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ORIGINAL ARTICLE

Molecular and phenotypic characterization of human amniotic fluid cells and their differentiation potential

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The main goal of the study was to identify a novel source of human multipotent cells, overcoming ethical issues involved in embryonic stem cell research and the limited availability of most adult stem cells. Amniotic fluid cells (AFCs) are routinely obtained for prenatal diagnosis and can be expanded *in vitro*; nevertheless current knowledge about their origin and properties is limited. Twenty samples of AFCs were exposed in culture to adipogenic, osteogenic, neurogenic and myogenic media. Differentiation was evaluated using immunocytochemistry, RT-PCR and Western blotting. Before treatments, AFCs showed heterogeneous morphologies. They were negative for MyoD, Myf-5, MRF4, Myogenin and Desmin but positive for osteocalcin, PPARgamma2, GAP43, NSE, Nestin, MAP2, GFAP and beta tubulin III by RT-PCR. The cells expressed Oct-4, Rex-1 and Runx-1, which characterize the undifferentiated stem cell state. By immunocytochemistry they expressed neural-glial proteins, mesenchymal and epithelial markers. After culture, AFCs differentiated into adipocytes and osteoblasts when the predominant cellular component was fibroblastic. Early and late neuronal antigens were still present after 2 week culture in neural specific media even if no neuronal morphologies were detectable. Our results provide evidence that human amniotic fluid contains progenitor cells with multi-lineage potential showing stem and tissue-specific gene/protein presence for several lineages.

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Introduction

Recent studies suggest that adult stem cells, capable of generating and replacing differentiated cells within their own specific tissues, may be more flexible than previously thought, giving rise to cells of unrelated tissues. Indeed, adult bone marrow, brain, skeletal muscle, liver, pancreas, fat and skin have all been shown to contain stem and progenitor cells capable of differentiating into cell types other than their tissue of origin [1, 2]. This possibility has raised enormous hopes that stem cells could be used in the treatment of diabetes, Parkinson's disease, spinal cord injuries, myocardial infarction and degenerative diseases caused by death or loss-of-function of specific cell types. Up to now, some reports exist regarding the characterization of amniotic fluid cells (AFCs) that should be considered as an interesting new source of prenatal stem cells devoid of ethical issues involved in embryonic stem cell research [3]. These cells are routinely obtained utilizing minimally invasive technique for prenatal diagnosis of fetal abnormalities

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but, despite their widespread and well-established use in prenatal genetic testing, current knowledge about origin and properties is quite limited. The amniotic fluid, contained in the sac of membranes known as the amnion, surrounds the embryo or fetus, protects from outside injury by cushioning sudden blows or movements and acts as vehicle for the exchange of body chemicals with the mother. In this fluid differentiated and undifferentiated cells arising from all three germ layers (ectoderm, mesoderm and endoderm) are present and its composition mainly depends on the gestational age [3-5]. Viable adherent cells from amniotic fluid may be classified into three major groups based on morphological, biochemical and growth characteristics: epithelioid (E-type), amniotic fluid (AF-type) and fibroblastic (F-type) cells. E-type cells have been thought to derive from fetal skin and urine, AF-type cells from fetal membranes and trophoblast and F-type cells from fibrous connective tissue and dermal fibroblasts [4-6]. Amniotic epithelial cells obtained from cesarean sections have been shown to express markers for neuronal, glial and progenitor cells and to differentiate into neuron-like cells in the ischemic brain of adult rats [7-9]. Moreover, there is evidence that amniotic fluid contains fetal mesenchymal stem cells with a multilineage differentiation potential [10-13]. In this study we extensively characterized unselected amniotic cells and tested their multilineage differentiation capacity *in vitro*. Interestingly AFCs were found positive for some markers characteristic of immature, lineage-committed and mature/functional phenotype and showed a differentiation potential towards different lineages.

Materials and methods

Cell collection and culture

Amniotic fluid samples were obtained after informed consent from 20 pregnant women (age ranging from 35-42 years) at 16 to 21 weeks of gestation. Cells were first grown for one week in a specific medium for amniocytes (Amniomed, Euroclone, UK) at 37 ^{o}C and 5% CO_2 in a fully humidified atmosphere. The supernatant of these cultures was then harvested, centrifuged at 1500 rpm for 10 min and the cells plated in 25 cm^2 flasks. Amniocytes were grown to confluence in Amniomed, or alternatively in DMEM (BioWhittaker, Belgium) supplemented with 20% fetal calf serum (FCS) (Hyclone, USA) or M199 medium (BioWhittaker) supplemented with 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin (Sigma-Aldrich, USA), 20 μg/mL of Endothelial Cell Growth Factor (ECGF) (Roche Diagnostics, Germany), trypsinized and replated at least 2 times. The cells were then cultured in specific media for adipogenic, osteogenic, muscular and neuro-glial differentiation.

Immunocytochemical analysis

Immunocytochemical analysis was performed on cytospin preparations and in situ on cells grown in Lab Tech tissue culture chamber slides (Nalge Nunc International, USA) using a DakoCytomation EnVision+System-HRP (DAB) (DAKO, USA) following the manufacturer's instructions.

In other experiments, fluorescent immunocytochemistry pre- and post-treatment was performed on cells grown until confluence. After fixation for 20 min at room temperature with 4% paraformaldehyde/PBS (pH 7.4), a permeabilization step (5 min with 0.3% Triton X-100 in PBS, Sigma Aldrich) was performed and in order to exclude false positive staining all antibody incubation and washing steps were conducted in the presence of a blocking reagent (10% goat serum in PBS) [14]. Fluorescent secondary antibodies (Cy2 and Cy3, Jackson ImmunoResearch Laboratories, USA) were used for double labeling of each field in combination with a nuclear counterstaining (4'-6-Diamidino-2-phenylindole DAPI) (Sigma Aldrich). A negative control (without primary antibody) was included.

Before and after culture in differentiating media, cells were analyzed with antibodies to the following markers: Nestin (Becton Dickinson, USA), p75NGFR (Upstate Biotechnology, USA), CD133/2 (Miltenyi Biotec Germany), SH2 (Serotex, UK), Actin (Novocastra, UK), Desmin (Novocastra), TE7 (Harlan Sera Lab, UK), CD44 (Sigma), NCAM (DAKO), CD45 (DAKO), CD34 (Becton Dickinson), MyoD (DAKO), Myogenin (SantaCruz, USA), O4 (Chemicon, USA), GFAP (polyclonal) (DAKO), GFAP (monoclonal) (Chemicon), beta tubulin III (Promega, USA) and cytokeratin AE1/AE3 (DAKO).

Western blot analysis

Samples showing comparable morphologies were collected and pooled. Briefly, protein extracts were obtained after treatment with 100 μl of lysis buffer (150 mM NaCl, 20 mM TRIS, 1% Triton X-100, 400 U/ml RNase inhibitor, pH 8, Roche) and precipitation with methanol. Forty μg of proteins were separated and blotted onto nitrocellulose membranes [15]. Sequential incubations with a polyclonal anti-human GFAP (1:1 000, DAKO), secondary peroxidase-conjugated antibody (1:15 000, Amersham Bioscience, UK) and chemiluminescent technique (ECL, Amersham Bioscience) were utilized for specific protein identification.

RNA isolation and RT-PCR

Using the RNeasy Mini Kit (QIAGEN, Germany) we extracted total RNA from 3×10^5 to 1×10^6 human AFCs. The contaminating genomic DNA was further eliminated by DNase digestion, total RNA was eluted in a final volume of 40 μl and its OD was measured at 260 nm. First strand cDNA synthesis was performed using the TaqMan Reverse Transcription using recombinant Moloney murine leukemia virus (rMoMuLV) and random hexameres as primers (Applied Biosystems, USA). One μg of total RNA was used for each reverse transcription and used for polymerase chain reaction using the PCR Master Mix 2x (Promega). A PTC-200 thermal cycler (MJ Research, USA) was programmed for 35 cycles as follows: 1 cycle at 95 ^{o}C for 2 min, 35 cycles at 95 ^{o}C for 30 s, followed by a specific melting temperature for 30 s for each gene analyzed (at 72 ^{o}C for 30s) and finally 1 cycle at 72 ^{o}C for 5 min. GAPDH was used as a normalizing housekeeping gene. To identify the expression of embryonic, myogenic, osteogenic, adipogenic and neural markers, primers were constructed based on published human sequences (see Table 1).

Primers were selected using the Primer Express (version 1.5) software available by Applied Biosystems.

Myogenic differentiation

To induce myogenic differentiation, AFCs were grown in the

following differentiating medium: DMEM 4.5 g/L glucose (BioWhittaker) supplemented with 2% FCS and 10 ng/ml Epidermal Growth Factor (EGF), 10ng/ml Platelet Derived Growth Factor (PDGF-BB) (both by Peprotech, USA), 3 μM 5-azacytidine (Sigma). After 24 h of culture, the myogenic medium was replaced without adding 5-azacytidine. The cells were also cultured in a commercial skeletal muscle cell growth medium (PromoCell, Germany). The medium was replaced weekly and cultures were observed for the presence of multinucleated cells (myotubes). After 14 day culture, RT-PCR and immunocytochemical analysis for the presence of myogenic markers were performed.

Adipogenic differentiation

To induce adipogenic differentiation AFCs were cultured for 2-3 weeks in DMEM 4.5 g/L glucose (BioWhittaker) supplemented with 10% FCS, 0.5 mM isobutyl-methylxanthine, 200 μM indomethacin, 10^{-6} M dexamethasone and 10 μg/ml insulin or 0.5 μM hydrocortisone, 60 mM indomethacin and 100 ng/ml of insulin (all by Sigma). The medium was replaced weekly. Before and after culture, cells were stained for 15 min with fresh Oil-Red O solution (Sigma) (3 parts of a stock solution 0.5% in isopropanol and 2 parts of distilled water), washed three times with distilled water and air dried. The percentage of adipocytes was determined by counting cells in multiple fields.

Osteogenic differentiation

Osteogenic differentiation was performed by culturing the cells with DMEM 4.5 g/L glucose supplemented with 10% FCS, 10^{-8} M dexamethasone, 0.2 mM ascorbic acid, and 10 mM ß-glycerol phosphate (all by Sigma-Aldrich) for 2-3 weeks. The medium was replaced weekly. To assess calcium accumulation, cultures were stained with Alizarin Red (Sigma-Aldrich) and scored for areas of mineralization. RT-PCR was also performed using osteocalcin specific primers, constructed based on published human sequences (see Table 1).

Neuro-glial differentiation

AFCs were exposed for two weeks to several neural media (used for neural stem cell, neuroblast or astroblast maintenance in culture)

Table 1 Primers used for RT-PCR.

Osteocalcin	F TGCAGAGTCCAGCAAAGGTG	Map2	F TAGCTATCCCAGGACCCCTCACAC
	R GATGTGGTCAGCCAACTCGTC		R GCCTAAATATTATTATTTCAATGC
PPARgamma2	F GCTGAATCCAGAGTCCGCTG	Nestin	F CAGCTGGCGCACCTCAAGATG
	R GCAAACTCAAACTTGGGCTCC		R AGGGAAGTTGGGCTCAGGACTGG
MyoD	F AGCACTACAGCGGCGACT	NSE	F CCCACTGATCCTTCCCGATACAT
	R GCGACTCAGAAGGCACGTC		R CCGATCTGGTTGACCTTGAGCA
Myf-5	F CAGTCCTGTCTGGTCCAGAA	TrkA	F GGGACCTCAACCGCTTCCTC
	R GGAACTAGAAGCCCCTGGAG		R ATTCCGGCTAACCACTCCCA
Desmin	F TGATGGAATACCGACACCAGA	Oct-4	F ACATGTGTAAGCTGCGGCC
	R GGTAGGTGGCAATCTCCACA		R GTTGTGCATAGTCGCTGCTTG
MRF4	F GGCTCTCCTTTGTATCCAGG	Sox-2	F ACCAGAAAAACAGCCCGGA
	R CCTTAGCCGTTATCACGAGC		R TCATGAGCGTCTTGGTTTTCC
Myogenin	F CAGCGAATGCAGCTCTCACA	hTERT	F GAGAACAAGCTGTTTGCGGG
	R AGTTGGGCATGGTTTCATCTG		R GGCATCTGAACAAAAGCCGT
GFAP	F GTGACTCATCCT CTTGAAGATGC	Rex-1	F AAACATGAGCCAGCAACTGAAG
	R ACAGATCCCACCAGTCTGCTCAC		R AGAAATCATCCCCTCCGAGAG
β tubulin III	F AGATGTACGAAGACGACGAGGAG	FGF-4	F CTACTGCAACGTGGGCATC
	R GTATCCCCGAAAATATAAACACAAA		R ACATGCCGGGGTACTTGTAG
GAP43	F TGATGCTGCCACAGAGCAGG	Runx-1	F TCACTGTGATGGCTGGCAAT
	R TGGGAAAGGACAGACTCACAGACGTG		R CTGCATCTGACTCTGAGGCTGA
PDX1	F CTGCCTTTCCCATGGATGAA	ABCG2	F GGCTTGCAACAACTATGACGAA
	R CAAGTTCAACATGACAGCCAGC		R GCCAGTTGTAGGCTCATCCAA
GAPDH	F GCTTGTCATCAATGGAAATCCC		
	R TCCACACCCATGACGAACATG		

in order to induce a neuro-glial fate. In particular AFCs were treated with a modified NS-A basal serum-free medium (Euroclone) specific for neural stem cells, alone or supplemented with 50% culture medium conditioned by mouse neural stem cells or astroblasts. A commercial embryonic stem cell serum-free medium (KnockOut™, D-MEM with specific KnockOut Serum Replacement) (both from Invitrogen, USA), normally used to maintain and amplify undifferentiated stem cells in culture was also tested.

Results

AFC morphology, phenotype and mRNA expression before differentiating treatments

Morphology: after culture we did not observe any difference between all the tested media and AFCs showed very heterogeneous sizes and morphologies: small epithelium-like cells, larger cells with irregular and swelling borders, sometimes bi-nucleated or with a high nuclear/cytoplasmic ratio and fibroblastic cells (Figure 1A).

Immunocytochemistry and Oil-Red O, Alizarin Red stainings: AFCs were not stained by Oil-Red O and Alizarin Red but were found to express some markers of adult mesenchymal stem cells (SH2, TE7, CD44) and epithelial cells (cytokeratins AE1/AE3) by immunocytochemistry. Cells also expressed at low frequency p75NGFR and CD133. Before treatment, AFCs showed diffuse staining for beta tubulin III and a localized GFAP signal that did not appear related to a specific cell morphology. Interestingly, nearly 100% of amniotic cells were strongly positive for

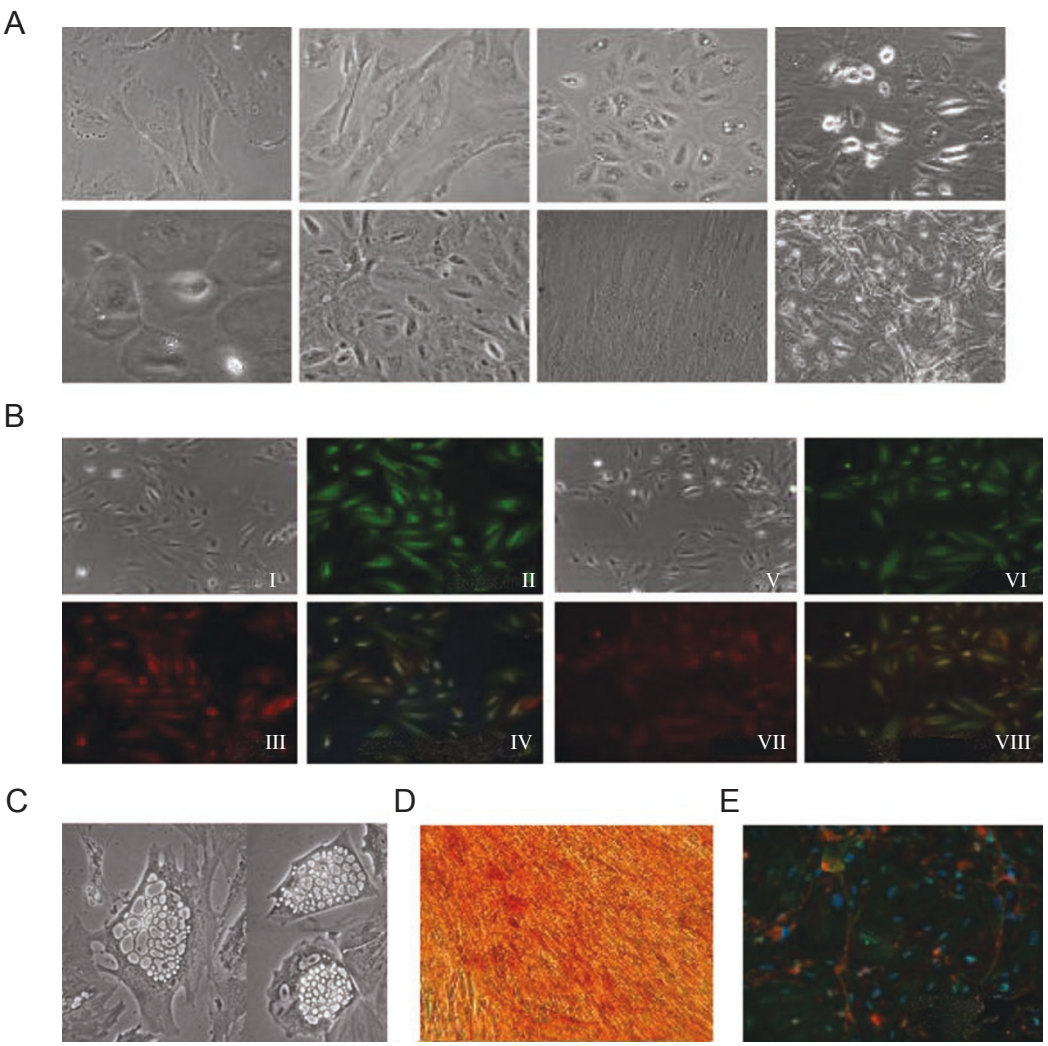

Figure 1 (A) The heterogeneous morphologies displayed by human amniotic fluid cells (AFCs). **(B)** Immunocytochemical analysis for progenitor and neuro-glial markers of AFC pre-treatments using the following specific antibodies: beta tubulin III (II), GFAP (III) and merge (IV); GFAP (VI), Nestin (VII) and merge (VIII). **(C)** Morphological changes of AFCs after adipogenic treatment showing the accumulation of lipid vacuoles. **(D)** Alizarin Red staining showing mineralization area on AFCs cultured in osteogenic differentiating medium. **(E)** Immunofluorescent analysis of AFCs after exposition to astrocyte conditioned medium for neuro-glial specific markers (GFAP and beta tubulin III).

nestin. Nestin labeling was performed with both mono and polyclonal antibodies in order to confirm this observation (data not shown). Other neural cell markers were not detected by immunocytochemistry. The percentages of positive cells and staining intensities are shown in Table 2 and Figure 1B.

RT-PCR and Western blot analysis: RT-PCR analysis detected the expression of osteocalcin, a marker for osteocytes, PPARgamma2, a marker for adipocytes and of some embryonic and stem cell markers (Oct-4, Rex-1, Runx-1, ABCG2). No expression of MyoD, Myf-5, MRF4, Myogenin, Desmin, Sox-2, hTERT, FGF-4 and PDX1 was

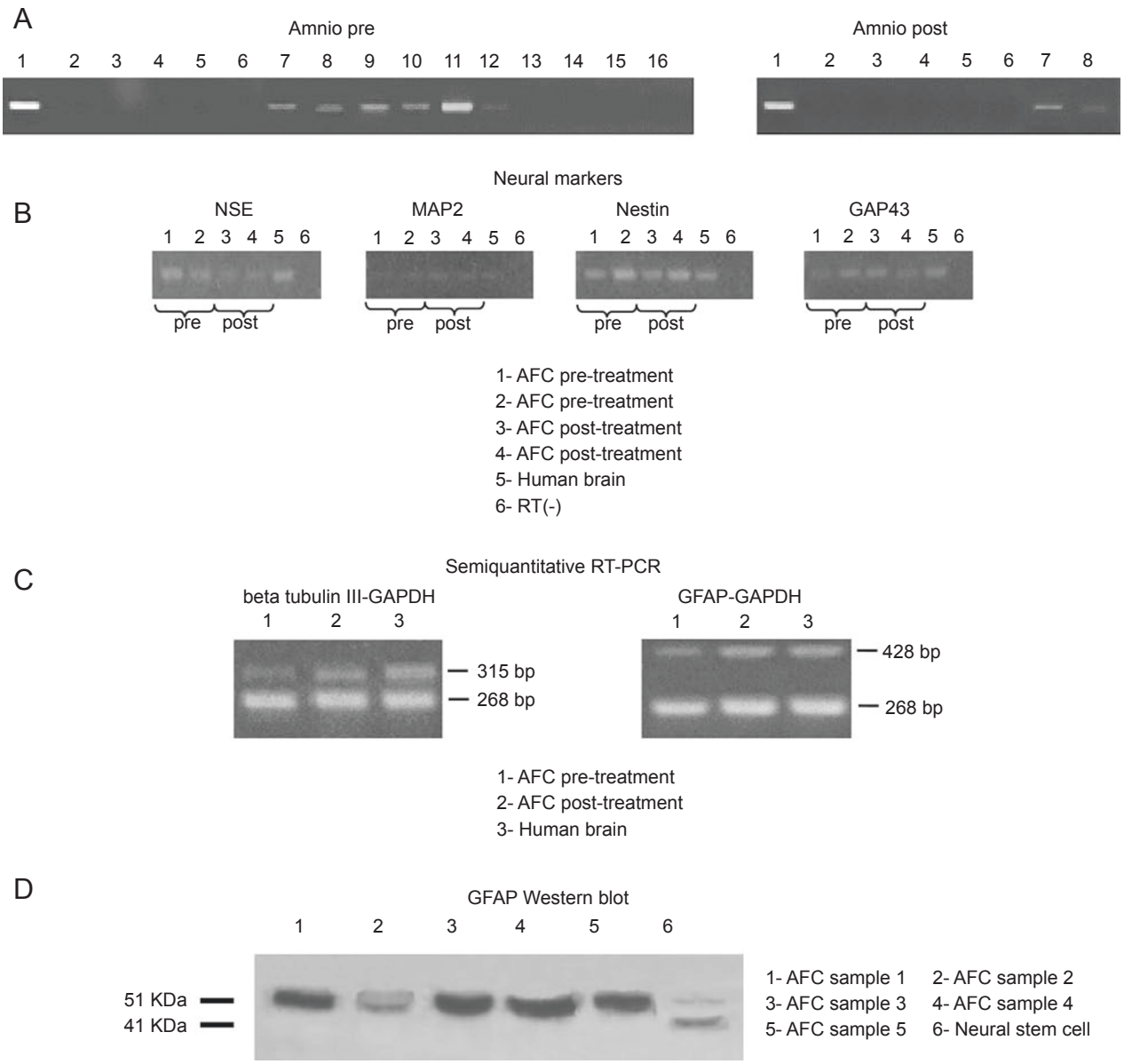

Figure 2 (A) Specific osteogenic (osteocalcin), adipogenic (PPARgamma2) and some embryonic stem cell markers (Oct-4, Rex-1, Runx-1) were present pre-treatment in our samples. No muscle expression (MyoD, Myogenin, MRF4, Desmin and Myf-5) was detected both before and after specific induction. Amnio Pre: Lane 1: GAPDH. Lane 2: MyoD. Lane 3: Myogenin. Lane 4: MRF4. Lane 5: Desmin. Lane 6: Myf-5. Lane 7: PPARgamma2. Lane 8: Osteocalcin. Lane 9: Oct-4. Lane 10: ABCG2. Lane 11: Runx-1. Lane 12: Rex-1. Lane 13: hTERT. Lane 14: SOX-2. Lane 15: FGF-4. Lane 16: PDX-1.Amnio Post: Lane 1: GAPDH. Lane 2: MyoD. Lane 3: Myogenin. Lane 4: MRF4. Lane 5: Desmin. Lane 6: Myf-5. Lane 7: PPARgamma2. Lane 8: Osteocalcin. **(B)** the neural markers NSE, MAP2, Nestin and GAP43 showed no differences both pre- and post-treatment by RT-PCR. **(C)** a semiquantitative RT-PCR showed an increase of beta tubulin III and GFAP expression post-treatment induction. **(D)** Western blot analysis revealed the presence of GFAP neural specific isoform (50±1 kDa). As a control we used neural stem cell derived protein extract showing the presence of both the isoforms (40±1 and 50±1 kDa).

Table 2 Immunocytochemical analysis of amniotic fluid cells before and after differentiating treatments

Marker	Before treatments	After treatments
MyoD	-	-
Myogenin	-	-
Desmin	<5%	-
Actin	-	nd
CD133	<5%	-
Nestin	100%	nd
SH2	80%	nd
CD45	-	nd
CD34	-	nd
TE7	70%	nd
NGFR	<0.1%	nd
CD44	100%	nd
NCAM	-	nd
AE1/AE3	99%	nd
O4	-	nd
GFAP	54%	70%
beta tubulin III	89%	95%

Results are expressed as percentage of positive cells on the total number of counted cells; negative results (-); not done (nd).

detected (Figure 2A). Interestingly, at the mRNA level AFCs expressed all the neural markers that we investigated (GAP43, NSE, Nestin, MAP2, GFAP and beta tubulin III) with the exception of TrkA. GFAP expression was also analyzed by Western blot and we detected the 50±1 kDa band (Figure 2D) corresponding to the central nervous system specific isoform of the protein [16].

AFC morphology, phenotype and mRNA expression after differentiating treatments

During adipogenic differentiation adipocytes were detected sporadically by light microscopy (1-5% of cells showed accumulation of lipid vacuoles) (Figure 1C) and staining for Oil-Red O. By RT-PCR, expression of PPAR-gamma2 was revealed both before and after adipogenic differentiation (Figure 2A).

Regarding osteogenic differentiation, only when AFCs showed a fibroblastic morphology we were able to obtain extended areas of mineralization as shown by staining with Alizarin Red (Figure 1D). Cells were positive for osteocalcin by RT-PCR (Figure 2A).

The cells grown in two muscle-specific media were unable to give rise to multinucleated cells and were negative for MyoD, Myogenin and Desmin by immunocytochemistry. In addition, no expression of MyoD, Myf-5, MRF4, Myogenin and Desmin was revealed by RT-PCR (Figure 2A).

After culture with neural media (NSA media or Knock-Out medium) we were not able to detect major specific morphological changes towards neuroglial phenotypes (Figure 1E). A slight increase was observed in the expression of beta tubulin III and GFAP after treatment by immunocytochemistry (Table 2). In order to confirm these enhancements, a semiquantitative RT-PCR was assessed to relatively quantify beta tubulin III and GFAP levels (Figure 2C). No differences were detected for the other neural genes by RT-PCR (Figure 2B).

Discussion

In this study, we explored AFC characteristics and multilineage potential. Before differentiating treatments, the cells isolated from different samples showed a highly heterogeneous morphology with a predominance of epithelial cells. This heterogeneity was also confirmed by immunocytochemistry and RT-PCR. Indeed, the cells expressed mesenchymal and epithelial cell markers such as SH2, CD44, TE7 and AE1/AE3 while they did not express a hematopoietic cell phenotype (CD45 and CD34). Osteocyte and adipocyte markers were detected by RT-PCR. No muscle specific markers were detected.

Regarding the neuronal markers, AFCs expressed mRNA and proteins involved in neural differentiation and function. Our characterization is in accordance with previously published data, although most reports refer to human amniotic epithelial cells (HAEC), known to be part of the cellular content of amniotic fluid. In this regard, Sakuragawa *et al.* [7] and Ishii *et al.* [8] reported that this epithelial cell subpopulation derived from placenta may give rise to neurons and glial cells. In our hands, after the exposition to neural cell specific media, AFCs do not alter their morphology and no major modifications of neural cell markers were detected by RT-PCR. Some authors have related HAEC neurotrophic function (i.e. acetylcholine and catecholamine synthesis and release) to their ability to sustain the early phases of neuroepithelium formation and neural development of the embryo [17]. In addition these cells can survive after implantation into a rat model of Parkinson's disease [18] or after spinal cord injury [19] and differentiate into neuron-like cells after transplantation [9]. Only a recent article by Prusa *et al.* reports neuronal differentiation of AFCs after exposition to a medium containing 2% serum and 1.25% dimethyl sulfoxide (DMSO) [20]. This effect could be induced by the presence of DMSO in their culture, which can cause cell shrinkage and neuron-like appearance as reported by some authors [21, 22].

In our study we focused on two neuronal markers. Firstly, AFCs were strongly positive for nestin, an intermediate

filament protein primarily expressed in the cerebrum during embryonic and early postnatal development, in dermatomal cells and myoblasts during myogenesis. Moreover, recently nestin has been found also in other non-neural cell types (such as cells in the pancreatic islets of Langerhans) [23, 24], cells of human limbal epithelia [25] and of the developing rat cochlea sensory epithelia [26], suggesting that its expression is a property of multipotent progenitor cells [27].

Secondly, we demonstrated the presence of GFAP in AFCs by immunocytochemistry and RT-PCR. Recently this intermediate filament protein has been also detected in various tissues with no specific glial function such as liver, gut, kidney [28, 29] and human umbilical vein endothelial cells [30]. In this regard we demonstrated by Western blot analysis that the protein found in AFCs is the central nervous system isoform specific for astrocytes.

Moreover, the multilineage differentiation capacity of the cultured AFCs was also tested by culturing these cells under specific osteogenic, adipogenic and myogenic culture conditions. After these treatments, AFCs gave rise to adipocytes and osteoblasts, when fibroblasts were the predominant cell population. In this regard, it has been reported [10, 12, 13] that amniotic fluid mesenchymal stem cells display a multilineage differentiation potential into fibroblasts, adipocytes and osteocytes. We suggest that the limited differentiation potential towards mesenchymal lineages that we found in our AFC cultures could be due to the cell heterogeneity with high percentage of epithelial cells. In this regard, the differentiation potential of AFCs could be improved in future studies by using additional cell selection procedures based on CD133 antigen expression. In fact, we found that AFCs are interestingly positive for this novel marker of stemness, that defines a population of primitive and immature cells.

Finally, it is noteworthy that AFCs were found positive for some genes (Oct-4, Runx-1, Rex-1, ABCG2, Nestin) characteristic of the embryonic and adult stem cells, thus suggesting their possible multipotentiality. The co-expression of these stemness genes with lineage specific differentiation markers confirm that AFCs include multipotent stem, committed and differentiated cells as very recently supported by a proteomic analysis showing the protein profile characteristic of embryonic, fetal and pluripotent cells [31].

In conclusion, our study supports the flexibility of AFCs and their promising potential as a multipotent cell source for regenerative somatic cell therapy.

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