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Original Article

Isolation and culture exploration of *Anas platyrhynchos* amniotic fluid stem cells *in vitro*

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ABSTRACT

Objective: This research was designed to establish the system of isolation and culture of *Anas platyrhynchos* (duck) amniotic fluid stem cells (DAFSCs), and to explore its biological characteristics and differentiation ability *in vitro*.
Material and methods: Main experimental reagents contained L-DMEM, fetal bovine serum, chicken serum, EGF, bFGF, L-glutamine, trypsin, rabbit anti-chicken CD44, CD73, CD105, nanog and SSEA-4 (Abcam, USA), FITC conjugated goat anti-rabbit secondary antibody IgG, DAPI, Trizol, inverse transcription kit, Propidium iodide, IBMX, INS, dexamethasone and indometacin. Cultivation system included L-DMEM with 10% FBS, 5% chicken serum, EGF 10 ng/mL, bFGF 10 ng/mL and 1% L-glutamine, and was cultured under 37°C, 5% CO_2 and saturated humidity. Immunofluorescent detection is used to detect cell surface markers, while RT-PCR was used to detect related gene expression. Cell cycle was detected with Flow Cytometer and was analyzed by ModFitLT 2.0, induced differentiation, and Oil Red O staining.
Results: More DAFSCs were gained via super-centrifugation and thermoelectric methods cost effectively. DAFSCs could go down to the future generation at passage 23(P23). CD44, CD73, CD105 and SSEA-4 were detected as positive with immunofluorescence histochemistry. *GAPDH*, *GDNF*, *rex*1 and *JAG*1 were detected as positive with RT-PCR. Cell cycle was detected on flow cytometer. Tentative exploration of differentiation ability that DAFSCs could be induced into adipocyte *in vitro*.
Conclusion: DAFSCs can be isolated from matrix that have strong self-renewal capacity *in vitro*. DAFSCs can be induced into adipocyte *in vitro*. These testify that DAFSCs can be an ideal seeded cells having potentials for preservation and utilization of rare genetic resources.

KEYWORDS

Anas platyrhynchos; DAFSC; Rare genetic resources; Stem cell

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INTRODUCTION

With the vigorous development of stem cell research, it has been ranked among the forefront in biomedical sciences. The stem cell technology might be an important method in current perspective of wild rare resources conservation and production (Telugu et al., 2012). Amniotic fluid stem cells (AFS) is a new type of stem cells reported in recent years, which is considered as one of the top 10 medical breakthroughs during 2007. AFS not only express Oct-4 and SSEA-4 but also display mesenchymal stem cell markers such as vimentin (De Coppi et al., 2007; Kim et al., 2007; Chiavegato et al., 2007; You et al., 2007).

AFS possess multi-directional differentiation potential. It can be differentiate into multiple cell lineages including adipogenic, osteogenic, myogenic, endothelial, neurogenic, hepatic, and embryonic germ (EG) layers *in vitro* (Tsai et al., 2006; Parolini et al., 2009). No feeder cells are needed in the culture. Moreover, as compared with establishing ES cells (He et al., 2006; Talbot and Blomberg, 2008) and induced pluripotent stem (iPS) cells, AFS can be gained easily and cost effectively. Strong ability of self-renewal and multipotential differentiation *in vitro* indicates the possibilities of AFS in rare biological conservation and utilization of resources and regenerative medicine. A recent research indicated that AFS was applied to develop cloned porcine embryos (Zhao and Zheng, 2010; Zheng et al., 2008), which raised the possibilities about the AFS application in assisted reproduction in other species. The information is little whether AFS could be isolated and cultured, and could serve as significant resources in *Anas platyrhynchos* now, a kind of rare bird in the world. The objective of this study was to establish an *in vitro* system to isolate and culture of *A. platyrhynchos* (duck) amniotic fluid stem cells (DAFSCs), and to assess its biological activities and differentiation abilities.

MATERIALS AND METHODS

Experimental animal: Total 8-12 day old fertilized eggs of *A. platyrhynchos* were selected at the Poultry Experimental Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing. All the *A. platyrhynchos* that provided eggs were treated in accordance with NIH and USDA guidelines for the use of animals in research and all experimental procedures involving *A. platyrhynchos* were conducted in accordance with the protocols and guidelines for agricultural animal research codified by the Committee for Ethics of Beijing, China.

Experimental reagents: The reagents used in this srudy were included L-DMEM (Gibco, USA), fetal bovine serum (Biochrom, Germany), chicken serum (Sigma, USA), EGF, bFGF, L-glutamine (peprotech, USA), trypsin (Gibco, USA), rabbit anti-chicken CD44, CD73, CD105, nanog and SSEA-4 (Abcam, USA), FITC conjugated goat anti-rabbit secondary antibody IgG (Abcam, USA), DAPI (Gibco, USA), Trizol (Invitrogen, USA), inverse transcription kit (Takara, Japan), Propidium iodide (Sigma, USA), IBMX, INS, dexamethasone and indomethacin (Beijng Zhong Shan Jin Qiao).

Isolation and culture system of DAFSCs *in vitro*: The collected fertilized eggs were disposed with thermoelectric method for 30 min. Egg shell was opened with curved scissors and amniotic fluid was collected. The amniotic fluid was centrifuzed at 3000 rpm for 30 min. Cells were inoculated in terms of 1.0×10^5/mL per 12 well plates. Culture system contained L-DMEM with 10% FBS, 5% chicken serum, EGF 10 ng/mL, bFGF 10 ng/mL and 1% L-glutamine,cultured under 37°C, 5% CO_2, saturated humidity. Medium was changed after 1 day of inoculation, then changing medium on every 2 days. Cells could be passaged when reached to 70-80% healing.

Characterization of DAFSCs

RT-PCR detection: We followed the trizol extracting method to isolate total RNA from the cells. Total RNA was used for the synthesis of cDNA using inverse transcription kit (Takara, Japan). Reaction condition contained: room temperature for 10 min, 42°C for 1 h, inactivation at 99°C for 5 min, ice-bath for 2 min. Information of gene specific primer sequences included DAFSCs; the induced cells were displayed in **Table 1**. PCR reaction system consisted of 20 μL reaction that contained 2xMixture 10 μL, cDNA 5 μL, mass free H_2O 7 μL, forward primer 1 μL, reverse primer 1 μL. Reaction conditions were an initial denaturation at 94°C for 5 min followed by 30 cycles of 30 Sec at 94°C, 30 Sec at 50-60°C as annealing, and 2 min at 72°C for extension as described by Boyer et al. (2005) and Hummon et al. (2007).

Immunofluorescence histochemistry: We selected DAFSCs of passages 6 reaching 60-70% healing for detection. The cells were washed with PBS and fixed in 4% (m/v) paraformaldehyde for 15 min followed by washing 2 times with PBS. Cells are permeabilized with 0.2% (v/v) Triton X-100 for 20 min and washed for 3 times with PBS. The cells were then blocked in 10% (v/v) goat serum for 1 h at room temperature, and were incubated with antibodies rabbit anti chicken CD44,

CD73, CD105, nanog and SSEA-4 for 8 h at 4°C. After that, the cells were washed 2 times with PBS, and incubated in FITC conjugated goat anti-rabbit secondary antibody IgG for 1 h at room temperature. After incubation, the cells were washed 2 times with PBS. Conducting nuclear staining with DAPI dye for 15-20 min, then washing the cells 2 times with PBS. The cells were observed using confocal microscope (Nikon TE-2000-E Nikon, Japan), and images were acquired.

Examination of cell cycle: We collected DAFSCs of P5 and P15 achieving 90% concrescence and in good growth situation with conventional method and added cells to flow cytometry tube respectively according to cell population of 2.0×10^5 at least, centrifuged at 1200 rpm for 8 min. Supernatant was abandoned and precipitation was washed with PBS for 3 times. The cells were resuspended with 1 mL 70% ethanol and incubated for 8 h at 4°C. Then, centrifugation was done at 1200 rpm for 8 min, and the cell were collected. Supernatant was abandoned and precipitation was washed with PBS for 2 times. Then, 0.5 mL PI dye liquor was added and the cells were incubated for 30 min. After filtrated with 80 μM cell strainer, the cells were placed on Flow cytometer for computer operation. PI(3,8-Diamino-5-[3-(diethylmethylammonio) propyl]-6-phenylphenanthridinium diiodide) is a reagent that stains DNA, and releases the red fluorescence after embedding double-stranded DNA.

Adipocyte induced differentiation: DAFSCs of P5 reaching 70-80% concrescence and in good growth situation were selected, and were divided into two groups; experimental groups and control group. We used adipocyte inductive medium containing 10% FBS, 0.5 mmol/L IBMX, 10 mg/L INS, 1.0 μmol/L dexamethasone and 200 μmol/L indometacin to culture experimental group, while control group was remained in normal medium. Medium are refreshed every 3 days. Oil Red O staining was applied after 20 days of induction. We examined adipocyte specific markers *LPL* and *PPARG* by RT-PCR. Control groups were used for comparing with experimental group. Oil Red O is a fat soluble dye; it can dissolve fat, and stain triglycerides easily.

RESULTS AND DISCUSSION

Morphology observation of DAFSCs culture: Primary cells migrated towards each other forming cell group gradually after vaccination. The cells proliferated rapidly and spreaded on petri dishes after 24 h of inoculation (**Figure 1A**). Passage after 1 day, the cell morphology started to be rotunded, and developed strong refraction, full cells, stronger stereo sense later and every generation for about 1-2 days (**Figure 1B**). The cells were cultured up to 23 passages, and most cells showed signs of slow cell proliferation and vacuolization (Mauro et al., 2010).

Characterization of DAFSCs: Gene and cell surface markers detection with RT-PCR and immunofluorescence histochemistry surface markers CD44, CD73, CD105, nanog and SSEA-4 were detected positive with immunofluorescence histochemistry (**Figure 2**), while gene *GDNF*, *rex*1 and *JAG*1 were detected positive with RT-PCR (**Figure 3**). Similar findings were reported by Hummon et al. (2007) and Thomas and Fenech (2009). These were all specific indications that AFS expressed indicating that the isolated cells were AFS.

Examination of cell cycle: We applied cell cycle examination to analyze cell cycle changes between different passages using Flow Cytometer. P5 and P15 of DAFSCs were chosen to detect their cell quantities and proportion in each cell cycle (**Figure 4**). Formula G2/M+S reflected

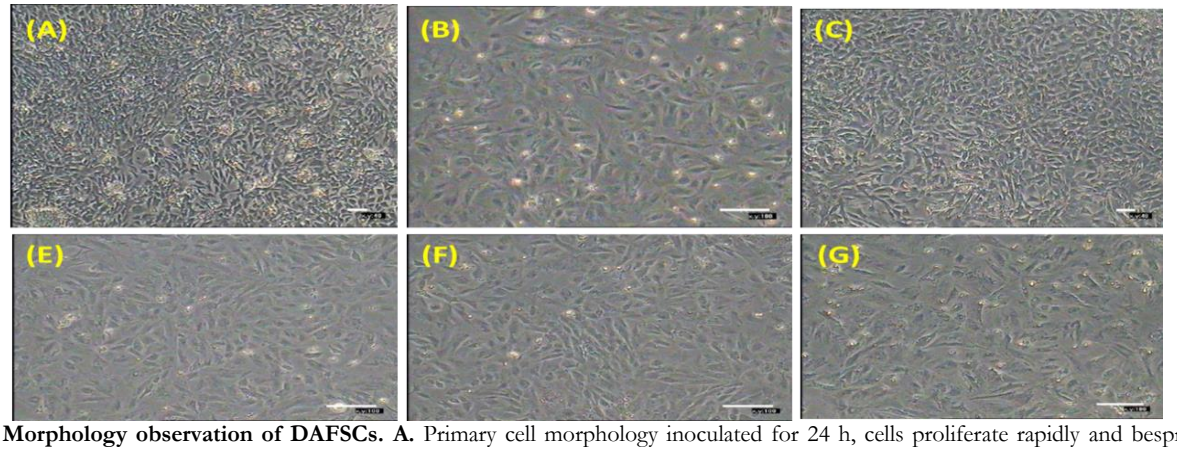

Figure 1. Morphology observation of DAFSCs. A. Primary cell morphology inoculated for 24 h, cells proliferate rapidly and bespread petri dish. White spots come from amniotic fluid. **B.** P1 of DAFSCs, cell morphology come to the development of rotundity, strong refraction, full cells, stronger stereo sense, most white spots disappear. **C.** P5 of DAFSCs. **D.** P10 of DAFSCs. **E.** P15 of DAFSCs. **F.** P20 of DAFSCs, most cells showing signs of senescence such as slow cell proliferation,vacuolization, size and flat and stereo feeling disappear.

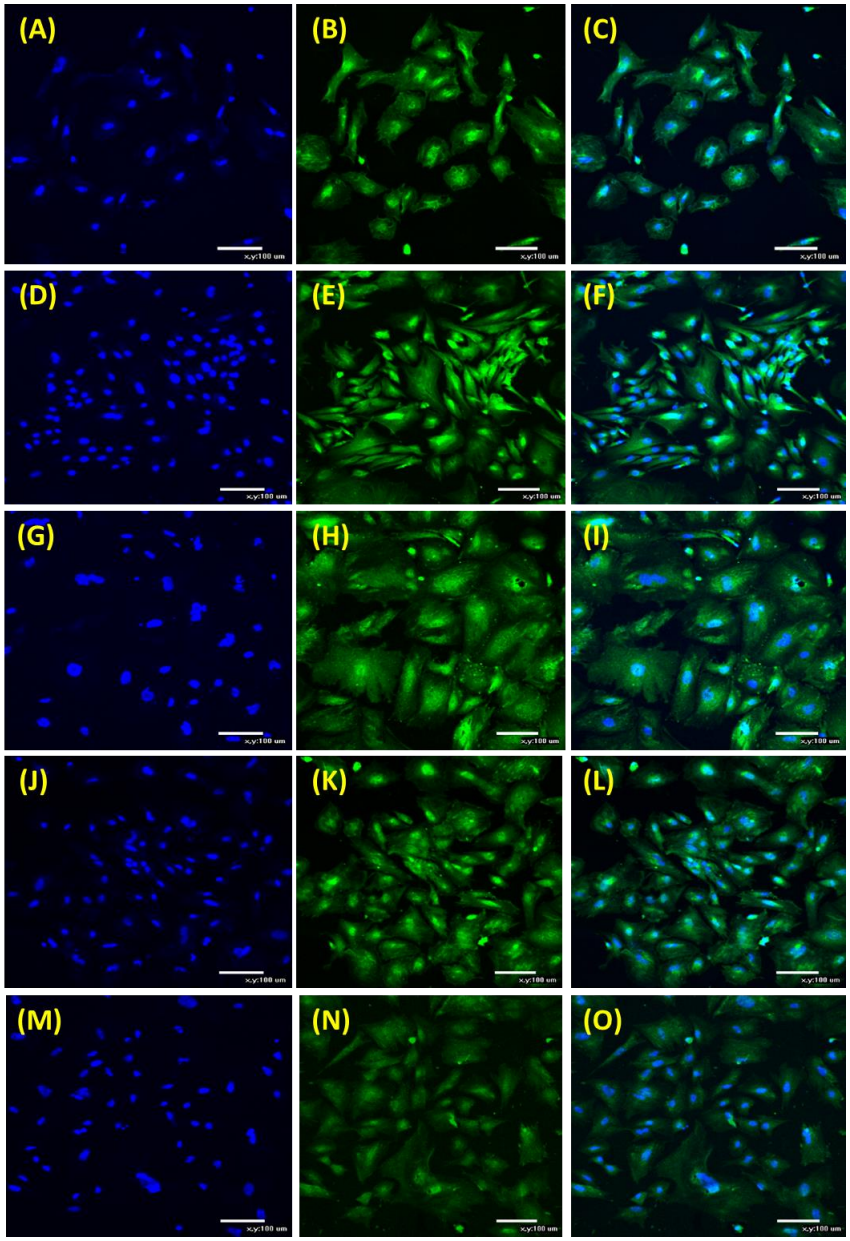

Figure 2. Immunofluorescence histochemical detection of DAFSCs. Surface markers CD44, CD73, CD105, nanog and SSEA-4 are detected positive with immunofluorescence histochemistry. **A, D, G, J** and **M**: nuclei is stained by DAPI. **B:** CD44+; **E:** CD73+; **H:** CD105+; **K:** nanog+; **N:** SSEA-4+. **C, F, I, L** and **O**: Merged.

cell proliferation vigor of different passages (**Table 2**). We found that proportion G2/M+S of P5 was the highest, P15 took the second place; this was a normal phenomenon that reflected biological regularity.

Induced differentiation of DAFSCs *in vitro*

Adipocyte induced differentiation: We adopted the subsequent morphological and characteristics analysis after inducing the DAFSCs differentiated into adipocytes. Cells grew slowly but less apoptosis existed in early days. Cell volume became bigger, lipid droplet were observed under inverted microscope after culturing for 8 days and become more and bigger with the culturing time increased (**Figure 5A-C**). Oil red O staining was carried out using culture for 20 days and lipid droplets showed orange red colors (**Figure 5D-F**). There were no morphological changes appeared in control groups. Adipocyte specific markers *LPL* and *PPARG* were detected positive by RT-PCR.

Thermoelectric method was used to isolate DAFSCs that helped the cells for suspending adequately in amniotic fluid. This is a new attempt to isolate DAFSCs. It is known that AFSCs are gianed little with technology current, although there are many isolation methods of

amniotic fluid nowadays (Priest et al., 1978; Gosden, 1983; Sessarego et al., 2008; Yeh et al., 2010; Ghaderi et al., 2011; Dev et al., 2012), however, these were not suitable for proliferating extensively *in vitro* and application in regenerative medicine. In 2014, a Thailand scientific research group declared that they found a new

Table 1. Primer sequences used for RT-PCR

Gene	Primer sequence (5′-3′)	Tm (°C)	Product length (bp)
GAPDH	F: GCAGATGCTGGTGCTGAATA R: CGGAGATGATGACACGCTTA	60	106
GDNF	F: ACTGTCAAGGCTGAGAACGG R: CCACTGCAATACCGGAAGAT	60	214
*rex*1	F: ATCCGCAAAGAGGAGTGTGT R: CCATCAGTCGTAGGCAACTTC	60	203
*JAG*1	F: CAGTTCCAGGAGAGTGCAGG R: GTAACCCTCAGGGCAGGAAC	60	248
LPL	F: AACTTGGGAGAAGCTCTGCG R: CAGGAATGGCTGGTTGGTCT	60	360
PPARG	F: AGCCTCCTTCTCCTCCCTATT R: GCTTCTCCTTCTCCGCTTG	60	336

Table 2. Percent counting for every stage of cell cycle

	G0/G1	G2/M	S	G2/M+S
P5	54.03%	28.79%	17.18%	45.97%
P15	62.00%	24.14%	13.86%	38.00%

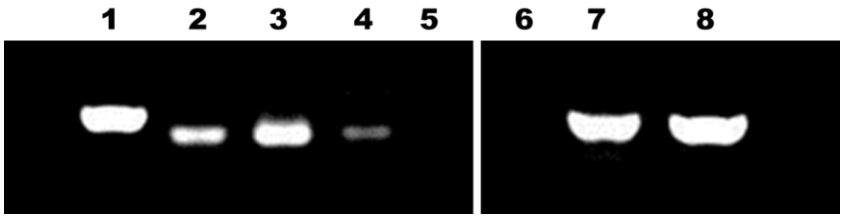

Figure 3. RT-PCR detection of DAFSCs and induced cells (adipocyte). Lane 1-4: Gene *GAPDH*, *GDNF*, *rex*1 and *JAG*1 are detected postive of DAFSCs. *GAPDH* is selected as internal control. **Lane 5** and **6**: *LPL* and *PPARG* are detected negative of control group. **Lane 7** and **8**: *LPL* and *PPARG* are detected postive of induced cells (adipocyte).

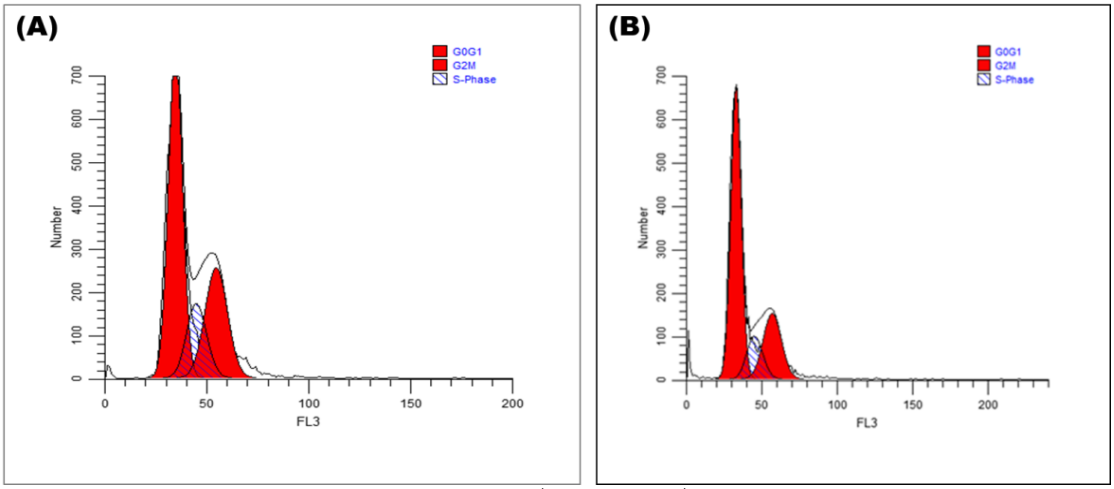

Figure 4. Examination of cell cycle. A. Cell quantities that lie in G0/G1, S and G2/M of P5 DAFSCs respectively. **B.** Cell quantities that lie in G0/G1, S and G2/M of P15 DAFSCs, respectively. S phase represent DNA replication stage, G2/M represent cell division stage, G2/M+Scan reflect cell proliferation vigour.

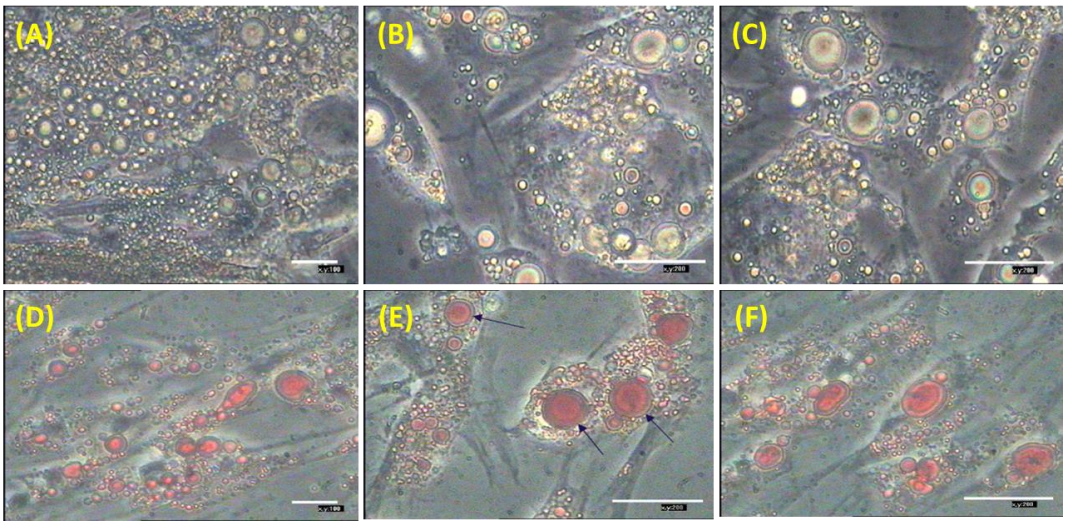

Figure 5. Adipocyte induced differentiation of DAFSCs *in vitro*. **A**, **B** and **C**: Morphology observation of induced cells under inverted microscope, lipid droplet become brighter after culture for 20 days. Lipid droplet distribute densely in **A**, which demonstrate a higher rate of induction. **C**, **D** and **E**: Lipid droplet present orange red after oil red O staining. Blue arrow stamp lipid droplet,which is typical morphological marker of adipocyte.

method to get high-concentration of AFSCs, but they had not made this method public yet. Although more number of cells were found in our thermoelectric method that we attempted in this research, still there existed limitations containing major kinds of cells, as reported by Atala (2006). Thus, improvements are needed in near future.

CONCLUSION

Super-centrifugation and thermoelectric methods can be used to isolate more DAFSCs simply and conveniently in cost effectively, which is applicable for plentiful augmentation *in vitro*. DAFSCs can be transfered to 23 passages nowadays keeping in good self-renewal capability and growth *in vitro*. DAFSCs express the genes *GDNF*, *rex*1 and *JAG*1 and specific surface markers CD44, CD73, CD105, nanog and SSEA-4. Cell cycle examination indicates that proliferation of low-passaged DAFSCs shows superior ability as compared to that of high-passaged.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

MN carried out the isolation and culture of *A. platyrhynchos* amniotic fluid stem cells and participated in the immunofluorescence histochemistry, RT-PCR, differentiation and drafted the manuscript. YW polishes English of the manuscript. MJ carried out cell cycle analysis. WG conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

REFERENCES

1. Atala A (2006). Recent developments in tissue engineering and regenerative medicine. Current Opinion in Pediatrics, 18: 167-171. https://doi.org/10.1097/01.mop.0000193294.94646.be
2. Boyer L, Lee T, Cole M, Johnstone S, Levine S, Zucker J, Guenther M, Kumar R, Murray H, Jenner R, Gifford D, Melton D, Jaenisch R, Young R (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell, 122: 947-956. https://doi.org/10.1016/j.cell.2005.08.020
3. Chiavegato A, Bollini S, Pozzobon M, Callegari A, Gasparotto L, Taiani J, Piccoli M, Lenzini E, Gerosa G, Vendramin I, Cozzi E, Angelini A, Iop L, Zanon G, Atala A, De Coppi, Sartore S (2007). Human amniotic fluid-derived stem cells are rejected after transplantation in the myocardium of normal,

ischemic, immuno-suppressed or immuno-deficient rat. Journal of Molecular and Cellular Cardiology, 42: 746-759. https://doi.org/10.1016/j.yjmcc.2006.12.008
4. De Coppi P, Bartsch G, Siddiqui MM, Xu T, Santos CC, Perin L, Mostoslavsky G, Serre AC, Snyder EY, J Yoo JJ, Furth ME, Soker S, Atala A (2007). Isolation of amniotic stem cell lines with potential for therapy. Nature Biotechnology, 25: 100-106. https://doi.org/10.1038/nbt1274
5. Dev K, Gautam SK, Giri SK, Kumar A, Yadav A, Verma V, Kumar P, Singh B (2012). Isolation, culturing and characterization of feeder-independent amniotic fluid stem cells in buffalo (*Bubalus bubalis*). Research in Veterinary Science, 93: 743-748. https://doi.org/10.1016/j.rvsc.2011.09.007
6. Ghaderi S, Soheili ZS, Ahmadieh H, Davari M, Jahromi FS, Samie S, Rezaie-Kanavi M, Pakravesh J, Deezagi A (2011). Human amniotic fluid promotes retinal pigmented epithelial cells' trans-differentiation into rod photoreceptors and retinal ganglion cells. Stem Cells and Development, 20(9): 1615-1625. https://doi.org/10.1089/scd.2010.0390
7. Gosden C (1983). Amniotic fluid cell tipes and culture. British Medical Bulletin, 39(4): 348-354. https://doi.org/10.1093/oxfordjournals.bmb.a071847
8. He S, Pant D, Schiffmacher A, Bischoff S, Melican D, Gavin W, Keefer C (2006). Developmental expression of pluripotency determining factors in caprine embryos: novel pattern of NANOG protein localization in the nucleolus. Molecular Reproduction and Development, 73: 1512-1522. https://doi.org/10.1002/mrd.20525
9. Hummon AB, Lim SR, Difilippantonio MJ, Ried T (2007). Isolation and solubilization of proteins after TRIzol extraction of RNA and DNA from patient material following prolonged storage. BioTechniques, 42: 467-470. https://doi.org/10.2144/000112401
10. Kim J, Lee Y, Kim H, Hwang K, Kwon H, Kim S, Cho D, Kang S, You J (2007). Human amniotic fluid-derived cells have characteristics of multipotent stem cells. Cell Proliferation, 40: 75-90. https://doi.org/10.1111/j.1365-2184.2007.00414.x
11. Mauro A, Turriani M, Ioannoni A, Russo V, Martelli A, Di Giacinto O, Nardinocchi D, Berardinelli P (2010). Isolation, characterization, and *in vitro* differentiation of ovine amniotic stem cells. Veterinary Research, 34(Suppl. 1): S25-S28. https://doi.org/10.1007/s11259-010-9393-2
12. Parolini O, Soncini M, Evangelista M, Schmidt D (2009). Amniotic membrane and amniotic fluid-derived cells: potential tools for regenerative medicine? Regenerative Medicine, 4: 275-291. https://doi.org/10.2217/17460751.4.2.275
13. Priest RE, Marimuthu KM, Priest JH (1978). Origin of cells in human amniotic fluid cultures: Ultrastructural features. Laboratory Investigations, 39(2): 106-109.
14. Sessarego N, Parodi A, Podestà M, Benvenuto F, Mogni M, Raviolo V, Lituania M, Kunkl A, Ferlazzo G, Bricarelli FD, Uccelli A, Frassoni F (2008). Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. Haematologica, 93(3): 339-346. https://doi.org/10.3324/haematol.11869
15. Talbot NC, Blomberg LA (2008). The pursuit of ES cell lines of domesticated ungulates. Stem Cell Reviews, 4: 235-254. https://doi.org/10.1007/s12015-008-9026-0
16. Telugu BP, Ezashi T, Roberts RM (2012). The promise of stem research in pigs and other ungulate species. Stem Cell Reviews, 6(1): 31-41. https://doi.org/10.1007/s12015-009-9101-1
17. Thomas P, Fenech M (2009). Buccal micronucleus cytome assay. Methods in Molecular Biology, 682: 235-248. https://doi.org/10.1007/978-1-60327-409-8_17
18. Tsai M, Hwang S, Tsai Y, Cheng F, Lee J, Chang Y (2006). Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells. Biology of Reproduction, 74: 545-551. https://doi.org/10.1095/biolreprod.105.046029
19. Yeh YC, Wei HJ, Lee WY, Yu CL, Chang Y, Hsu LW, Chung MF, Tsai MS, Hwang SM, Sung HW (2010). Cellular cardiomyoplasty with human amniotic fluid stem cells: in vitro and *in vivo* studies. Tissue Engineering. Part A, 16(6): 1925-1936. https://doi.org/10.1089/ten.tea.2009.0728
20. You Q, Cai L, Zheng J, Tong X, Zhang D, Zhang Y (2008). Isolation of human mesenchymal stem cells from third-trimester amniotic fluid. International Journal of Gynaecology and Obstetrics, 103: 149-152. https://doi.org/10.1016/j.ijgo.2008.06.012
21. Zhao X, Zheng Y (2010). Development of cloned embryos from porcine neural stem cells and amniotic fluid-derived stem cells. Animal, 4(6): 921-929. https://doi.org/10.1017/S1751731110000121
22. Zheng YB, Gao ZL, Xie C, Zhu HP, Peng L, Chen JH, Chong YT (2008). Characterization and hepatogenic differentiation of mesenchymal stem cells from human amniotic fluid and human bone marrow: a comparative study. Cell Biology International, 32: 1439-1448. https://doi.org/10.1016/j.cellbi.2008.08.015

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