even after extensive expansion in culture [52,53].

Analysis of AFMSC transcriptome demonstrated that: (1) AFMSCs' gene expression profile, as well as that of other MSC populations, remains stable between passages in culture, enduring cryopreservation and thawing well; (2) AFMSCs share with MSCs derived from other sources a core set of genes involved in extracellular matrix remodeling, cytoskeletal organization, chemokine regulation, plasmin activation, TGF- β and Wnt signaling pathways; (3) in comparison to other MSCs, AFMSCs show a unique gene expression signature that consists of the upregulation of genes involved in signal transduction pathways (e.g. HHAT, F2R, F2RL) and in uterine maturation and contraction (e.g. OXTR, PLA2G10), thus suggesting a role of AFMSCs in modulating the interactions between the fetus and the uterus during pregnancy [54].

The cell-surface antigenic profile of human AFMSCs has been determined through flow cytometry by different investigators. Cultured human AFMSCs are positive for mesenchymal markers (i.e. CD90, CD73, CD105, CD166), for several adhesion molecules (i.e. CD29, CD44, CD49e, CD54), and for antigens belonging to the major histocompatibility complex I (MHC-I). They are negative for hematopoietic and endothelial markers (e.g. CD45, CD34, CD14, CD133 and CD31).

AFMSCs exhibit a broad differentiation potential towards mesenchymal lineages. Under specific *in vitro* inducing conditions, they are able to differentiate towards the adipogenic, osteogenic, and chondrogenic lineage

Despite not being pluripotent, AFMSCs can be efficiently reprogrammed into pluripotent stem cells (iPS) via retroviral transduction of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc). Strikingly, AFMSC reprogramming capacity is significantly higher (100-fold) and much quicker (6 days vs. 16-30 days) in comparison to that of somatic cells such as skin fibroblasts. As iPS derived from adult cells, AF-derived iPS generate embryoid bodies (EBs) and differentiate towards all three germ layers *in vitro* and *in vivo* form teratomas when injected into SCID mice [54].

Pre-clinical studies

After AFMSC identification, various studies investigated their therapeutic potential in different experimental settings. Different groups demonstrated that AFMSCs are able not only to express cardiac and endothelial specific markers under specific culture conditions, but also to integrate into normal and ischemic cardiac tissue, where they differentiate into cardiomyocytes and endothelial cells [55-58]. In a rat model of bladder cryo-injury, AFMSCs show the ability to differentiate into smooth muscle and to prevent the compensatory hypertrophy of surviving smooth muscle cells [59].

AFMSCs can be a suitable cell source for tissue engineering of congenital malformations. In an ovine model of diaphragmatic hernia, repair of the muscle deficit using grafts engineered with autologous mesenchymal amniocytes leads to better structural and functional results in comparison to equivalent fetal myoblast-based and acellular

implants [60]. Engineered cartilaginous grafts have been derived from AFMSCs grown on biodegradable meshes in serum-free chondrogenic conditions for at least 12 weeks; these grafts have been successfully used to repair tracheal defects in foetal lambs when implanted in utero [61]. The surgical implantation of AFMSCs seeded on nanofibrous scaffolds and predifferentiated *in vitro* towards the osteogenic lineage into a leporine model of sternal defect leads to a complete bone repair in 2 months' time [62].

Intriguingly, recent studies suggest that AFMSCs can harbor trophic and protective effects in the central and peripheral nervous systems. Pan showed that AFMSCs facilitate peripheral nerve regeneration after injury and hypothesized that this can be determined by cell secretion of neurotrophic factors [63,64]. After transplantation into the striatum, AFMSCs are capable of surviving and integrating in the rat adult brain and migrating towards areas of ischemic damage [65]. Moreover, the intra-ventricular administration of AFMSCs in mice with focal cerebral ischemia-reperfusion injuries significantly reverses neurological deficits in the treated animals [66].

Remarkably, it has also been observed that AFMSCs present *in vitro* an immunosuppressive effect similar to that of bone marrow-derived MSCs [67]. Following stimulation of peripheral blood mononuclear cells with anti-CD3, anti-CD28, or phyto-hemagglutinin, irradiated AFMSCs determine a significant inhibition of T-cell proliferation with dose-dependent kinetics [68].

Amniotic Fluid Stem Cells (AFSCs)

The first evidence that the AF could contain pluripotent stem cells was provided in 2003 when Prusa described the presence of a distinct subpopulation of proliferating AF cells (0.1% to 0.5% of the cells present in the AF) expressing the pluripotency marker Oct4 at both transcriptional and proteic levels [69]. Oct4 (i.e. octamer binding transcription factor 4) is a nuclear transcription factor that plays a critical role in maintaining ES cell differentiation potential and capacity of self-renewal [70-72]. Other than by ES cells, Oct4 is specifically expressed by germ cells, where its inactivation results in apoptosis, and by embryonal carcinoma cells and tumors of germ cell origin, where it acts as an oncogenic fate determinant [73-76]. While its role in stem cells of fetal origin has not been completely addressed, it has been recently demonstrated that Oct4 is neither expressed nor required by somatic stem cells or progenitors [77-79].

After Prusa, different groups confirmed the expression of Oct4 and of its transcriptional targets (e.g. Rex-1) in the AF [80,81]. Remarkably, Karlmark transfected human AF cells with the green fluorescent protein gene under either the Oct4 or the Rex-1 promoter and established that some AF cells were able to activate these promoters. Several authors subsequently reported the possibility of harvesting AF cells displaying features of pluripotent stem cells [82,83]. Thereafter, the presence of a cell population able to generate clonal cell lines capable of differentiating into lineages representative of all three embryonic germ layers was definitively demonstrated [84]. These cells, named AF stem (AFS) cells, are characterized by the expression of the surface antigen c-kit (CD117), which is the type III tyrosine kinase receptor of the stem cell factor [85].

Isolation and culture

The proportion of c-kit⁺ cells in the amniotic fluid varies over the course of gestation, roughly describing a Gaussian curve; they appear at very early time points in gestation (i.e. at 7 weeks of amenorrhea in humans and at E9.5 in mice) and present a peak at mid-gestation

equal to 90×10^4 cells/fetus at 20 weeks of pregnancy in humans and to 10,000 cells/fetus at E12.5 in mice [86]. Human AFS cells can be derived either from small volumes (5 ml) of second trimester AF (14-22 weeks of gestation) or from confluent back-up amniocentesis cultures. Murine AFS cells are obtainable from the AF collected during the second week of gestation [87-89].

Cells and tissue development

Cell isolation is based on a two-step protocol consisting of the prior immunological selection of c-kit positive cells from the AF (approximately 1% of total AF cells) and of the subsequent expansion of these cells in culture [90-95]. Isolated AFS cells can be expanded in feeder layer-free, serum-rich conditions without evidence of spontaneous differentiation *in vitro*. Cells are cultured in basic medium containing 15% of fetal bovine serum and Chang supplement.

Characterization

Karyotype analysis of human AFS cells deriving from pregnancies in which the fetus was male revealed the fetal origin of these cells [96].

AFS cells proliferate well during *ex vivo* expansion. When cultivated, they display a spectrum of morphologies ranging from a fibroblast-like to an oval-round shape. As demonstrated by different authors, AFS cells possess a great clonogenic [97,98]. Clonal AFS cell lines expand rapidly in culture (doubling time 1/4 36 h) and, more interestingly, maintain a constant telomere length (20 kbp) between early and late passages. Almost all clonal AFS cell lines express markers of a pluripotent undifferentiated state: Oct4 and NANOG [99-103]. However, they have been proved not to form tumors when injected in severe combined immunodeficient (SCID) mice [104]. The cell-surface antigenic profile of AFS cells has been determined through flow cytometry by different investigators. Cultured human AFS cells are positive for ES cell (e.g. SSEA-4) and mesenchymal markers (e.g. CD73, CD90, CD105), for several adhesion molecules (e.g. CD29, CD44), and for antigens belonging to the MHC-I. They are negative for hematopoietic and endothelial markers (e.g. CD14, CD34, CD45, CD133, CD31) and for antigens belonging to the major histocompatibility complex II (MHC-II).

As stability of cell lines is a fundamental prerequisite for basic and translational research, AFS cell capacity of maintaining their baseline characteristics over passages has been evaluated based on multiple parameters. Despite their high proliferation rate, AFS cells and derived clonal lines show a homogeneous, diploid DNA content without evidence of chromosomal rearrangement even after expansion to 250 population doublings [105,106]. Moreover, AFS cells maintain constant morphology, doubling time, apoptosis rate, cell cycle distribution, and marker expression (e.g. Oct4, CD117, CD29, CD44) up to 25 passages. During *in vitro* expansion, (A) Human AFS cells mainly display a spindle-shaped morphology during *in vitro* cultivation under feeder layer-free, serum-rich conditions. (BeC) Clonal human AFS cell lines retain long telomeres and a normal karyotype after more than 250 cell divisions. (B) Conserved telomere length of AFS cells between early passage (20 population doublings, lane 3) and late passage (250 population doublings, lane 4). Short length (lane 1) and high length (lane 2) telomere standards provided in the assay kit. (C) Giemsa band karyogram showing chromosomes of late passage (250 population doublings) cells adapted from De Coppi et al. However, cell volume tends to increase and significant fluctuations of proteins involved in different networks (i.e. signaling, antioxidant, proteasomal, cytoskeleton, connective tissue, and chaperone proteins) can be observed using a gel-based proteomic approach [107]; the significance

of these modifications warrants further investigations but needs to be taken into consideration when interpreting experiments run over several passages and comparing results from different groups.

AFS cells and, more importantly, derived clonal cell lines are able to differentiate towards tissues representative of all three embryonic germ layers, both spontaneously, when cultured in suspension to form EBs, and when grown in specific differentiation conditions.

EBs consists of three-dimensional aggregates of ES cells, which recapitulate the first steps of early mammalian embryogenesis [108-110]. As ES cells, when cultured in suspension and without anti-differentiation factors, AFS cells harbor the potential to form EBs with high efficiency: the incidence of EB formation (i.e. percentage of number of EB recovered from 15 hanging drops) is estimated to be around 28% for AFS cell lines and around 67% for AFS cell clonal lines. Similarly to ES cells, EB generation by AFS cells is regulated by the mTor (i.e. mammalian target of rapamycin) pathway and is accompanied by a decrease of Oct4 and Nodal expression and by an induction of endodermal (GATA4), mesodermal (Brachyury, HBE1), and ectodermal (Nestin, Pax6) markers [111,112].

In specific mesenchymal differentiation conditions, AFS cells express molecular markers of adipose, bone, muscle, and endothelial differentiated cells (e.g. LPL, desmin, osteocalcin, and V-CAM1). In the adipogenic, chondrogenic, and osteogenic medium, AFS cells respectively develop intracellular lipid droplets, secrete glycosaminoglycans, and produce mineralized calcium [113]. In conditions inducing cell differentiation towards the hepatic lineage, AFS cells express hepatocyte-specific transcripts (e.g. albumin, alpha-fetoprotein, multidrug resistance membrane transporter 1) and acquire the liver-specific function of urea secretion [114]. In neuronal conditions, AFS cells are capable of entering the neuroectodermal lineage.

Cells and tissue development

AFS cells differentiation into lineages representative of the three embryonic germ layers. (A) Hepatogenic differentiation: urea secretion by human AFS cells before (rectangles) and after (diamonds) hepatogenic *in vitro* differentiation. (B) Neurogenic differentiation: secretion of neurotransmitter glutamic acid in response to potassium ions. (C) Osteogenic differentiation: mouse micro CT scan 18 weeks after implantation of printed constructs of engineered bone from human AFS cells; arrow head: region of implantation of control scaffold without AFS cells; rhombus: scaffolds seeded with AFS cells. Adapted from de Coppi et al. [114] neuronal markers (e.g. GIRK potassium channels), exhibit barium-sensitive potassium current, and release glutamate after stimulation. (Ongoing studies are investigating AFS cell capacity to yield mature, functional neurons [115-118]. AFS cells can be easily manipulated *in vitro*. They can be transduced with viral vectors more efficiently than adult MSCs, and, after infection, maintain their antigenic profile and the ability to differentiate into different lineages [119]. AFS cells labeled with super-paramagnetic micrometer-sized iron oxide particles (MPIOs) retain their potency and can be non-invasively tracked by MRI for at least four weeks after injection *in vivo* [119].

Preclinical studies

Despite the very recent identification of AFS cells, several reports have investigated their potential applications in different settings.

Bone: Critical-sized segmental bone defects are one of the most challenging problems faced by orthopaedic surgeons. Autologous and

heterologous bone grafting are limited respectively by the small amount of tissue available for transplantation and by high refracture rates. Tissue engineering strategies that combine biodegradable scaffolds with stem cells capable of osteogenesis have been indicated as promising alternatives to bone grafting. However, bone regeneration through cell-based therapies has been limited so far by the insufficient availability of osteogenic cells. The potential of AFS cells to synthesize mineralized extracellular matrix within porous scaffolds has been investigated by different groups. After exposure to osteogenic conditions in static two-dimensional cultures, AFS cells differentiate into functional osteoblasts (i.e. activate the expression of osteogenic genes such as Runx2, Osx, Bsp, Opn, and Ocn, and produce alkaline phosphatase) and form dense layers of mineralized matrix. As demonstrated by clonogenic mineralization assays, 85% of AFS cells versus 50% of MSCs are capable of forming osteogenic colonies. When seeded into three-dimensional biodegradable scaffolds and stimulated by osteogenic supplements (i.e. rhBMP-7 or dexamethasone), AFS cells remain highly viable up to several months in culture and produce extensive mineralization throughout the entire volume of the scaffold [80-110]. *In vivo*, when subcutaneously injected into nude rodents, pre-differentiated AFS cell-scaffold constructs are able to generate ectopic bone structures in four weeks' time. AFS cells embedded in scaffolds, however, are not able to mineralize *in vivo* at ectopic sites unless previously predifferentiated *in vitro*. These studies demonstrate the potential of AFS cells to produce three-dimensional mineralized bioengineered constructs and suggest that AFS cells may be an effective cell source for functional repair of large bone defects. Further studies are needed to explore AFS cell osteogenic potential when injected into sites of bone injury [100-120].

Cartilage: Enhancing the regeneration potential of hyaline cartilage is one of the most significant challenges for treating damaged cartilage. The capacity of AFS cells to differentiate into functional chondrocytes has been tested *in vitro*. Human AFS cells treated with TGF- β 1 have been proven to produce significant amounts of cartilaginous matrix (i.e. sulfated glycosaminoglycans and type II collagen) both in pellet and alginate hydrogel cultures.

Skeletal muscle: Stem cell therapy is an attractive method to treat muscular degenerative diseases because only a small number of cells, together with a stimulatory signal for expansion, are required to obtain a therapeutic effect. The identification of a stem cell population providing efficient muscle regeneration is critical for the progression of cell therapy for muscle diseases [121-130].

AFS cell capacity of differentiating into the myogenic lineage has recently started to be explored. Under the influence of specific induction media containing 5-Aza-20-deoxycytidine, AFS cells are able to express myogenic-associated markers such as Mrf4, Myo-D, and desmin 231 both at a molecular and proteic level. However, when transplanted undifferentiated into damaged skeletal muscles of SCID mice, despite displaying a good tissue engraftment AFS cells did not differentiate towards the myogenic lineage. Further studies are needed to confirm the results of this single report.

Heart: Cardiovascular diseases are the first cause of mortality in developed countries despite advances in pharmacological, interventional, and surgical therapies. Cell transplantation is an attractive strategy to replace endogenous cardiomyocytes lost by myocardial infarction. Fetal and neonatal cardiomyocytes are the ideal cells for cardiac regeneration as they have been shown to integrate structurally and functionally into the myocardium after transplantation. However, their application is limited by the ethical restrictions involved in the use of fetal and neonatal cardiac tissues.

Chiavegato [20] investigated human AFS cell plasticity towards the cardiac lineage. Undifferentiated AFS cells express cardiac transcription factors at a molecular level (i.e. Nkx2.5 and GATA-4 mRNA) but do not produce any myocardial differentiation marker. Under *in vitro* cardiovascular inducing conditions (i.e. co-culture with neonatal rat cardiomyocytes), AFS cells express differentiated cardiomyocyte markers such as cTnI, indicating that an *in vitro* cardiomyogenic-like medium can lead to a spontaneous differentiation of AFS cells into cardiomyocyte-like cells. *In vivo*, when xenotransplanted in the hearts of immunodeficient rats 20 minutes after creating a myocardial infarction, AFS cell differentiation capabilities were impaired by cell immune rejection. More recently, we have proved that we could activate the myocardial gene program in GFP-positive rat AFS (GFP-rAFS cells) [131-140] by co-culture with rCMs. The differentiation attained via a paracrine/contact action was confirmed using immunofluorescence, RT-PCR, and single-cell electrophysiological tests. Moreover, despite only a small number of Endorem-labeled GFP-rAFS cells acquired an endothelial or smooth muscle phenotype and to a lesser extent CMs in an allogeneic acute myocardial infarction (AMI) context, and there was still improvement of ejection fraction as measured by magnetic resonance imaging (MRI) three weeks after. This could be partially due to a paracrine action perhaps mediated by the secretion of thymosin β 4 by Bollini et al. [8]

Hematopoietic system: Hematopoietic stem cells (HSCs) lie at the top of hematopoietic ontogeny and, if engrafted in the right niche, can theoretically reconstitute the organism's entire blood supply. Thus, the generation of autologous HSCs from pluripotent, patient-specific stem cells offer real promise for cell-therapy of both genetic and malignant blood disorders.

The hematopoietic potential of c-kit⁺ hematopoietic lineage negative cells present in the amniotic fluid (AFKL cells) has been recently explored. *In vitro*, human and murine AFKL cells exhibit strong multilineage hematopoietic potential. Cultured in semisolid medium, these cells are able to generate erythroid, myeloid, and lymphoid colonies. Moreover, murine cells exhibit the same clonogenic potential (0.03%) as hematopoietic progenitors present in the liver at the same stage of development. *In vivo*, mouse AFKL cells (i.e. 2×10^4 cells intravenously injected) are able to generate all three hematopoietic lineages after primary and secondary transplantation into immunocompromised hosts (i.e. sublethally irradiated Rag-/- mice), demonstrating their ability to self-renew. These results clearly show that c-kit⁺ cells present in the amniotic fluid have true hematopoietic potential both *in vitro* and *in vivo* [100-144].

Kidney: The incidence and prevalence of end stage renal disease (ESRD) continues to increase worldwide. Although renal transplantation represents a good treatment option, the shortage of compatible organs remains a critical issue for patients affected by ESRD. Therefore, the possibility of developing stem cell-based therapies for both glomerular and tubular repair has received intensive investigation in recent years. Different stem cell types have shown some potential in the generation of functional nephrons but the most appropriate cell type for transplantation is still to be established.

The potential of AFS cells in contributing to kidney development has been recently explored. Using a mesenchymal/epithelial differentiation protocol previously applied to demonstrate the renal differentiation potential of kidney stem cells, Siegel demonstrated that AFS cells and clonal-derived cell lines can differentiate towards the renal lineage; AFS cells sequentially grown in a mesenchymal differentiation medium containing EGF and PDGF-BB, and in an

epithelial differentiation medium containing HGF and FGF4, reduce the expression of pluripotency markers (i.e. Oct4 and c-Kit) and switch on the expression of epithelial (i.e. CD51, ZO-1) and podocyte markers (i.e. CD2AP, NPHS2). AFS cells have also been shown to contribute to the development of primordial kidney structures during *in vitro* organogenesis; undifferentiated human AFS cells injected into a mouse embryonic kidney cultured *ex vivo* are able to integrate in the renal tissue, participate in all steps of nephrogenesis, and express molecular markers of early kidney differentiation such as ZO-1, claudin, and GDNF. Finally, very recent *in vivo* experiments show that AFS cells directly injected into damaged kidneys are able to survive, integrate into tubular structures, express mature kidney markers, and restore renal function. These studies demonstrate the nephrogenic potential of AFS cells and warrant further investigation of their potential use for cell-based kidney therapies.

Lung: Chronic lung diseases are common and debilitating; medical therapies have restricted efficacy and lung transplantation is often the only effective treatment. The use of stem cells for lung repair and regeneration after injury holds promise as a potential therapeutic approach for many lung diseases; however, current studies are still in their infancy.

AFS cell ability to integrate into the lung and to differentiate into pulmonary lineages has been elegantly investigated in different experimental models of lung damage and development. *In vitro*, human AFS cells injected into mouse embryonic lung explants engraft into the epithelium and into the mesenchyme and express the early pulmonary differentiation marker TFF1, *in vivo*, in the absence of lung damage, systemically administered AFS cells show the capacity to home to the lung but not to differentiate into specialized cells; while, in the presence of lung injury, AFS cells not only exhibit a strong tissue engraftment but also express specific alveolar and bronchiolar epithelial markers (e.g. TFF1, SPC, CC10). Remarkably, cell fusion phenomena were elegantly excluded and long-term experiments confirmed the absence of tumor formation in the treated animals up to 7 months after AFS cell injection [100-145].

Intestine: To date, very few studies have considered the employment of stem cells in gastroenterological diseases. Although still at initial stages and associated with numerous problems, ever-increasing experimental evidence supports the intriguing hypothesis that stem cells may be possible candidates to treat and/or prevent intestinal diseases.

In a study evaluating AFS cell transplantation into healthy newborn rats, Ghionzoli demonstrated that, after intraperitoneal injection, AFS cells (1) diffuse systemically within a few hours from their administration in 90% of the animals, (2) engraft in several organs of the abdominal and thoracic compartment and (3) localize preferentially in the intestine colonizing the gut in 60% of the animals. Preliminary *in vivo* experiments investigating the role of AFS cells in a neonatal rat model of necrotizing enterocolitis show that intraperitoneally-injected AFS cells are able not only to integrate into all gut layers but also to reduce bowel damage, improve rat clinical status, and lengthen animal survival.

Conclusion

Many stem cell populations (e.g. embryonic, adult, and fetal stem cells) as well as methods for generating pluripotent cells (e.g. nuclear reprogramming) have been described to date. All of them carry specific advantages and disadvantages and, at present, it has yet to be established which type of stem cell represents the best candidate for

cell therapy. However, although it is likely that one cell type may be better than another, depending on the clinical scenario, the recent discovery of easily accessible cells of fetal derivation, not burdened by ethical concerns, in the AF has the potential to open new horizons in regenerative medicine. Amniocentesis, in fact, is routinely performed for the antenatal diagnosis of genetic diseases and its safety has been established by several studies documenting an extremely low overall fetal loss rate (0.06% to 0.83%) related to this procedure. Moreover, stem cells can be obtained from AF samples without interfering with diagnostic procedures.

Two stem cell populations have been isolated from the AF so far (i.e. AFMSCs and AFS cells) and both can be used as primary (not transformed or immortalized) cells without further technical manipulations. AFMSCs exhibit typical MSC characteristics: fibroblastic-like morphology, clonogenic capacity, multilineage differentiation potential, immunosuppressive properties, and expression of a mesenchymal gene expression profile and of a mesenchymal set of surface antigens. However, ahead of other MSC sources, AFMSCs are easier to isolate and show better proliferation capacities. The harvest of bone marrow remains, in fact, a highly invasive and painful procedure, and the number, the proliferation, and the differentiation potential of these cells decline with increasing age. Similarly, UCB-derived MSCs exist at a low percentage and expand slowly in culture.

AFS cells, on the other hand, represent a novel class of pluripotent stem cells with intermediate characteristics between ES cells and AS cells. They express both embryonic and mesenchymal stem cell markers, are able to differentiate into lineages representative of all embryonic germ layers, and do not form tumors after implantation *in vivo*. However, AFS cells have only recently identified and many questions need to be answered concerning their origin, epigenetic state, immunological reactivity, and regeneration and differentiation potential *in vivo*. AFS cells, in fact, may not differentiate as promptly as ES cells and their lack of tumorigenesis can be argued against their pluripotency.

Although further studies are needed to better understand their biologic properties and to define their therapeutic potential, stem cells present in the AF appear to be promising candidates for cell therapy and tissue engineering. In particular, they represent an attractive source for the treatment of perinatal disorders such as congenital malformations (e.g. congenital diaphragmatic hernia) and acquired neonatal diseases requiring tissue repair/regeneration (e.g. necrotizing enterocolitis). In a future clinical scenario, AF cells collected during a routinely performed amniocentesis could be banked and, in case of need, subsequently expanded in culture or engineered in acellular grafts. In this way, affected children could benefit from having autologous expanded/engineered cells ready for implantation either before birth or in the neonatal period.

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