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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 12 February 2010
Final form accepted: 17 May 2010
Published online: 24 May 2010

Volume 15 (2010) pp 424-439
DOI: 10.2478/s11658-010-0017-1

Research article

THE HETEROGENEITY OF CELL SUBTYPES FROM A PRIMARY CULTURE OF HUMAN AMNIOTIC FLUID

SHENGLI ZHANG[1,2], HONGQUAN GENG[3], HUA XIE[1], QIQUAN WU[1,2], XIAORONG MA[1,2], JUNMEI ZHOU[1,3]* and FANG CHEN[1,3]*

[1]Department of Urology, Shanghai Children's Hospital, Shanghai Jiao Tong University School of Medicine, 1400 Beijing Road West, Shanghai, 200040, China, [2]Department of Urology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China, [3]Tissue Engineering Laboratory, Xinhua Hospital, Shanghai Institute for Pediatric Research, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Abstract: Heterogeneous human amniotic fluid contains various cell types. Herein, we report on the possibility of simultaneously isolating three subtypes of cells from one primary culture. Using a stainless steel instrument named a colony poculum, two of the three cell subtypes could be efficiently cultured, and these were further characterized. The results indicated that these two cell subtypes had different morphologies and were characterized by different cell marker expression profiles, including the differential expression of CD105, CD117 and EBAF. Furthermore, their gene expression array data revealed their different gene expression profiles. Although both cell types expressed several embryonic stem cell-specific markers, they were non-tumorigenic *in vivo*. This paper not only provides new insight into the heterogeneity of human amniotic fluid, it also presents a simple yet efficient cell isolation method. These results will contribute to the thorough investigation of the properties and potential future applications of human amniotic fluid-derived cells.

Key words: Human amniotic fluid, Cell subtypes, Isolation, Colony poculum

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* Authors for correspondence. e-mail addresses: Fang Chen: cdbxh@yahoo.cn, Junmei Zhou: jemyzh@vip.sina.com, tel.: 86-21-62470059, fax: 86-21-62790494

Abbreviations used: AF-type – amniotic fluid-specific; DAPI – dipeptidyl aminopeptidase; EC cells – embryonal carcinoma cells; EG cells – embryonic germ cells; ES cells – embryonic stem cells; E-type – epitheloid type; FITC – fluorescein isothiocyanate; F-type – fibroblastic type; GO – gene ontology; hAFC – human amniotic fluid-derived cells; PBS – phosphate-buffered saline; PE – phycoerythrin; RT – room temperature; RT-PCR – reverse transcriptase polymerase chain reaction

INTRODUCTION

Human amniotic fluid cells are used for the prenatal diagnosis of a wide range of fetal abnormalities caused by genetic mutations. These cells can be obtained from disparate fetal tissues including bladder, lung, skin, gastroinestinal and oral cavity tissues [1]. As amniotic fluid cells are so diverse, there is considerable literature concerning their varied nature. However, their properties have not yet been fully elucidated. Several research groups have obtained long-term propagating cell lines with various properties from human amniotic fluid [2-7]. These results do show some common features of human amniotic fluid-derived cells (hAFC): they primarily express mesenchymal markers [3, 4], although some hAFC also express Oct4 [2, 6], which is a POU transcription factor that is considered to be a marker for pluripotent embryonic stem (ES) cells, embryonal carcinoma (EC) cells and embryonic germ (EG) cells [8]. Further research showed that these cells can be induced to differentiate into distinct cell lineages such as adipocytes, osteocytes and neuronal cells under specific conditions [3, 4, 6, 7]. As a result, hAFC seem to be a unique cell population in an intermediate stage between embryonic and adult stem cells in terms of their properties [9], making them an appealing stem cell source for future cell therapeutic strategies.

Amniotic fluid is heterogeneous and contains various types of cells. In terms of its morphological aspects and growth potential, three cell types have been identified from different amniotic fluid culture systems [10, 11]: epitheloid-type (E-type), amniotic fluid-specific (AF-type) and fibroblastic-type (F-type) cells. AF-type and E-type cells are present at the outset of cultivation, while F-type cells usually appear later and cannot be identified in every amniocentesis sample. However, the inherent mechanism responsible for the cell variation of specific hAFC cultured by different research groups remains unclear [10, 11]. In addition, there are inconsistencies in reports on the hAFC surface markers. Some laboratories found that hAFC are negative for CD117 [4, 12], while another group was able to isolate hAFC with CD117-bound magnetic microspheres [6]. The expression levels of CD105 was also found to differ. Some groups found that CD105 is strongly expressed in hAFC [6, 12], while another group reported that CD105 is only weakly expressed [4]. The fact that such studies yielded significant differences in the expression profiles of hAFC indicates the compelling need for further investigation.

In this study, we addressed the issues discussed above by developing a novel method to simultaneously isolate the three different cell subtypes from human amniotic fluid at the primary culture stage. A colony poculum made of stainless steel was used to separately select cell colonies with different morphologies. The high density of the stainless steel made the colony poculum easy to handle during the isolation procedure, making it superior to the commercially available plastic cloning cylinders. This paper not only presents a simple yet efficient cell isolation method, it also provides new insight into the properties of human amniotic fluid. The results herein contribute to a more thorough understanding

of the properties of hAFC, and may provide important information regarding the potential therapeutic applications of these cells.

MATERIALS AND METHODS

Preparation of the colony poculum

A cylindrical colony poculum is made of stainless steel with a diameter of 0.6 to 0.8 cm. The wall is smooth, and its height is no more than the height of a culture dish (Fig. 1A). One day prior to performing the experiment, the colony poculums were sonicated for 60 min and then autoclaved. Vaseline was also autoclaved for these experiments.

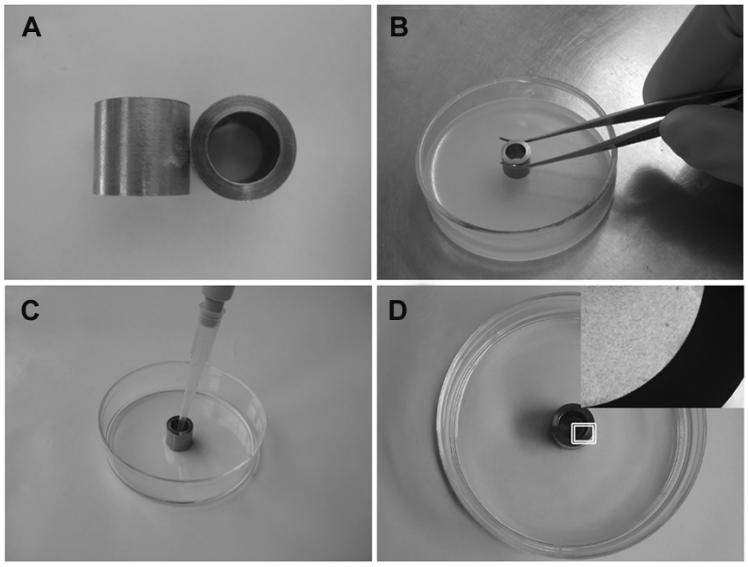

Fig. 1. The isolation procedure using the colony poculum. A – The colony poculum, top and side view. B – The bottom of the colony poculum was gently smeared with Vaseline and then the poculum was applied to the desired cell colony. C – The cells in the colony were trypsin-digested and aliquoted with a 200-µl micropipette. D – The cell suspension within the colony poculum. The insert shows an enlarged photo of the area marked with a white square. The red portion is the cell suspension and the black portion is the wall of the colony poculum.

Collection of human amniotic fluid samples

This study was carried out with the approval of the Ethics Committee at the Xin Hua hospital, which is affiliated with Shanghai Jiaotong University, and informed consent forms were obtained from all the pregnant women who donated samples of their amniotic fluid for this scientific research. The samples of amniotic fluid were collected through amniocentesis under sterile conditions using a 22-G needle during the gestational period between 16 and 18 weeks. Nine samples with no genetic abnormalities (Down Syndrome, Edwards Syndrome, etc.) or structural disorders (neural tube defects, congenital heart disease, etc.) were used in this study.

Primary culture of the human amniotic fluid cells

Amniotic fluid samples (generally 25 ml for each sample) were filtered using 100-μm filters, and then centrifuged at 400 g (4ºC) for 10 min. The supernatant was then discarded, and the precipitates were seeded in 60-mm tissue culture dishes that were pre-coated with 0.2% gelatin. The dishes were incubated at 37ºC with 5% humidified CO_2 using hAFC medium that consisted of a-MEM medium (Gibco, Invitrogen) containing 15% ES-grade FBS, 1% glutamine and 1% penicillin/streptomycin (Gibco, Invitrogen) supplemented with 18% Chang B and 2% Chang C (Irvine Scientific). Non-adhering cells, along with the original medium, were removed on the fifth day after seeding. The adherent cells were maintained in the original dishes, and new medium was added. Small colonies with different morphologies were observed at this time, and they were labeled on the back side of the dish. They gradually grew larger in size. The medium used to culture these colonies was changed every other day. Each colony was photographed on a daily basis using phase-contrast microscopy.

Isolation of the colony-forming cells

One day prior to performing the isolation procedure, a new 24-well plate was precoated with 0.2% gelatin. On the day that the cells were isolated, the gelatin solution was replaced with 1 ml of hAFC medium. After removing the old medium, the dish was rinsed once with phosphate-buffered saline (PBS; Gibco, Invitrogen), and then 2 ml of PBS was added to the dish. The bottom of the sterile colony poculum was gently smeared with Vaseline to keep the poculum immobilized during the entire procedure (Fig. 1B), and it was then carefully placed around a colony. The PBS within the colony poculum was drawn off using a 200-μl micropipette, and then 40 μl of 0.05% trypsin-EDTA was added to the poculum. After approximately 2 min, the trypsin-EDTA solution was neutralized by the addition of 160 μl of hAFC medium, and then the solution was mixed gently with a 200-μl micropipette at least 5 to 10 times in order to dissociate the cells (Fig. 1C). The cell suspension within the poculum (Fig. 1D) was carefully transferred to the prepared 24-well plate. Using this procedure, cell colonies could be individually isolated and continuously propagated. There was still a large amount of remaining cells in the culture dish after the colony selection; these were used for routine clinical diagnosis. Only those samples with normal clinical diagnoses were used in the subsequent experiments.

Flow cytometry analysis

To verify the antigen expression, cells from 4 of the samples at passage 5 were analyzed using a Becton Dickinson FACScan flow cytometer. The cells were detached and stained with fluorescein isothiocyanate- or phycoerythrin-conjugated antibodies against CD10, CD14, CD34, CD44, CD45, CD90, CD105, CD117, HLA-A, HLA-B, HLA-C, HLA-DR, HLA-DP and HLA-DQ, all of which were purchased from the Becton Dickinson company.

Reverse transcriptase polymerase chain reaction

All of the solutions for reverse transcriptase polymerase chain reaction (RT-PCR) were prepared using distilled water that had been treated with 0.1% diethylpyrocarbonate. The total RNA was extracted from the cultured cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. RT-PCR was performed using a Two-Step RT-PCR kit and specific DNA primers (Tab. 1). The amplified DNA fragments were resolved and visualized using gel electrophoresis. The gel was stained with ethidium bromide, and the DNA in the gel was imaged under ultraviolet light using a Bioprofile image analysis system.

Tab. 1. The primers used in the RT-PCR experiments.

Gene	Accession No.	Primer sequence	Annealing temp. (ºC)	Product size (bp)
Oct-4	NM_002701.1	S 5'TGGGGGTTCTATTTGGGAAGG 3' A 5'GTTCGCTTTCTCTTTCGGGC 3'	55	193
Nanog	NM_024865	S 5'TGATTCTTCCACCAGTCC 3' A 5'TGGAGGCTGAGGTATTTC 3'	60	171
TDGF1	NM_003212	S 5'ATGGCCCGCTTCTCTTAC 3' A 5'GTTCCGTCCGTAGAAGGAG 3'	55	300
Sox2	NM_003106	S 5'CAACGGCAGCTACAGCA 3' A 5'GGAGTGGGAGGAAGAGGT 3'	60	283
EBAF	NM_003240	S 5'TGTCTTTCCCGTCCATCA 3' A 5'AGCCCTTCATCCTTCCTC 3'	58	346
Thy-1	NM_006288	S 5'CCCCGCAATCCCTCAAAC 3' A 5'CCCAATCCTGGCTTCCCT 3'	55	227
FGF4	NM_002007	S 5'GGGCGTGGTGAGCATCTT 3' A 5'CTTCTTGGTCTTCCCATTCTTG 3'	50	199
Rex1	AF450454	S 5'GCGTACGCAAATTAAAGTCCAGA 3' A 5'CAGCATCCTAAACAGCTCGCAGAAT 3'	58	306
GADPH	NM_002046.3	S 5' AGCCACATCGCTCAGACACC 3' A 5' GTACTCAGCGCCAGCATCG 3'	60	303

Microarray analysis

The total RNA was harvested using TRizol Reagent (Invitrogen) and an RNeasy kit (Qiagen) according to the manufacturer's instructions. The samples were amplified and labeled using the Agilent Quick Amp labeling kit, and hybridized with the Agilent whole genome oligo microarray in Agilent's SureHyb Hybridization Chambers. The arrays were scanned with an Agilent DNA microarray scanner (part number G2505B), and the data was analyzed using the GeneSpring GX software (Agilent). Differentially expressed genes were identified using fold-change screening. In addition, gene ontology (GO) analysis and pathway analysis were performed on these genes.

Immunocytochemical staining

Cells were fixed in 4% paraformaladehyde for 10 min at room temperature (RT), washed three times with PBS, and treated with 0.05% TritonX-100 for 15 min at RT. The cells were then blocked with 3% bovine serum albumin/PBS for 30 min at RT prior to being incubated with the primary antibodies. The primary

antibodies included mouse antibodies against Oct4 (1:200, Chemicon) and SSEA-4 (1:100, Chemicon) and rabbit anti-TRA-1-81 (1:100, Chemicon). All of the antibodies were diluted in PBS and incubated with the samples overnight at 4ºC. On the following day, the cells were washed three times with PBS and incubated with FITC- or Cy3-conjugated secondary antibodies (1:100, Jackson, West Grove, PA) at RT for one hour. The nuclei were counterstained with dipeptidyl aminopeptidase (DAPI). Fluorescent signals were visualized and captured using a confocal microscope (FV500, Olympus, Japan).

Tumorigenic observation

Approximately 2-3 x 10^7 cells were injected into the thigh muscles of 4- to 6-week old NOD-SCID mice. The mice were examined for teratoma formation 14 to 16 weeks later.

Cell cycle and karyotype analysis

Cells were harvested and stained with propidium iodide. After staining, the samples were analyzed for DNA content using a flow cytometer (Becton Dickinson, Mountain View, CA). The data was analyzed using the Multicycle software program, which uses the polynomial S-phase algorithm. For the karyotype analysis, the cells were incubated in growth medium supplemented with 0.2 mg/ml colchicine for 2 h at 37ºC, 5% CO_2. After washing, the cells were disaggregated with trypsin/EDTA solution for 2 min, and resuspended in 75 mM KCl. The cells were fixed in a series of cool methanol:acetic acid (3:1, 2:1, and 3:1) and drops of the cell suspension were spread on clean microscope slides. The chromosomes were stained with 5% Giemsa for 40 minutes and examined at 1,000×. At least 20 photographs of 6 metaphase spreads were counted.

RESULTS

Observation of the different morphological cell colonies that grew simultaneously in one primary culture dish

Cell colonies were observable on the fifth day after primary seeding. Three distinct types of cell colony could be simultaneously observed in the primary culture dish. The first type of cell colony appeared to be round in shape, had a relatively tight morphology and was characterized by a well-defined border (Fig. 2A, i). These colonies grew rapidly in size during the cell propagation phase (Fig. 2A, ii). After the first passage, these cells had a short spindle-like shape with large nuclei. The cells exhibited characteristics very similar to those of the previously described AF-type cells. The second type of cell colony, which lacked a defined border, was observed later, after primary seeding, and appeared significantly looser in overall morphology compared to the AF-type (Fig. 2B, i). These cells gradually formed a separate colony, after which these colonies proliferated rapidly (Fig. 2B, ii). After the first passage, these cells had a long spindle-like shape with large nuclei. The characteristics of these cells were very

similar to the previously described features of F-type cells. The third type of cells appeared as early as the aforementioned AF-type cells. Although these colonies were round in shape, they were not as tight in overall cell morphology as the AF-type cells (Fig. 2C, i). In addition, most of these cells were shorter than the AF-type cells, and they were not in contact with other cells (Fig. 2C, ii). Their characteristics were in accordance with those previously described for E-type cells, which could not be propagated continuously.

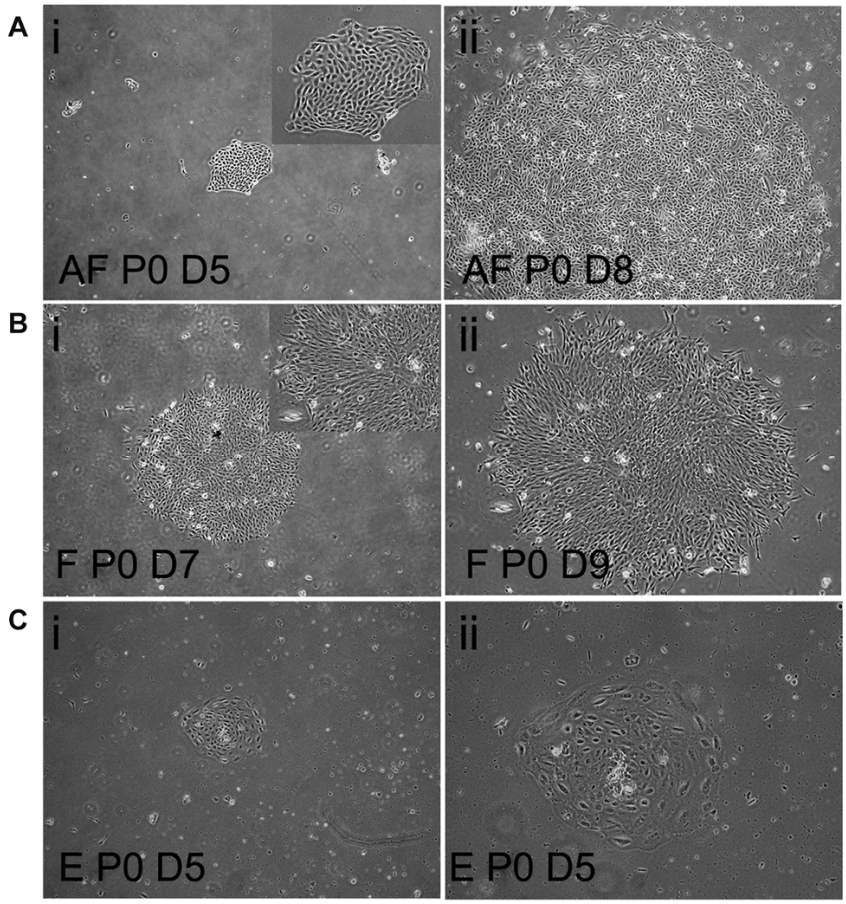

Fig. 2. Morphological differences in the primary cells. A – The morphology of the AF-type cells. i. A phase-contrast image of a primary AF-type cell colony on the fifth day after seeding the amniotic fluid (magnification: 100x). ii. A typical AF-type cell colony on the eighth day after seeding (magnification: 40x). B – The morphology of the F-type cells. i. A phase-contrast image of a primary F-type cell colony on the seventh day after seeding (magnification: 100x). ii. A typical AF-type cell colony on the ninth day after seeding (magnification: 40x). C – The morphology of the E-type cells. i. A phase-contrast image of a primary E-type cell colony on the fifth day after seeding (magnification: 40x). ii. An enlarged photo of Fig. 1Ci (magnification: 100x). P = passage; D = day; AF = AF-type; F = F-type; E = E-type.

Three types of cell colonies were simultaneously isolated using the colony poculum, and two types could be efficiently propagated

Each cell colony was marked on the back of the culture dish. When the colonies had grown to sizes suitable for isolation with the colony poculum, they were isolated and propagated. After the primary culture (passage 0), a total of 85 cell colonies containing three cell types were isolated from nine different amniotic fluid samples into 24-well plates (passage 1). To facilitate proliferation, these colonies were passaged and initially grown in 12-well plates (passage 2). Once

Tab. 2. The number of colonies obtained from each of the three types of cell isolated from nine human amniotic fluid samples.

Sample No.	Type	Numbers of colonies in passage 1 in a 24-well plate		Numbers of colonies in passage 2 in a 12-well plate		Numbers of colonies in passage 3 in 35-mm dishes	
		Total		Total		Total	
	E-type		0		0		0
1	AF-type	8	6	8	6	8	6
	F-type		2		2		2
	E-type		0		0		0
2	AF-type	7	4	7	4	7	4
	F-type		3		3		3
	E-type		2		0		0
3	AF-type	10	5	8	5	8	5
	F-type		3		3		3
	E-type		1		0		0
4	AF-type	9	6	8	6	8	6
	F-type		2		2		2
	E-type		0		0		0
5	AF-type	7	4	7	4	7	4
	F-type		3		3		3
	E-type		0		0		0
6	AF-type	12	8	9	6	9	6
	F-type		4		3		3
	E-type		0		0		0
7	AF-type	11	8	11	8	11	8
	F-type		3		3		3
	E-type		3		0		0
8	AF-type	12	6	9	6	9	6
	F-type		3		3		3
	E-type		2		0		0
9	AF-type	9	7	7	7	7	7
	F-type		0		0		0
Total		85		74		74	

they had achieved approximately 80 to 90% confluence, they were transferred first to 35-mm dishes (passage 3), and then to 60-mm (passage 4) and 100-mm culture dishes (passage 5) when the cells had reached the same confluence at each passage. Most of the AF-type and F-type cell colonies could be isolated and cultured from each amniotic fluid sample assayed, while only eight E-type cell colonies could be identified from the primary culture of the amniotic fluid, and of these, none could be continuously propagated (Tab. 2). Therefore, we only analyzed the marker expression of the AF- and F-type cells. Both the AF- and F-types of cells could be passaged over 20 times without obvious morphological changes.

AF-type and F-type cells displayed different marker expressions

Using flow cytometry analysis, RT-PCR and immunocytochemcal staining, we characterized the gene expression profiles of the cultured AF- and F-type cells. The flow cytometry results indicated that 37.4% of the F-type cells were positive for CD117, while only 12.92% of the AF-type cells were positive for CD117. Moreover, while CD105 was strongly expressed in the F-type cells (96.11%), CD105 was only weakly detected in the AF-type cells (2.88%). In addition, most of the AF- and F-type cells expressed CD44, CD90 and HLA-A,B,C, and most were negative for CD10, CD14, CD34, CD45 and HLA-DR,DP,DQ (Fig. 3).

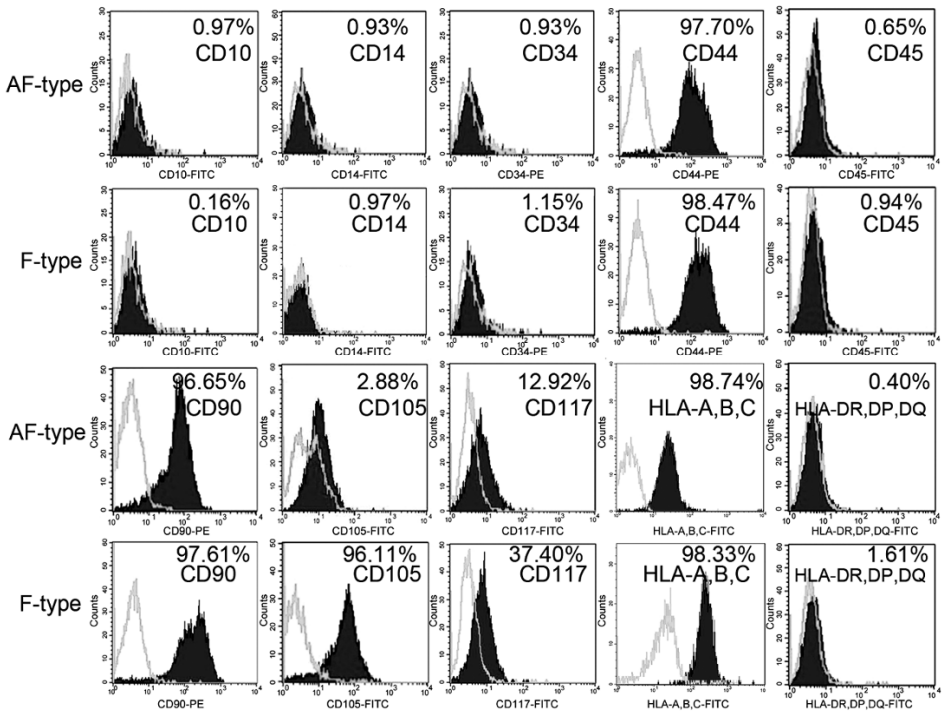

Fig. 3. The results of a flow cytometry analysis of AF-type and F-type hAFC in passage 5.

RT-PCR using eight ES cell-specific genes showed that both the AF- and F-type cells expressed Oct-4, Nanog, Sox2, Rex-1 and Thy-1 at different levels, but neither of the cell types expressed FGF4 or TDGF1. We also found that only the F-type cells expressed EBAF (Fig. 4A). Immunocytochemical staining indicated that the AF-type cells (Fig. 4B, i) were positive for Oct4 in the nucleus (Fig. 4B, ii) and were positive for SSEA-4 and TRA-1-81 in the cytoplasm and cell membrane, respectively (Fig. 4B, iii and iv). The F-type cells were also positive for these ES cell-specific markers, and notably, no significant differences were found between the AF-type and F-type cells (data not shown).

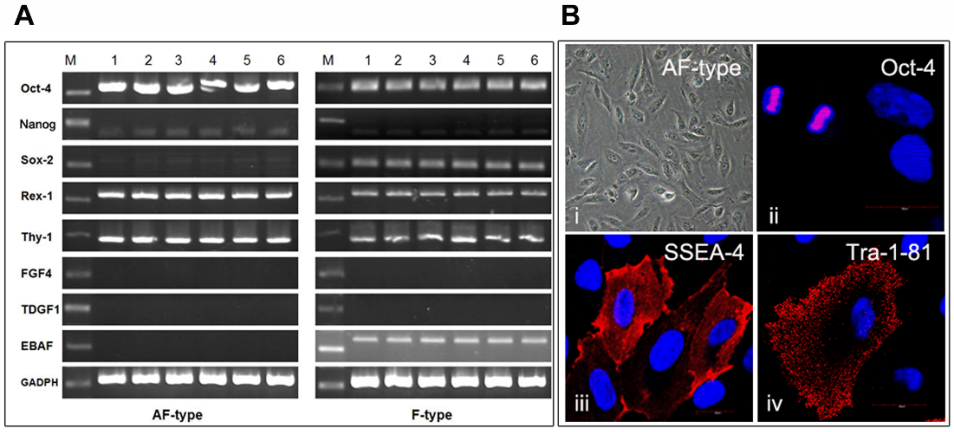

Fig. 4. ES cell-specific marker expression of hAFC. A – The RT-PCR results indicated that both the AF-type and F-type cells expressed Oct4, Nanog, Sox2, Rex-1 and Thy-1 at different levels. Neither type of cells expressed FGF4 or TDGF1. Only the F-type cells expressed EBAF. M refers to the DNA marker, and the numbers 1-6 indicate different samples of AF-type or F-type, as labelled. B – Immunocytochemcal staining of AF-type cells. i. A phase-contrast image of AF-type cells (magnification: 200x). ii. Oct4 immunostaining of AF-type cells (magnification: 1200x). iii. SSEA-4-immunostaining of AF-type cells (magnification: 1200x). iv. TRA-1-81-immunostaining of AF-type cells (magnification: 1200x).

Microarray data demonstrated different global gene expression profiles between the AF-type and F-type cells

The microarray data revealed that the AF-type and F-type cells had different global gene expression profiles (Fig. 5). The scatter plots created using this data demonstrated 2-fold changes in the gene expression levels between these two groups of cells (Fig. 5A). The hierarchical clustering of the global gene expression indicated a similar gene expression profile within the AF- and F-type groups, and a distinguished gene expression profile between the AF- and F-type groups (Fig. 5B). GO enrichment analysis between these two groups demonstrated dramatic differences in their biological processes (Fig. 5C), cellular components (Fig. 5D) and molecular functions (Fig. 5E). This data

confirmed that the simultaneously isolated AF- and F-type cells were not identical cell types. Detailed microarray information is provided in the supplemental materials (http://dx.doi.org/10.2478/s11658-010-0017-1).

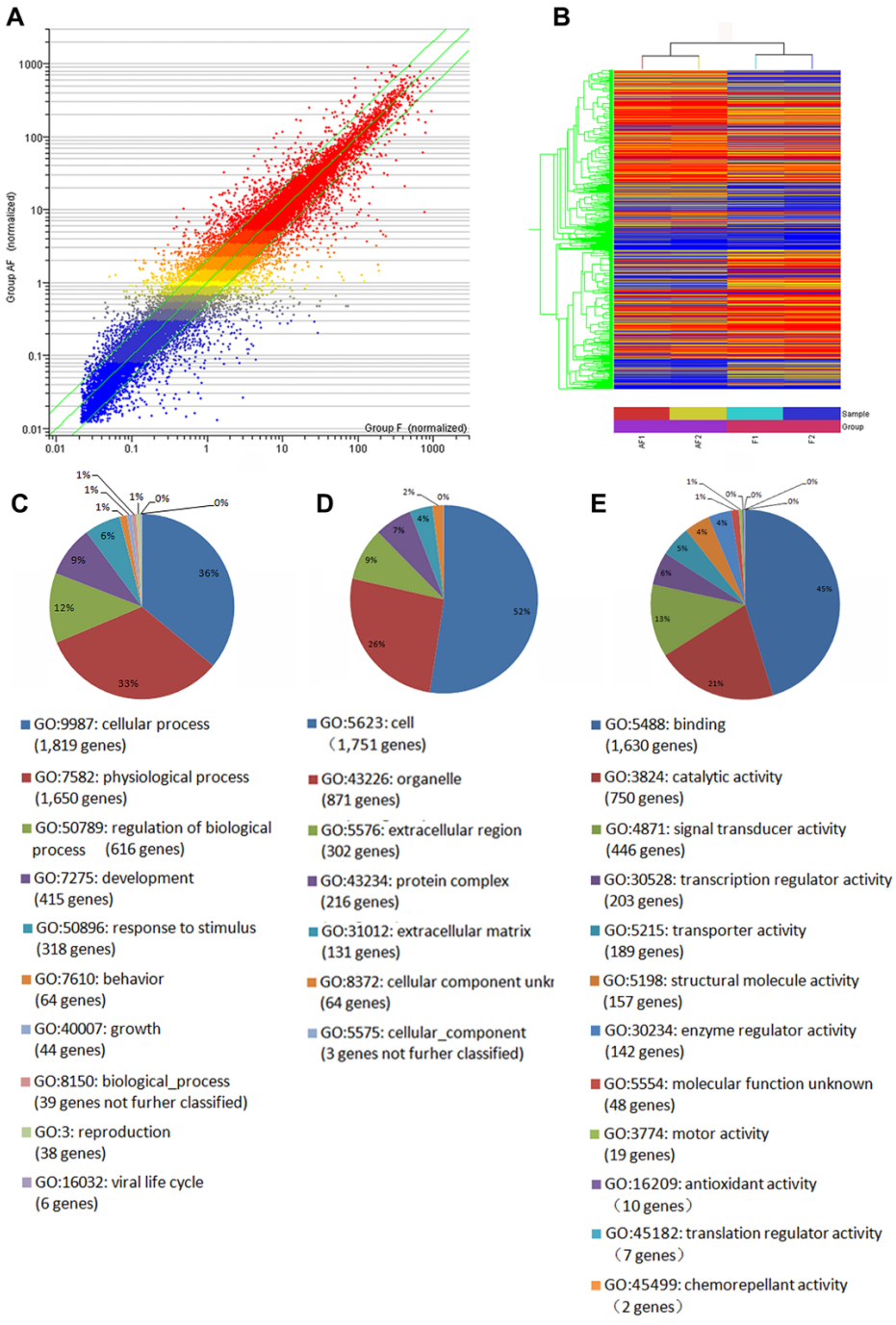

Fig. 5. Global gene expression microarray analysis. A – Scatter plots of the global gene expression profiles comparing the AF-type cells with the F-type cells via cDNA microarrays. The green lines indicate two-fold changes in the gene expression levels between the two groups. B – Hierarchical clustering of the global gene expression levels demonstrate a similar gene expression profile within the AF-type group (comparing the panels 1 and 2 on the left) and the F-type group (comparing panels 1 and 2 on the right), in addition to a distinguished gene expression profile pattern between the AF-type group and the F-type group (comparing panels 1 and 2 on the left with panels 1 and 2 on the right. C – Biological processes of gene ontology (GO) enrichment analysis between the AF-type group and the F-type group. The different colors indicate different gene expression clustering belonging to a different GO number of biological processes. D – The cellular components of the GO enrichment analysis between the AF-type group and the F-type group. The different colors indicate different gene expression clustering belonging to a different GO number of the cellular components. E – The molecular function of the GO enrichment analysis between the AF-type group and the F-type group. The different colors indicate different gene expression clustering belonging to a different GO number of molecular function.

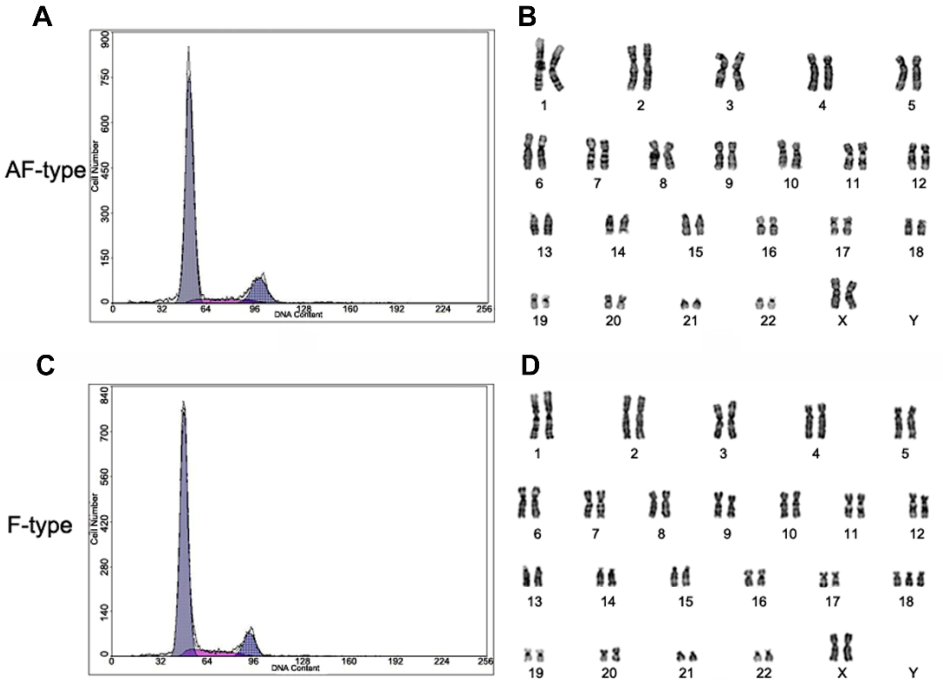

Fig. 6. The results of the cell cycle and karyotype analysis of hAFC. A – Cell cycle analysis of AF-type cells. B – Karyotype analysis of AF-type cells. C – Cell cycle analysis of F-type cells. D – Karyotype analysis of F-type cells.

Both cell subtypes displayed a normal cell cycle and karyotype and were non-tumorigenic *in vivo*

Cell cycle analysis indicated that both the AF- and F-type cells displayed a typically normal cell cycle profile, and there was no difference in the cell cycle profiles between them (Fig. 6A and C). Karyotyping of the two cell populations at passage 10 showed they were normal diploid as judged by Giemsa banding (Fig. 6B and D). Three AF-type cell populations and four F-type cell populations were injected into NOD-SCID mice. No teratomas were observed after 14 to 16 weeks.

DISCUSSION

This study demonstrated that three distinct cell subtypes from heterogeneous human amniotic fluid could be simultaneously isolated from a primary culture dish. The two culturable cell subtypes were found to be distinct from each other on the basis of their morphological appearance and marker expression, which further provided new insight into the heterogeneity of hAFC and helped to elucidate the inherent reasons for the distinct characteristics of previously reported hAFC from different research groups.

Three morphologically distinct cell subtypes could be simultaneously isolated from human amniotic fluid at the primary culture stage, which is a finding that is significantly different from that reported previously [3-7]. Previously published heterogeneous cell population data was obtained via the enzymatic digestion of a whole primary culture dish containing various colonies, whereas our new purification procedure aimed to isolate and characterize the distinct cell types at the primary hAFC stage in a simple yet efficient way. We initially used the commercially available plastic cloning cylinders for colony picking, but found that they were prone to moving during the isolation procedure due to their low density. We then made the colony poculum using high density stainless steel, and it proved more stable. In addition, the colony poculum was smeared with vaseline to aid in immobilizing it during the isolation procedure. These innovations helped to simultaneously isolate all three cell subtypes from the primary culture of human amniotic fluid, and two of these cell subtypes could be efficiently cultured. After consecutive observations of colony growth from a small number of cells to a large group of cells with uniform morphology, the cells of each colony were passaged separately without contamination of cells from another colony thanks to the segregation capabilities of the vaseline-smeared colony poculum, resulting in the uniform isolation of each cell subtype from the first generation. This novel separation procedure will greatly facilitate further investigation of the different cell subtypes inherent to amniotic fluid, and will guarantee the reliability of experimental results that originate from a uniform cell subtype. In our protocol, the E-type cells could not be propagated. Human amniotic fluids are truly heterogeneous and might contain more than the 3 types of cell morphologically observed in our system. We speculate that our

E-type cells were different from those described by other authors. In addition to this morphological difference, the two expanded cell subtypes expressed different markers, including CD117, CD105 and EBAF, which further confirmed the necessity of separating different cell sub-types of hAFC at the primary stage.

As described above, the expression levels of CD117 and CD105 in hAFC have differed in various reports [4, 6, 12]. We hypothesized that these variations were due to the enzymatic digestion of the whole primary culture dish, which contains various colonies, in addition to the specific culture conditions used by different research groups. This resulted in the differential characterization of hAFC. The solution to this problem was to isolate different cell populations at the primary hAFC stage, thus providing a uniform cell subtype for further sorting, characterization and in-depth analysis. Our proposed method provided an ideal solution to this issue. Through our approach, two subtypes of hAFC were efficiently cultured showing different expression levels of CD117 and CD105, which in turn led to an elucidation of the previously reported variability. The inherent mechanism of this intriguing issue will be investigated in our future research.

Using RT-PCR, the two culturable cell populations were found to express ES cell-specific markers such as Oct4, Nanog, Sox2, Rex-1 and Thy-1, while neither of the strains expressed FGF4 or TDGF1. Some researchers have also found that hAFC cultured under specific experimental conditions express Oct4 and Rex1 [7, 13]. We found that only the F-type cells expressed EBAF, a finding that has not been reported previously. EBAF (also named LEFTY) encodes a member of the TGF-beta family of proteins, and this protein plays an important role in the determination of the left-right asymmetry of organ systems during development [14]. This gene is also expressed in human ES cells [15]. In addition, during the spontaneous *in vitro* differentiation of ES cells, the expression of EBAF appears to serve an important role in the establishment of cell populations of mesodermal origin [16]. The inherent mechanism yielding the different expression levels of EBAF between AF-type and F-type hAFC and its possible function in cell fate determination during the *in vitro* differentiation process of hAFC require additional investigation.

High-throughput, genome-wide analyses of the transcriptome have been widely used in stem cell biology. In addition to FACS and RT-PCR analysis, our global gene expression array also demonstrated different gene expression profiles between the AF-type group and the F-type group, which further confirmed the necessity for distinguishing between different cell subtypes. This will be considered in future differentiation experiments.

The isolation, characterization and controlled differentiation of human stem cells from various sources will allow for the establishment of new therapeutic strategies for a wide variety of diseases. While there are ethical concerns regarding ES cells [17], and technical obstacles to the generation of inducible pluripotent stem cells [18-20], clinically discarded amniotic fluid has arisen as

a promising source of stem cells due to the cells' high proliferation potential and multi-lineage differentiation abilities [3-7]. In addition, these cells are non-tumorigenic *in vivo* and contain no chromosomal abnormalities as judged by Giemsa banding. Furthermore, cell cycle analysis indicates that both cell subtypes display a typically normal cell cycle profile. All of these results suggest their advantages for use in therapeutic applications.

In summary, this study not only provides new insight into the heterogeneity of human amniotic fluid, but also presents a simple yet efficient cell isolation method. All of these results provide an in-depth investigation of heterogeneous of hAFC, which may have various therapeutic applications in the near future.

Acknowledgements. We would like to thank Dr. Jianjun Shi for his helpful comments, Hua Shao, Jun Pan and Baisong Chen for their technical support, and Xiangliang Yuan for his support regarding the flow cytometry experiments.

This research was supported by grants from the National Natural Science Foundation of China (No. 30672108), the Science and Technology Commission of Shanghai Municipality (07XD14024, 08JC1416000) and the Shanghai Municipal Health Bureau (2008054).

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