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Original Article

Human amniotic fluid stem cells have better potential in early second trimester of pregnancy and can be reprogramed to iPS

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ABSTRACT

Objective: To study the difference of amniotic fluid stem cell potential at different gestational age.
Materials and methods: Second trimester amniocentesis was performed during 15 to 22nd week of gestational age in a single medical center from 2015 to 2016. Early second trimester amniotic fluid stem cells (E-AFS) and later one (L-AFS) were defined 15–18th week, and 19–22nd week, respectively. Cell characteristics, surface markers and ability to form induced pluripotent stem cells (iPS) were studied.
Results: All the amniotic fluid stem cells samples could be isolated and cultured from second trimester amniocentesis. E-AFS showed more Ckit + cell, shorted doubling time, smaller cell size and higher cell density compared to L-AFS. Both groups had the same stem cell surface markers with highly expression of CD44, CD73, CD90, and CD105, negative for CD45. They can easily be reprogramed into amniotic fluid stem cell derived iPS via standard induction.
Conclusion: Human amniotic fluid stem cells could be isolated from early or late second trimester amniocentesis with the similar stem cell surface markers presentation, especially in mesenchymal stem cells markers. However, the cells from early second trimester amniocentesis have more Ckit + number and more potential characteristics compared to late second trimester amniocentesis. Both E-AFS and L-AFS could form the iPS easily which lead to the future disease modeling study.

Introduction

Human amniotic fluid and the cells within the fluid has been used for prenatal diagnosis such as fetal karyotyping or DNA analysis for decades. The main aim of second trimester amniocentesis in fetal medicine is to diagnose chromosomal abnormalities or genetic defects prenatally and the reasons why couples decide to undergo this invasive procedure include elevated risk for Down's syndrome after screening tests, certain markers or structural fetal abnormalities noted on ultrasound which make chromosomal aneuploidy or genetic disease suspicious, and previous history of an affected child [1]. Due to the widespread availability of prenatal screening and diagnosis, amniocentesis is performed routinely in clinic and the amniotic fluid obtained not only facilitates cytogenetic and molecular diagnosis but also provides a promising source of mesenchymal stem cells (MSC) or other pluripotent stem cells [2] without the ethical controversy of embryonic stem cells [3].

It was confirmed in 2003 that MSC exist in human amniotic fluid (AF) [4]. MSC can be retrieved from human second-trimester AF with good efficiency (around 0.9–1.5%) that is superior to human bone marrow and cord blood. Several pluripotent markers (Oct-4, NANOG and SSEA-4) are always positive on human AF MSC [5]. Distinguishing them from other amniotic fluid cell types is simple because they adhere to plates during cultivation. These MSC are pluripotent in differentiation. For example, they may differentiate *ex vivo* into cardiomyocyte-like cells and engraft in rat heart with cardiomyocyte-like characteristics [6]. Although the majority of stem cells from AF are more phenotypically similar to MSC than to hematopoietic stem cells [5], currently cells with hematopoietic potential have been identified in a subpopulation (Lin-/Ckit + stem cells) of human and murine Amniotic fluid stem cells (AFS) [7].

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Induced pluripotent stem cells (iPS) could be generated with Yamanaks's factors firstly reported decade ago [8]. Amniotic fluid stem cells could be reprogrammed into iPS with different formula as some researchers reported [9,10]. Also, the potential of iPS derived from AFS is better and more effectively than adult stem cell origin like bone marrow [11,12]. In this study, we will firstly divide second trimester amniocentesis (15–22nd weeks) into early and late groups to see the difference/comparison of iPS ability, stem cell potential and characteristics of AFS.

Method

Collection of human amniotic fluid by amniocentesis

Human amniotic fluid samples were collected from the patients having amniocentesis in Fetal Medicine Center of Chang Gung Memorial Hospital (CGMH) in Linkou, Taiwan from 2015 to 2016. All enrolled cases were consented by Dr. Sheng-Wen Steven Shaw under ethical approval by CGMG IRB. Human amniocentesis was performed with sterile condition and under ultrasound guidance. The 22G echotip needle (Cock, USA) was inserted through maternal abdominal wall into the amniotic cavity. A total of 25 ml of amniotic fluid was withdrawn (20 ml for karyotyping and extra 5 ml for this study). The collected amniotic fluid was placed at room temperature. Multiple pregnancy, chromosomal anomalies, abnormal ultrasound findings were excluded in this study. We divided the study groups into early second trimester amniotic fluid stem cells (E-AFS, 15–18th weeks) and late second trimester amniotic fluid stem cells (L-AFS, 19–22nd weeks), and analyzed the differences between groups.

Isolation and characterization of human AFS cells

The Human amniotic fluid stem cells were obtained from the freshly collected amniotic fluid by routine amniocentesis from healthy pregnant donors with 15–22 gestational weeks as previously reports [13,14]. The cells in the suspension were implanted in TPP Tissue Culture Petri Dishes (TPP, Switzerland). The amniotic culture medium is composed of Chang medium B and Chang medium C (Irvine Scientific, USA), MEM Alpha solution (63%, Life Technology, UK), fetal bovine serum (15%, Invitrogen, UK), antibiotics (penicillin and streptomycin) and L-glutamine (1%, Invitrogen, UK). The dishes were incubated in an incubator that was constantly under 37 °C, 95% air and 5% carbon dioxide. Once 60–80% confluence was achieved the adherent cells were detached from the plate by 0.05% trypsin and 0.02% sodium-EDTA (Life Technologies, UK) and were passaged into a bigger dish or dispensed evenly into 2 dishes.

The specific surface antigens of hAFSCs were characterized by flow cytometry analyses. The cells were stained with phycoerythrin (PE)-conjugated antibodies against Ckit (CD117), CD44, CD73, CD90, CD105, and CD45 (BD PharMingen, CA) and protected from light for 30 min at 4 °C. Following incubation, the cells were washed twice with PBS. Thereafter, the cells were analyzed using the Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany).

Retroviral reprogramming and culture of human iPSCs

Human AFS cells were reprogrammed by a retroviral system encoding four factor combinations, including OCT4 (POU class 5 homeobox 1), SOX2 (SRY-box 2), KLF4 (Kruppel-like factor 4) and MYC (myelocytomatosis oncogene) as previously reported [15]. Mouse embryonic fibroblasts (MEFs) feeder cells from 13.5 to 14.5 dpc CD1 mice embryos were prepared. The reprogrammed human iPSCs were maintained on mitomycin C-treated MEF feeder cells in Knockout Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 20% Knockout Serum Replacement (KSR, Invitrogen), 8 ng/ml bFGF (Propetech), 50 U/ml penicillin, 50 mg/ml streptomycin, 1 mM sodium pyruvate, 1X MEM non-essential amino acid, 2 mM L-glutamine and 0.1 mM 2-mercaptoethanol, and passaged with 0.1% EDTA (Invitrogen) in PBS at a ratio of 1:3 every week.

Alkaline phosphatase and trypan blue exclusion assays

The colonies showing alkaline phosphatase activity were tested using an alkaline phosphatase detection kit (Millipore) according to the manufacturer's instructions. For cell viability assay, the cultured cell suspensions were treated with H_2O_2 (1 mM, Sigma), MG132 (2.5 mM, Sigma), benzamil (50 mM, Sigma) or genipin (500 nM, Wako). After 24 h, 20 ml 0.4% trypan blue (Gibco) were mixed with 20 ml cell suspension sampled from each well for 1 h. Cell viability was determined by light microscopy. Cells that excluded trypan blue were considered viable.

Immunohistochemistry study of the iPS cells

The primary antibodies used for immunocytochemistry in this study were: rabbit anti-OCT4 (1:500, Abcam), mouse anti-TRA-1-60 (Podocalyxin; 1:250, Santa Cruz), and mouse anti-TRA-1-81 (Podocalyxin; 1:250, BD Pharmingen). The secondary antibodies were Alexa594-conjugated donkey anti-rabbit IgG (1:200, Invitrogen), and Alexa488-conjugated donkey anti-mouse IgG (1:200, Invitrogen).

Data analysis

To determine the number of antibody-labeled cells, positively stained cells were counted manually. Each set of values was expressed as means ± standard deviation. Differences between groups were determined using a two sample Student's t-test or one-way ANOVA with Bonferroni post-hoc test. All *p*-values were two-tailed, and a p value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using Statistical Program for SPSS version 16.

Results

All the amniotic fluid stem cells could be isolated and cultured from amniocentesis

There were 22 cases from early second trimester (15–18th week of gestational age) and 5 cases from late second trimester (19–22nd weeks of gestational age). The indications of amniocentesis were mostly advance maternal age, following by high risk of Down syndrome, abnormal ultrasound finding, previous history of chromosomal abnormalities, and elective request. There was no statistically significant difference in maternal age between women having early second or later second trimester amniocentesis (33.45 ± 1.21, and 34.13 ± 1.90 year-old, respectively). The mean gestational age of amniocentesis for E-AFS was (16.15 ± 2.53), which was significantly earlier than the mean gestational age of L-AFS (19.09 ± 2.46).

Early second trimester amniotic fluid stem cells (E-AFS) has greater potential compared to L-AFS.

Fig. 1A and Table 1 showed that, the percentage of Ckit + cells were higher in cultured E-AFS (5.2%), compared to cultured L-AF (1.2%) ($p < 0.05$). The cell size was measured as described in the methods section. Isolated E-AFS had the significantly smaller cell diameter, higher cell density, and shorter cell doubling time compared to isolated L-AFS ($p < 0.05$ in all three parameters) (Fig. 1B, C, D).

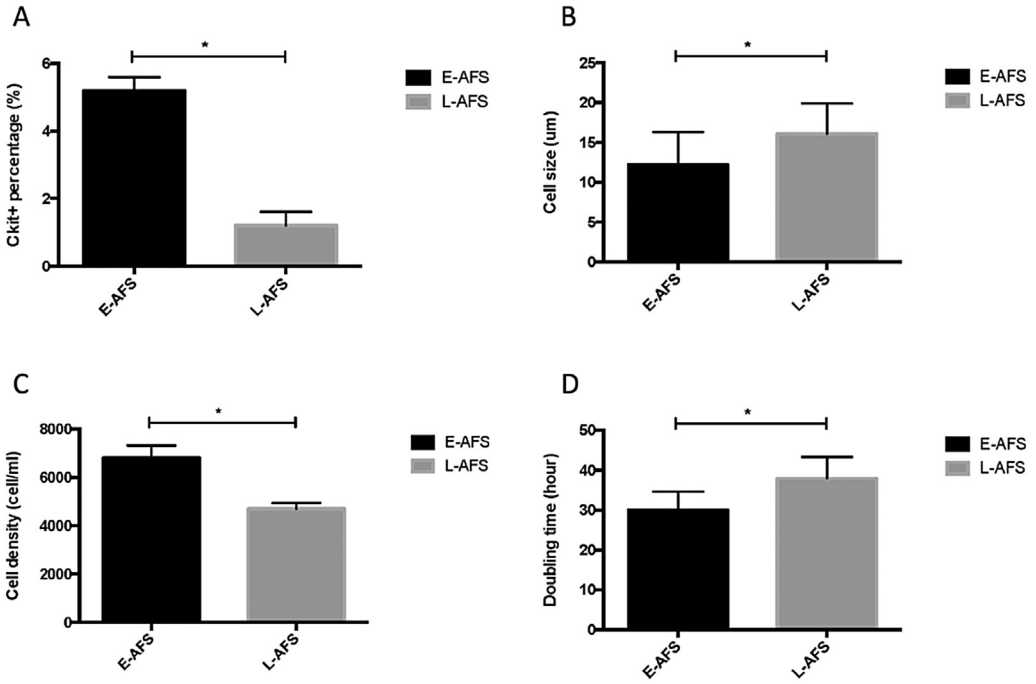

Fig. 1. The difference between E-AFS and L-AFS in cell potential. (A) The percentage of Ckit + cells in E-AFS is significantly more than L-AFS. (B) The cell size of E-AFS is smaller. (C) The cell density of E-AFS is higher than L-AFS. (D) The doubling time of cell culture in E-AFS is shorter. *: $p < 0.05$, error bar: standard deviation.

Table 1
Characteristics of human amniotic fluid stem cells derived from early second (E-AFS) or late trimester amniotic fluid stem cells (L-AFS) (n = 5, each group).

	E-AFS (n = 5)	L-AFS (n = 5)
Cell size (um)	12.2 ± 4.1	16.1 ± 3.8
Cell density (cell/ml)	6800 ± 520	4700 ± 250
Doubling time (hours)	30 ± 4.7	38 ± 5.3
Ckit + population (%)	5.2 ± 0.4	1.2 ± 0.4

Both E-AFS and L-AFS showed the stable mesenchymal stem cell surface markers

We successfully obtained both E-AFS and L-AFS with high expressions of CD44 (cell migration marker) and mesenchymal stem cell markers including CD73, CD90, and CD105. These cells did not express CD45 (hematopoietic stem cell marker) (Fig. 2). The expression level of these surface markers in both groups did not show the statistic difference.

Human amniotic fluid stem cells from early or later second trimester can be both induced into iPS.

The standard four factors were introduced to both E-AFS and L-AFS. These cells could be reprogramed into iPS and show the pluripotent markers including Alkaline phosphatase, OCT4, TRA-1-60, and TRA-1-81 as described in Fig. 3. The successful rate to reprogram iPS is 100%. And all the E-AFS, L-AFS or iPS could be differentiated into three germ layers (data not shown).

Discussion

In this study, we demonstrated the difference between early second trimester amniotic fluid stem cells (15–18th week) and late second trimester amniotic fluid stem cells (19–22nd week). E-AFS showed the better stem cell potential compared to L-AFS in more percentage of C-Kit+ (CD117) positive cells, smaller cell size, better cell density, and shorter doubling time. The more powerful stem cell potential usually has smaller cell size, and shorter culture doubling time [16]. More cells could also be found in E-AFS as we could assume the late second trimester having more amniotic fluid which lead to diluted cell number. Second trimester amniocentesis is a very standard procedure in Taiwan between 15 and 22nd weeks with reported low miscarriage rate [1,17], but this is the first studying to divide into early and late second trimester amniocentesis. According to these results, we believe the earlier gestation of AFS has better stem cell potential.

Secondly, the surface markers of stem cells were strongly positive in CD44, CD73, CD90 and CD105 which are all mesenchymal stem cell markers, but negative in CD45 (hematopoietic stem cell marker) that is identical to our previously report [18]. These results are similar in each cell samples from E-AFS or L-AFS. We previously reported AFS is a good fetal MSC source as well as placenta and umbilical cord for either fetal stem cell therapy or regenerative medicine [19–21]. Therefore, no matter which gestational age we obtained the amniotic fluid, these cells could be cultured to mesenchymal stem cell and have the same characteristics in surface marker presentations.

In addition, this is not the first report that AFS could be reprogramed into iPS in the literature [11,12]. Our previously report also showed the AFS could be co-cultured with embryonic stem cells, also forming teratoma with pluripotent markers [14]. We here further proved the four factors could easily reprogram either E-AFS or L-AFS to iPS successfully. Amniotic fluid stem cell derived iPS has better clinical potential compared to bone marrow stem cell derived iPS. The unique time window to obtain second trimester amniotic fluid via amniocentesis is 15 to 22nd weeks, which means we have opportunity to transplantation these cell autologously back to the same fetus for fetal stem cell therapy [22]. The iPS is also the great source to study the disease modeling in vitro.

The case number on both study group is relative small that is the major limitation of this study. The E-AFS or L-AFS are all via

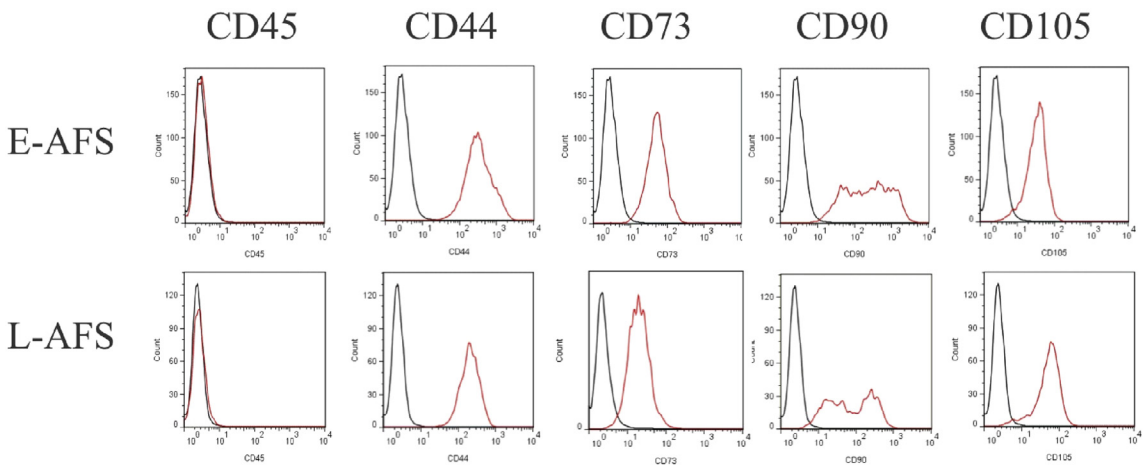

Fig. 2. The surface marker expression in both E-AFS (upper panel) and L-AFS (lower panel). Both groups showed high expressions of CD44 (cell migration marker) and mesenchymal stem cell markers including CD73, CD90, and CD105. These cells did not express CD45 (hematopoietic stem cell marker).

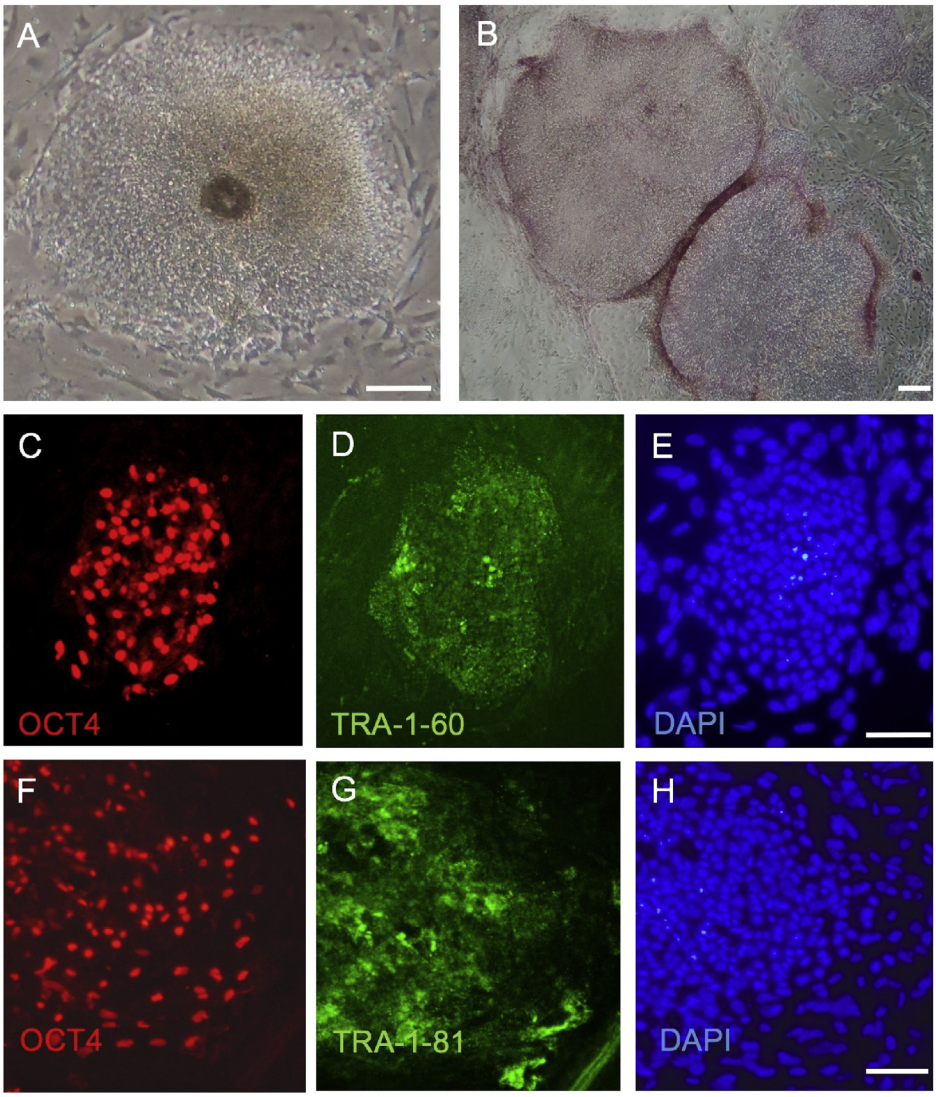

Fig. 3. Characterization of AFS-derived-iPS. (A) Morphology of AFS-derived-iPS. (B) Alkaline phosphatase assay in AFS-derived-iPS. (C–E) Immunobiochemical staining for OCT4 (red), TRA-1-60 (green) and DAPI (blue) in AFS-derived iPS. (F–H) Immunobiochemical staining for OCT4 (red), TRA-1-81 (green) and DAPI (blue) in AFS-derived iPS. Scale bar, 20 μm.

standard culturing system therefore the uncultured amniotic fluid stem cells might be worth to add in the future experiment to see the difference between cultured and uncultured cells.

In conclusion, human amniotic fluid stem cells could be isolated from early or late second trimester amniocentesis with the similar stem cell surface markers presentation, especially in MSC markers. However, the cells from early second trimester amniocentesis have more Ckit + number and more potential characteristics compared to late second trimester amniocentesis. Both E-AFS and L-AFS could form the iPS easily which lead to the future disease modeling study.

Conflicts of interest

There is no conflict of interest.

Acknowledgments

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