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Stem cells derived from amniotic fluid: new potentials in regenerative medicine

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Abstract

Human amniotic fluid cells have been used as a diagnostic tool for the prenatal diagnosis of fetal genetic anomalies for more than 50 years. Evidence provided in the last 5 years, however, suggests that they can also harbour a therapeutic potential for human diseases, as different populations of fetal-derived stem cells have been isolated from amniotic fluid. Mesenchymal stem cells were the first to be described, which possess the higher proliferation and differentiation plasticity of adult mesenchymal stem cells and are able to differentiate towards mesodermal lineages. Amniotic fluid stem cells have more recently been isolated. They represent a novel class of pluripotent stem cells with intermediate characteristics between embryonic and adult stem cells, as they are able to differentiate into lineages representative of all three germ layers but do not form tumours when injected *in vivo*. These characteristics, together with the absence of ethical issues concerning their employment, suggest that stem cells present in the amniotic fluid might be promising candidates for tissue engineering and stem cell therapy of several human disorders.

Keywords: *amniotic fluid, fetal cells, regenerative medicine, stem cells*

Introduction

Tissue regeneration after damage remains a major challenge. 'Regenerative medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause' (Daar and Greenwood, 2007). Strategies that have been applied include cell-based therapies, the use of biomaterials (scaffolds) alone and the use of scaffolds seeded with cells (Hipp and Atala, 2008). However, the use of cells for transplantation and tissue engineering has been restrained by the limited proliferation and differentiation capabilities of somatic differentiated cells.

In recent decades, major advancements have been facilitated by the discovery of cells, defined as 'stem cells', capable of widely expanding *ex vivo* and of differentiating to various cell lineages. Stem cells have been isolated from embryonic, fetal and adult tissues and, more recently, also from extra-embryonic adnexa such as umbilical cord, placenta, fetal membranes and amniotic fluid. Each different stem cell population carries particular features, values and limitations. According to The United States National Academies of Science report, 'Stem Cells and the Future of Regenerative Medicine', more than a hundred million patients in the USA are affected by diseases that, in the future, may be potentially treated with stem cell-based therapies (Commission on Life Sciences, 2002).

This paper provides an overview of the potential advantages and disadvantages of the main stem cell populations identified to date, and focus on the stem cell populations present in amniotic fluid, along with their properties and potential clinical applications.

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Stem cells: a variety of diverse populations

The term 'stem cell' identifies cells that share the dual ability to proliferate indefinitely (i.e. self-renewal) and to differentiate into one or more types of specialized cells (i.e. potency) (Mimeault and Batra, 2006). Based on their differentiation potential, stem cells are classified into pluripotent (i.e. able to differentiate into all derivatives of the three primary germ layers) and multipotent (i.e. able to give rise to multiple cell types deriving from a single germ layer) (Solter, 2006). There is still no consensus as to whether cells with the capacity to regenerate and/or contribute to only a single lineage should be referred to as unipotent stem cells, unipotent progenitors or simply cells that have retained a proliferative potential (Blanpain *et al.*, 2007).

Stem cells, depending on their origin, are further divided in two main groups: embryonic and adult stem cells. Embryonic stem cells (ESC) are pluripotent cells deriving from the inner cell mass of a blastocyst (Cole *et al.*, 1965, 1966; Evans and Kaufman, 1981; Martin, 1981; Bongso *et al.*, 1994; Thomson *et al.*, 1995, 1998). Because of their plasticity and potentially unlimited capacity for self-renewal (Niwa, 2007; Ying *et al.*, 2008), ESC have been proposed as a potential treatment for several disorders such as diabetes and Parkinson's disease. However, to date, no approved medical treatment has been derived from embryonic stem cell research (Pucéat and Ballis, 2007; Edwards, 2008). ESC clinical use has been mainly constrained so far by: (i) safety concerns regarding their observed tendency to form tumours when injected undifferentiated or only partially differentiated *in vivo* (Lawrenz *et al.*, 2004; Hanson and Caisander, 2005; Maitra *et al.*, 2005; Teramoto *et al.*, 2005; Kolossov *et al.*, 2006; Hentze *et al.*, 2007; Shih *et al.*, 2007); (ii) possible host immune rejection of cellular allografts (Kofidis *et al.*, 2005; Swijnenburg *et al.*, 2005; Nussbaum *et al.*, 2007; Grinnemo *et al.*, 2008; Sarić *et al.*, 2008); and (iii) ethical considerations concerning their obtainment from human embryos (Daley *et al.*, 2007; Edwards, 2007; Green, 2007). On the other side of the spectrum, adult stem cells (ASC) reside in specific locations, called niches, in adult tissues in which they are able to maintain their multipotency (Jones and Wagers, 2008). Their physiological role consists of constantly renewing and, in case of damage, repopulating the tissues in which they reside (Yamashita *et al.*, 2007). At present ASC have been identified in almost all organs and tissues other than the gonads (Bonner-Weir and Weir, 2005; Griffiths *et al.*, 2005; Guettier, 2005; Kim *et al.*, 2005; Gupta *et al.*, 2006; Barker *et al.*, 2007; Lyngbaek *et al.*, 2007; Scoville *et al.*, 2008). Although their differentiation potential and proliferative capacities are limited compared with that of ESC, as well as the possibility that genetic alterations occurring with ageing may lead to a loss of their functions, ASC represent a possible resource both for research and medical purposes as their derivation can be performed in an autologous setting and does not involve the destruction of human embryos (Mimeault *et al.*, 2007).

In order to overcome the limitations associated with both ESC and ASC, attempts have been made to identify alternative stem cell sources. On the one hand, methods capable of generating patient-specific pluripotent stem cells from adult cells have been developed. Among these, nuclear reprogramming has been recently reported (Takahashi and Yamanaka, 2006; Hanna *et al.*, 2007; Meissner *et al.*, 2007; Takahashi *et al.*, 2007); it consists in the de-differentiation of somatic cells through cell retroviral transduction of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc) (Maherali *et al.*, 2007; Yamanaka, 2008). The obtained cells, i.e. induced pluripotent stem cells, are molecularly and functionally indistinguishable from ESC in many respects, as they exhibit similar morphology and growth properties, express ESC markers, are able to generate germline-competent chimeras and form tumours when injected into nude mice. Current studies are investigating the safety profile of these cells for therapeutic application (Liu, 2008). On the other hand, different multipotent progenitors have been recently isolated from the fetus, i.e. fetal stem cells. These cells, although more lineage-committed than ESC, show better proliferation and differentiation capacities in comparison to adult progenitors, do not form teratomas *in vivo* and, if obtained before week 12 of gestation, are poorly associated with rejection when transplanted in immunocompetent mice (Campagnoli *et al.*, 2001; Guillot *et al.*, 2006, 2007; Mimeault and Batra, 2006). At present haematopoietic, mesenchymal, neural, pancreatic and lung progenitors have been obtained from fetal tissues (Rollini *et al.*, 2004; Gao *et al.*, 2006, 2007; Kenzaki *et al.*, 2006; Andrade *et al.*, 2007; Chan *et al.*, 2007; Brands *et al.*, 2008; Guillot *et al.*, 2008). However, the collection of fetal tissue during gestation is associated with high morbidity and mortality both for the fetus and the mother (Walsh and Adzick, 2000; Kohl, 2004; Deprest *et al.*, 2008).

Given the aforementioned limitations, attempts have been made to obtain stem cells from extra-embryonic tissues. Umbilical cord blood was the first to be investigated, and is now a well established source of transplantable haematopoietic stem cells that have a greater proliferative capacity and lower immunologic reactivity in comparison to those derived from bone marrow (Broxmeyer *et al.*, 1989; Yu *et al.*, 2001; Schoemans *et al.*, 2006; Brunstein *et al.*, 2007; Hwang *et al.*, 2007). Moreover, it has been recently demonstrated that umbilical cord blood also contains mesenchymal (Weiss and Troyer, 2006; Secco *et al.*, 2008) and multipotent stem cells (van de Ven *et al.*, 2007), as well as cells with ESC-like characteristics (Zhao *et al.*, 2006). In the last 10 years, the placenta, the fetal membranes (i.e. amnion and chorion) and amniotic fluid have also 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 (Marcus and Woodbury, 2008). Several populations of cells with multilineage differentiation potential and immunomodulatory properties have been isolated from the human placenta and fetal membranes; they have been classified by an international workshop (Parolini *et al.*, 2007) as human amniotic epithelial cells (Tamagawa *et al.*, 2004; Miki *et al.*, 2005; Miki and Strom, 2006; Kim *et al.*, 2007a; Marcus *et al.*, 2008), human amniotic mesenchymal stromal cells (Alviano *et al.*, 2007; Soncini *et al.*, 2007), human chorionic mesenchymal stromal cells (Igura *et al.*, 2004; In 't Anker *et al.*, 2004), and human chorionic trophoblastic cells. In the amniotic fluid, two main populations of stem cells have so far been isolated: (i) amniotic fluid mesenchymal stem (AFMS) cells; and (ii) amniotic fluid stem (AFS) cells. Although only recently described, given the easier accessibility of the amniotic fluid in comparison to other extra-embryonic tissues, these cells may hold much promise in regenerative medicine.

Amniotic fluid: function, origin and composition

Amniotic fluid 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 and acts as a vehicle for the exchange of body chemicals with the mother (Underwood *et al*., 2005).

In humans, the amniotic fluid starts to appear at the beginning of week 2 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 (Miki and Strom, 2006). Thereafter, it progressively increases in volume, completely surrounding the embryo after week 4 of pregnancy. Over the course of gestation, amniotic fluid volume changes markedly from 20 ml in week 7 to 600 ml in week 25, 1000 ml in week 34 and 800 ml at birth. During the first half of gestation, the amniotic fluid results from active sodium and chloride transport across the amniotic membrane and the non-keratinized fetal skin, with concomitant passive movement of water (Brace and Resnik, 1999). In the second half of gestation, the amniotic fluid is constituted by fetal urine, gastrointestinal excretions, respiratory secretions and substances exchanged through the sac membranes (Mescher *et al*., 1975; Lotgering and Wallenburg, 1986; Muller *et al*., 1994; Fauza, 2004).

The amniotic fluid is primarily composed of water and electrolytes (98–99%) 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. Amniotic fluid cells derive both from extra-embryonic structures (i.e. placenta and fetal membranes) and from embryonic and fetal tissues (Thakar *et al*., 1982; Gosden, 1983). Although amniotic fluid cells are known to express markers of all three germ layers (Cremer *et al*., 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, urinary and gastrointestinal tracts (Milunsky, 1979; von Koskull *et al*., 1984; Fauza, 2004). Amniotic fluid cells display a broad range of morphologies and behaviours, varying with gestational age and fetal development (Hoehn and Salk, 1982). In normal conditions the number of amniotic fluid cells increases with advancing gestation; if a fetal disease is present, amniotic fluid cell counts can be either dramatically reduced (e.g. intrauterine death, urogenital atresia) or abnormally elevated (e.g. anencephaly, spina bifida, exomphalos) (Nelson, 1973; Gosden and Brock, 1978). Based on their morphological and growth characteristics, viable adherent cells from the amniotic fluid are classified into three main groups: epithelioid (33.7%), amniotic fluid (60.8%) and fibroblastic type (5.5%) (Hoehn *et al*., 1975). In the event of fetal abnormalities other types of cells can be found in the amniotic fluid, e.g. neural cells in presence of neural tube defects and peritoneal cells in case of abdominal wall malformations (Gosden and Brock, 1978; Aula *et al*., 1980; von Koskull *et al*., 1981).

The majority of cells present in the amniotic fluid are terminally differentiated and have limited proliferative capabilities (Gosden and Brock, 1978; Siegel *et al*., 2007). In the 1990s, however, two groups demonstrated the presence of small subsets of cells in the amniotic fluid harbouring a proliferation and differentiation potential. First, Torricelli *et al*. (1993) reported the presence of haematopoietic progenitors in the amniotic fluid collected before week 12 of gestation. Then Streubel *et al*. (1996) was able to differentiate amniotic fluid cells into myocytes, thus suggesting the presence in the amniotic fluid of non-haematopoietic precursors. These results initiated a new interest in the amniotic fluid as an alternative source of cells for therapeutic applications.

Amniotic fluid mesenchymal stem cells

Mesenchymal stem cells (MSC) represent a population of multipotent stem cells able to differentiate towards mesoderm-derived lineages (i.e. adipogenic, chondrogenic, myogenic and osteogenic) (Pittenger *et al*., 1999). Initially identified in adult bone marrow where they represent 0.001–0.01% of total nucleated cells (Owen and Friedenstein, 1988), MSC 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) (Porada *et al*., 2006). As they exhibit the potential to repair and regenerate damaged tissues along with immunomodulatory properties, they are at present one of the most attractive tools for clinical applications based on cell therapy (Horwitz *et al*., 2002; Abdallah and Kassem, 2008; Le Blanc *et al*., 2008).

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

Isolation and culture

AFMSC can be easily obtained: in humans, from small volumes (2–5 ml) of second-trimester amniotic fluid (Tsai *et al*., 2004), where their percentage is estimated to be 0.9–1.5% of the total amniotic fluid cells (Roubelakis *et al*., 2007); and in rodents, from the amniotic fluid collected during the second or third week of pregnancy (De Coppi *et al*., 2007a; Nadri and Soleimani, 2008). Various protocols have been proposed for their isolation; all are based on the expansion of unselected populations of amniotic fluid cells in serum-rich conditions without feeder layers, allowing cell selection by culture conditions. The success rate of the isolation of AFMSC is reported by different authors to be 100% (Tsai *et al*., 2004; Nadri and Soleimani, 2008). AFMSC 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 AFMSC can also be cultured in the absence of animal serum without losing their properties (Kunisaki *et al*., 2007); this finding is a fundamental prerequisite for the beginning of clinical trials in humans.

Characterization

The fetal versus maternal origin of AFMSC has been investigated by different authors. Molecular human leucocyte antigen typing and amplification of the sex-determining region Y gene in amniotic fluid samples collected from male fetuses (In 't Anker *et al.*, 2003; Roubelakis *et al.*, 2007) demonstrated the exclusive fetal derivation of these cells. However, whether AFMSC originate from the fetus or from the fetal portion of extra-embryonic tissues is still a matter of debate (Kunisaki *et al.*, 2007).

AFMSC display a uniform spindle-shaped fibroblast-like morphology similar to that of other MSC populations and expand rapidly in culture (Tsai *et al.*, 2007). Human cells derived from a single 2 ml amniotic fluid sample can increase up to 180×10^6 cells within 4 weeks (three passages) and, as demonstrated by growth kinetics assays, possess a greater proliferative potential (average doubling time 25–38 h) in comparison to that of bone marrow-derived MSC (average doubling time 30–90 h) (In 't Anker *et al.*, 2003; Roubelakis *et al.*, 2007; Nadri and Soleimani, 2008; Sessarego *et al.*, 2008). Moreover, the clonogenic potential of AFMSC has been shown to exceed that of MSC isolated from the bone marrow (86 ± 4.3 versus 70 ± 5.1 colonies) (Nadri and Soleimani, 2008). Despite their high proliferation rate, AFMSC retain a normal karyotype and do not display tumourigenic potential even after extensive expansion in culture (Roubelakis *et al.*, 2007; Sessarego *et al.*, 2008).

Analysis of the AFMSC transcriptome demonstrated that: (i) AFMSC gene expression profile, as well as that of other MSC populations, remains stable between passages in culture, enduring cryopreservation and thawing well; (ii) AFMSC share with MSC derived from other sources a core set of genes involved in extracellular matrix remodelling, cytoskeletal organization, chemokine regulation, plasmin activation, TGF-β and Wnt signalling pathways; and (iii) in comparison to other MSC, AFMSC show a unique gene expression signature which consists of the up-regulation 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 AFMSC in modulating the interactions between the fetus and the uterus during pregnancy (Tsai *et al.*, 2007).

The cell-surface antigenic profile of human AFMSC has been determined through flow cytometry by different investigators (**Table 1**). Cultured human AFMSC 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 haematopoietic and endothelial markers (e.g. CD45, CD34, CD14, CD133, CD31).

AFMSC exhibit a broad differentiation potential towards mesenchymal lineages. Under specific in-vitro inducing conditions, they are able to differentiate towards adipogenic,

Table 1. Immunophenotype of culture-expanded second-trimester human AFMSC: results by different groups.

Markers	*Antigen*	*CD no.*	*Roubelakis* et al. *(2007)*	*Tsai* et al. *(2004)*	*In 't Anker* et al. *(2003)*
Mesenchymal	SH2, SH3, SH4	CD73	+	+	+
	Thy1	CD90	+	+	+
	Endoglin	CD105	+	+	+
	SB10/ALCAM	CD166	+	nt	+
Endothelial and	LCA	CD14	–	nt	–
haematopoietic	gp105–120	CD34	–	–	–
	LPS-R	CD45	–	–	–
	Prominin-1	CD133	–	nt	nt
Integrins	β1-integrin	CD29	+	+	nt
	α4-integrin	CD49d	–	nt	–
	α5-integrin	CD49e	+	nt	+
	LFA-1	CD11a	+	nt	–
Selectins	E-selectin	CD62E	+	nt	–
	P-selectin	CD62P	+	nt	–
Ig superfamily	PECAM-1	CD31	+	–	–
	ICAM-1	CD54	+	nt	+
	ICAM-3	CD50	–	nt	–
	VCAM-1	CD106	+	nt	–
	HCAM-1	CD44	+	+	+
MHC	I (HLA-ABC)	none	+	+	+
	II (HLA-DR,DP,DQ)	none	nt	–	–

nt = not tested.

osteogenic and chondrogenic lineages (In 't Anker *et al*., 2003; Tsai *et al*., 2007; Nadri and Soleimani, 2008).

Preclinical studies

After AFMSC identification, various studies investigated their therapeutic potential in different experimental settings. 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 grafts (Fuchs *et al*., 2004; Kunisaki *et al*., 2006). Zhao *et al*. (2005) demonstrated that human AFMSC are able not only to express cardiac-specific genes under specific culture conditions, but also to integrate into normal and ischaemic cardiac tissue where they differentiate into cardiomyocyte-like cells. In a rat model of bladder cryo-injury, AFMSC show the ability to differentiate into smooth muscle and to prevent the compensatory hypertrophy of surviving smooth muscle cells (De Coppi *et al*., 2007a).

Intriguingly, recent papers suggest that AFMSC can harbour trophic and protective effects in the central and the peripheral nervous systems. Pan *et al*. (2006, 2007) showed that AFMSC facilitate peripheral nerve regeneration after injury and postulated that this can be determined by cell secretion of neurotrophic factors. After transplantation into the striatum, AFMSC are capable of surviving and integrating in the rat adult brain and can migrate towards areas of ischaemic damage (Cipriani *et al*., 2007). Moreover, the intra-ventricular administration of AFMSC in mice with focal cerebral ischaemia-reperfusion injuries significantly reverses neurological deficits in the treated animals (Rehni *et al*., 2007).

Remarkably, it has also been recently observed that AFMSC present an immunosuppressive effect *in vitro* similar to that of bone marrow-derived MSC (Uccelli *et al*., 2007). Following stimulation of peripheral blood mononuclear cells with anti-CD3, anti-CD28 or phytohaemagglutinin, irradiated AFMSC determine a significant inhibition of T-cell proliferation with dose-dependent kinetics (Sessarego *et al*., 2008).

Amniotic fluid stem cells

The first evidence that the amniotic fluid could contain pluripotent stem cells was provided when Prusa *et al*. (2003) described the presence of a distinct sub-population of proliferating amniotic fluid cells (0.1–0.5%) expressing the pluripotency marker Oct4 at both transcriptional and protein levels. Oct4 (i.e. octamer binding transcription factor 4) is a nuclear transcription factor that plays a critical role in maintaining ESC differentiation potential and capacity for self-renewal (Schöler *et al*., 1989; Nichols *et al*., 1998; Niwa *et al*., 2000). Other than its expression by ESC, Oct4 is specifically expressed by germ cells, where its inactivation results in apoptosis, and by embryonal carcinoma cells and tumours of germ cell origin, where it acts as an oncogenic fate determinant (Donovan, 2001; Pesce and Schöler, 2001; Gidekel *et al*., 2003; Looijenga *et al*., 2003). 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 (Berg and Goodell, 2007; Lengner *et al*., 2007; Liedtke *et al*., 2007).

After Prusa *et al*. (2003), different groups confirmed the expression of Oct4 and of its transcriptional targets (e.g. Rex-1) in the amniotic fluid (Bossolasco *et al*., 2006; Stefanidis *et al*., 2008). Remarkably, Karlmark *et al*. (2005) transfected human amniotic fluid cells with the green fluorescent protein gene under either the Oct4 or the Rex-1 promoter and established that some amniotic fluid cells were able to activate these promoters. Several authors subsequently reported the possibility of harvesting amniotic fluid cells displaying features of pluripotent stem cells (Tsai *et al*., 2006; Kim *et al*., 2007b). 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 (De Coppi *et al*., 2007b). These cells, named amniotic fluid stem cells (AFS cells), are characterized by the expression of the surface antigen c-kit (CD117), the type III tyrosine kinase receptor of the stem cell factor (Zsebo *et al*., 1990).

Isolation and culture

AFS cells can be isolated from the amniotic fluid of humans and rodents. Human AFS cells can be derived either from small volumes (5 ml) of second-trimester amniotic fluid (14–20 weeks of gestation) or from confluent back-up amniocentesis cultures. Murine AFS cells are obtainable from the amniotic fluid collected during week 2 of pregnancy (E11.5–14.5) (Tsai *et al*., 2006; De Coppi *et al*., 2007b; Kim *et al*., 2007b). AFS cells isolation is based on a two-step protocol consisting in the prior immunological selection of c-kit positive cells from the amniotic fluid (approximately 1%) and in the subsequent expansion of these cells in culture (De Coppi *et al*., 2007b; Kolambkar *et al*., 2007; Perin *et al*., 2007). 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 (De Coppi *et al*., 2007b).

Characterization

Karyotype analysis of human AFS cells deriving from pregnancies in which the fetus was male revealed the fetal origin of these cells (De Coppi *et al*., 2007b).

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 (**Figure 1a**). As demonstrated by different authors, AFS cells possess a great clonogenic potential (Tsai *et al*., 2006; De Coppi *et al*., 2007b). Clonal AFS cells lines expand rapidly in culture (doubling time = 36 h) and, more interestingly, maintain a constant telomere length (20 kbp) between early and late passages (**Figure 1b**). Despite their high proliferation rate, clonal AFS cells show a homogeneous, diploid DNA content without evidence of chromosomal rearrangement even after expansion to 250 population doublings (**Figure 1c**). Almost all clonal AFS cells lines express markers of a pluripotent undifferentiated state: Oct4 and NANOG (Tsai *et al*., 2006; Chambers *et al*., 2007; De Coppi *et al*., 2007b). However, it has been shown that they do not form tumours when injected into severe combined immunodeficient (SCID) mice (De Coppi *et al*., 2007b).

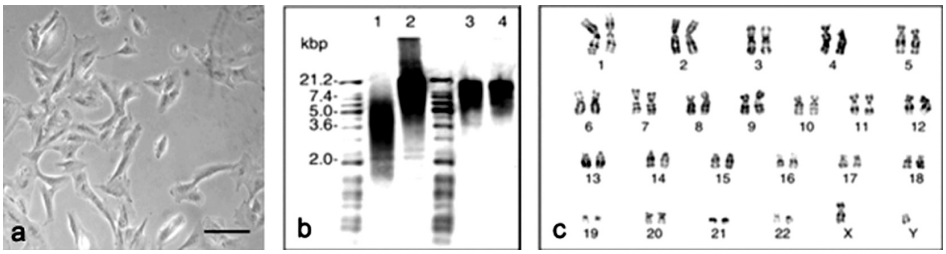

Figure 1. (**a**) Human AFS cells mainly display a spindle-shaped morphology during in-vitro cultivation under feeder layer-free, serum-rich conditions. (**b**,**c**) Clonal human AFS cell lines retain long telomeres and a normal karyotype after more than 250 cell divisions. (**b**) Conserved telomere 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 (4250 population doublings) cells (adapted from De Coppi *et al*., 2007b).

The cell-surface antigenic profile of AFS cells has been determined through flow cytometry by different investigators (**Table 2**). Cultured human AFS cells are positive for ESC (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 major histocompatibility complex I (MHC-I). They are negative for haematopoietic and endothelial markers (e.g. CD14, CD34, CD45, CD133, CD31), and for antigens belonging to the major histocompatibility complex II (MHC-II).

AFS cells and, more importantly, derived clonal cell lines are able to differentiate towards tissues representative of all three embryonic germ layers. 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 adipogenic, chondrogenic and osteogenic medium, AFS cells develop intracellular lipid droplets, secrete glycosaminoglycans and produce mineralized calcium, respectively (Tsai *et al*., 2006; Kim *et al*., 2007b). Moreover, when embedded in alginate/collagen scaffolds and implanted *in vivo*, human AFS cells are able to generate blocks of bone-like highly mineralized tissue over a period of 18 weeks *(***Figure 2a***)* (De Coppi *et al*., 2007b). 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 (**Figure 2b**) (De Coppi *et al*., 2007b). In neuronal conditions, AFS cells are capable of entering the neuro-ectodermal lineage. After induction, they express neuronal markers (e.g. GIRK potassium channels), exhibit a barium-sensitive potassium current, and release glutamate after stimulation (**Figure 2c**). Ongoing studies are investigating AFS cells capacity to yield mature, functional neurons (Santos *et al*., 2008; Toselli *et al*., 2008).

Preclinical studies

Because of the very recent identification of AFS cells, few papers exploring their differentiation potential have been published.

Chiavegato *et al*. (2007) investigated AFS cells differentiation potential towards cardiac and vascular lineages. The authors proved that, under in-vitro cardiovascular inducing conditions, human AFS cells express cardiomyocyte (i.e. Nkx2.5, MLC-2v, GATA-4, β-MyHC), endothelial (i.e. angiopoietin, CD146) and smooth muscle (i.e. smoothelin) markers. However, when xenotransplanted in a rat model of myocardial infarction, human AFS cells differentiation capabilities were impaired by cell immune rejection (Chiavegato *et al*., 2007; Dai and Kloner, 2007).

The potential of AFS cells in contributing to kidney development has also been explored. Human AFS cells injected into a mouse embryonic kidney 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 (Perin *et al*., 2007).

Very recently, AFS cells ability to integrate into the lung and to differentiate into pulmonary lineages has been elegantly investigated in different experimental models (Carraro *et al*., 2008). *In vitro*, human AFS cells injected into mouse embryonic lung explants engraft into the epithelium and 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, whilst 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 tumour formation in the treated animals up to 7 months after AFS cells injection.

The capacity of AFS cells to differentiate into functional chondrocytes has also been confirmed. Human AFS cells treated with TGF-β1 produce significant amounts of cartilaginous matrix (i.e. sulfated glycosaminoglycans and type II collagen) in both pellet and alginate hydrogel cultures (Kolambkar *et al*., 2007).

Conclusions

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

Table 2. Surface markers expressed by human AFS cells: results by different groups.

Markers	*Antigen*	*CD no.*	*De Coppi* et al. *(2007b)*	*Kim* et al. *(2007b)*	*Tsai* et al. *(2006)*
ESC	SSEA-3	none	–	+	nt
	SSEA-4	none	+	+	nt
	Tra-1–60	none	–	+	nt
	Tra-1–81	none	–	nt	nt
Mesenchymal	SH2, SH3, SH4	CD73	+	nt	+
	Thy1	CD90	+	nt	+
	Endoglin	CD105	+	nt	+
Endothelial and haematopoietic	LCA	CD14	nt	nt	–
	gp105–120	CD34	–	nt	–
	LPS-R	CD45	–	nt	nt
	Prominin-1	CD133	–	nt	nt
Integrins	β1-integrin	CD29	+	nt	+
Ig superfamily	PECAM-1	CD31	nt	+	nt
	ICAM-1	CD54	nt	+	nt
	VCAM-1	CD106	nt	+	nt
	HCAM-1	CD44	+	+	+
MHC	I (HLA-ABC)	none	+	+	+
	II (HLA-DR,DP,DQ)	none	–	–	–

nt = not tested.

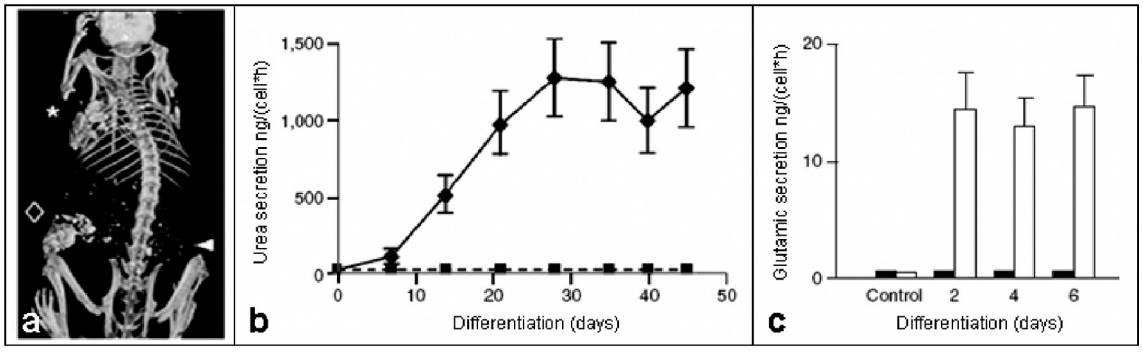

Figure 2. AFS cells differentiation into lineages representative of the three embryonic germ layers (picture adapted from De Coppi *et al.*, 2007b). (**a**) Osteogenic differentiation: mouse micro-CT scan 18 weeks after implantation of printed constructs of engineered bone from human AFS cells; arrow head: region of implantation of control scaffold without AFS cells; rhombus: scaffolds seeded with AFS cells. *(***b**) Hepatogenic differentiation: urea secretion by human AFS cells before (rectangles) and after (diamonds) hepatogenic in-vitro differentiation. (**c**) Neurogenic differentiation: secretion of neurotransmitter glutamic acid in response to potassium ions.

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 in the amniotic fluid, not burdened by ethical concerns, has the potential of opening 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 (0.06–0.83%) related to this procedure (Caughey *et al.*, 2006; Eddleman *et al.*, 2006). Moreover, stem cells can be obtained from amniotic fluid samples without interfering with diagnostic procedures.

The two stem cell populations have been isolated from the amniotic fluid so far (i.e. AFMSC and AFS cells) can both be used as primary (not transformed or immortalized) cells without further technical manipulations. AFMSC exhibit typical MSC characteristics: fibroblastic-like morphology, clonogenic capacity, multilineage differentiation potential, immunosuppressive properties, expression of a mesenchymal gene expression profile and of a mesenchymal set of surface antigens. However, ahead of other MSC sources, AFMSC are easier to isolate and show better proliferation capacities. The harvest of bone marrow remains a highly invasive and painful procedure, and the number, proliferation and differentiation potential of these cells decline with increasing age (D'Ippolito *et al.*, 1999; Kern *et al.*, 2006). Similarly, umbilical cord blood-derived MSC exist at a low percentage and expand slowly in culture (Bieback *et al.*, 2004).

AFS cells, on the other hand, represent a novel class of pluripotent stem cells with intermediate characteristics between ESC and ASC (Bajada *et al*., 2008; Siegel *et al*., 2007). 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 tumours after implantation *in vivo*. However, AFS cells have been only recently identified and many questions need to be answered concerning their origin, epigenetic state, immunological reactivity, regeneration and differentiation potential *in vivo*. AFS cells, in fact, may not differentiate as promptly as ESC and their lack of tumorigenesis can be argued against their pluripotency.

Although further studies are needed to better understand their biological properties and to define their therapeutic potential, stem cells present in the amniotic fluid 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, amniotic fluid cells collected during a routinely performed amniocentesis could be banked and, in case of need, subsequently expanded in culture or engineered in acellular grafts (Kunisaki *et al*., 2007; Siegel *et al*., 2007). 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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