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The Exploration of Beijing Fatty Chicken Amniotic Fluid Stem Cells with Multilineage Potential

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ABSTRACT

This test aim at exploring and optimizing the isolation and culture methods of chicken amniotic fluid stem cells (CAFSCs) *in vitro*, characterizing CAFSCs and inducing CAFSCs to differentiate into three layers. Methods and results include that more CAFSCs are gained via thermoelectric method with lower cost. Groping and optimizing culture system abiding by the idea of simulating the chicken body environment, involving adding proportion of chicken serum and some growth factors. CAFSCs can be transferred to 34 passages nowadays. Surface marker Oct4, CD105, nanog, CD73 and SSEA-4 on CAFSCs are detected positive with immunofluorescence histochemistry, Gene CD44, CD29, CD73 and SH2 are detected positive with RT-PCR. We carry karyotype analysis out and find that chromosome is 2n=78 and shows normal. CAFSCs in mid period of subculture propagate fastest from cell cycle examination on Flow Cytometer and growth curve using CCK-8 kit on ELIASA. CAFSCs are induced to neuron and epithelium (epiblast), adipocyte (mesoblast), hepatocyte (entoblast).

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Authors' Contribution
MN carried out all experiments and drafted the manuscript. YZ polished language of the manuscript. YD disposed anaphasis of karyotyping. WG and XL conceived the study, participated in its design and coordination.

Key words
Chicken genetic resources, Amniotic fluid stem cell, Multilineage potential.

INTRODUCTION

The research history of amniotic fluid stem cells can be traced back to the beginning of the 20th century. It was first isolated and showed quite strong proliferation capacity *in vitro* (Brace and Wolf, 1989; Kaviani *et al*., 2001). Amniotic fluid stem cells possess some advantages that researchers pay close attention to. AFSCs (amniotic fluid stem cells) can be collected during amniocentesis and isolated from amniotic fluid that would be otherwise throw away. Therefore, it is not limited to the ethical debate that compared with the utilization of embryonic stem cells. Separation approach is relatively simple, practicable and spend less as with other fetal derived stem cells. AFSC populations can be easily expanded *in vitro* and can be stored over long periods of time with no adverse effects, so amniotic fluid is a better source of pluripotent and multipotent stem cells for organ regeneration and endangered species conservation and adhibition.

We have not seen a report about isolation and culture of avian species amniotic fluid stem cells successfully nowadays. The amniotic fluid stem cells research mainly focus on people and cattle, pigs, sheep and other mammals (de Coppi *et al*., 2007; Gao *et al*., 2014; Cui *et al*., 2014). Despite the importance of chicken species as model of birds for *in vivo* studies, little is known about chicken AFSCs until now. Unlike ES (embryonic stem cells), AFSC do not form teratoma when injected subcutaneously into nude mice (Phermthai *et al*., 2010). The aim of this research is to isolate and get abundant CAFSCs, grope and optimize culture system and to characterize them in terms of morphology, specific mesenchymal or pluripotent markers, proliferation and differentiation potential to provide superior seed cells for regenerative medicine and rare species conservation.

MATERIALS AND METHODS

Experimental animal

Fertilized eggs of Beijing Fatty chicken (*Gallus gallus*) (7-9 day old) are selected by the Poultry Experimental Base Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing. All chicken that provide eggs are treated in accordance with NIH and USDA guidelines for the use of animals in research and all experimental procedures involving chicken are conducted in accordance with the protocols and guidelines for agricultural animal research codified by the Committee for Ethics of Beijing, China.

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Experimental reagents

DMEM / HAM'S F-12 (1:1) (DF12) (Gibco, USA), fetal bovine serum (Biochrom, Germany),chicken serum (Sigma, USA), EGF, bFGF, L-glutamine (Peprotech, USA), trypsin (Gibco, USA), rabbit anti chicken Oct-4, CD105, nanog, CD73, SSEA-4 (Abcam, USA), FITC conjugated goatanti rabbit secondary antibody IgG (Abcam, USA), DAPI (Gibco, USA), Trizol (Invitrogen, USA), inverse transcription kit (Takara, Japan), CCK-8 kit (Be Yun Tian, China).

Isolation and culture of CAFSCs

Fertilized eggs of Beijing Fatty chickens (7-9 day old) are selected. Taking out eggs from incubator then putting them immediately in ice water, lasting for 20 min in order to let cells suspend in amniotic fluid. Opening the eggs and spilling amniotic fluid enveloped by amniotic membrance lighty after sterilizing with alcohol. Extracting amniotic fluid with 10 mL needle tubing and decanting it into 15 mL centrifuge tube, centrifuge for 3000 rpm, 30 min, abandoning supernatant.

Cells are inoculated in terms of 5.0×10^6/mL density in DF12 with 10% FBS, 5% chicken serum, EGF 10ng/mL, bFGF 10ng/mL and 1% L-glutamine, cultured under 37°C, 5% CO_2, saturated humidity. Changing medium after 1 day of inoculation, then changing medium every 2 days. Cells can be passaged when go to 70-80% healing.

Characterization of CAFSCs

RT-PCR detection

Collecting the cells, total RNA is isolated with trizol extracting method. Total RNA is reverse transcribed using inverse transcription kit, reaction condition sustains at room temperature for 10 min, 42°C for 1 h, 99°C, inactivated AMV reverse transcriptase for 5 min, ice-bath for 2 min. Information of gene specific primer sequences are shown in Table I. PCR reaction system contains 20μL:2×Mixture for 10μL, cDNA for 5μL, rnase Free H_2O for 7μL, forward primer for 1μL, reverse primer for 1 μL. Reaction condition is consisted of initial 5 min at 94°C one cycle and then denaturation for 30 cycles of 30 s at 94°C, 30 s at 50-60°C for annealing, 2 min at 72°C for extension.

Immunofluorescence histochemistry

CAFSCs of passages 8 which go to 60-70% healing are selected. Cells are washed with PBS and then fixed in 4% (m/v) paraformaldehyde for 15 min (Tanekhy *et al*., 2016) then washed thrice with PBS. Cells are permeabilized using 0.2% (v/v) Triton X-100 for 20 min and washed a further three times with PBS. The cells are blocked in 10% (v/v) goat serum for 30 min, and subsequently incubated in following antibodies: Rabbit anti Chicken Oct-4, CD105, nanog, CD73 and SSEA-4 for 8h at 4°C. After that, cells are washed thrice with PBS, and incubated in appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 1 h at room temperature. After incubation, cells are washed thrice with PBS. Nuclear staining is carried out with DAPI dye for 15 min, then cells are washed thrice with PBS. The cells are observed by the Nikon TE-2000-E confocal microscope, images are acquired.

Table I.- Primer sequences used for RT-PCR.

Gene	Primer sequence (5'-3')	Temp. (°C)	Length (bp)
GAPDH	F:ACTGTCAAGGCTGAGAACGG	60	204
	R:AGCTGAGGGAGCTGAGATGA		
CD44	F:ACGAGGAGCAAAGCATGTGA	58	453
	R:TTCACCGTCTCCCTTGTGTG		
CD29	F:CACTCCCGTGCTGTGAATCT	58	207
	R:CTAGCAAGCAGAAGCCCAGT		
CD73	F:AAACTCCTTTCGGCGCACTA	58	449
	R:CCCTTGAAACGGGAGAACCA		
SH2	F:TCAACCCTGAGGAGGTGTCA	58	333
	R:CCGGCCCCAAATCTCTTTCT		
alb	F:ACCACTGCCTTCCATTATCC	60	342
	R:CAAGCACCGACATCACTCTC		
AFP	F:TCGCCATTCATTACCAGGAT	60	393
	R:TCACGCATACACTCCAGCA		
aq5	F:GGCTGGCATCCTCTATGGTG	60	203
	R:CGGAGAGGCCAATGGACAAT		
CK1	F:ATGGGTATTGGGCGTCACTG	60	465
	R:CTCTTCAAAACGCAGGCCAC		
MAP2	F:TGTGGAAGTGAAACCTGCTG	60	363
	R:GGCTGTGGAACTAAGGCATC		
NFKB1	F:GTTTCACACGGAGGCTTGAT	60	361
	R:GGCTTTGGTTCACTCGTTTC		
PPARG	F:AGCCTCCTTCTCCTCCCTATT	60	336
	R:GCTTCTCCTTCTCCGCTTG		
LPL	F:AAGCCTGTTGGACACATTGA	60	346
	R:CTTTGTAGGGCATCTGAGCA		

aq5., aquaporin5.

Karyotype analysis

CAFSCs that passaged after 5 h are selected, most cells are just attached and individual exists. (1) Adding up 20 μL colchicine of 10 μg/mL to 3 mL medium, cultured at 37°C, 5% CO_2 and saturated humidity for 72 h; (2) Cells are collected and added to centrifuge tube, centrifuge at 2000 rpm for 8 min; (3) Abandoning supernatant, adding to 10

mL KCl of 0.075 mol/L, water bath at 37°C, for 30 min. (4) Adding to 1 mL stationary liquid and blending, centrifuge at 2000 rpm for 8 min; (5) Abandoning supernatant, adding to 7 mL stationary liquid and blending, standing at room temperature for 15 min, centrifuge at 2000 rpm for 8 min; (6) Dropping then staining with Giemsa. (7) Images are taken from laser scanning confocal microscope (LSCM).

Self-renewal and proliferation assays

Drawing of growth curve

Collecting passage 5, 15 and 25 of CAFSCs and inoculated in 96-well plates respectively in terms of 2000/well density. 100 μL complete medium is added per well. Cells of 0, 24, 48, 72, 96, 120 and 144 h behind attached are selected to detect proliferation, three repetitions are set up each passage and averaging. Cells of 3 passages are detected every time point and 10 μL CCK-8 solution is added per well, then incubated in incubator for 2 h. Absorbancy (OD value) is detected with ELIASA at 450 nm, drawing growth curve finally (Sahin *et al*., 2016).

Examination of cell cycle

CAFSCs of P6, P16 and P26 attaining 90% confluence and in good growth status are collected with conventional method and added to flow cytometry tube according to 1.0×10^5-5.0×10^5 respectively, centrifuge at 1200 rpm for 8 min. Abandoning supernatant, washing precipitation with PBS for 3 times. Cells are re-suspended with 70% ethanol and incubated for 8 h at 4°C. Centrifuge at 1200 rpm for 8 min, collecting cells. Abandoning supernatant, washing precipitation with PBS for 2 times. 0.5 mL PI dye liquor is added and incubated for 30 min lucifugally. After filtrated with 80 μM cell strainer, cells are detected on Flow Cytometer.

Induced differentiation of CAFSCs in vitro

Neuron induced differentiation

CAFSCs of P8 attaining 50-60% confluence and in good growth status are selected. There are two groups divided. CAFSCs of the experimental group are cultured with neuron inductive medium containing 10%FBS, 1μM all-trans retinoic acid and 100 μM β-mercaptoethanol while CAFSCs of control group are remained in complete medium. Medium is refreshed every three days. Neuron specific markers NSE and nestin are detected 15 d later by immunofluorescence histochemistry, while MAP2 and NFKB1 are detected by RT-PCR. Control groups are used for comparison.

Epithelium induced differentiation

CAFSCs of P8 attaining 70%-80% confluence and in good growth status are selected. There are two groups divided. CAFSCs of the experimental group are cultured with epithelium inductive medium containing 10% FBS, 30ng/mL vEGF, and 10ng/mL IGF-1 while CAFSCs of control group are remained in complete medium. Medium is refreshed every three days. Epithelium specific markers aquaporin-5 and CK18 are detected 12 d later by immunofluorescence histochemistry, while Aquaporin-5 and CK1 are detected positive by RT-PCR. Control groups are used for comparison.

Adipocyte induced differentiation

CAFSCs of P8 attaining 70-80% confluence and in good growth status are selected. There are two groups divided. CAFSCs of the experimental group are cultured with mixed medium containing CAFSCs medium and adipocyte inductive medium in early days (adipocyte inductive medium: CAFSCs medium is 1:1, 2:1, 3:1 along with changing medium) to avoid marked apoptosis. This method let CAFSCs adapt to adipocyte inductive medium gradually. Adipocyte inductive medium is used lonely after 10 d culture containing 10% FBS, 0.5 mmol/L IBMX, 10mg/L INS, 1.0 μmol/L dexamethasone and 200 μmol/L indomethacin while CAFSCs of control group are remained in complete medium. Medium is refreshed every three days. Oil red O staining is carried out after 20 d induction. Adipocyte specific markers LPL and PPARG are detected by RT-PCR. Control groups are used for comparison (El-Shennawy *et al*., 2016).

Hepatocyte induced differentiation

CAFSCs of P25 attaining 70-80% confluence and in good growth status are selected. There are two groups divided. CAFSCs of the experimental group are cultured with hepatocyte inductive medium containing 5% FBS, 20ng/mL FGF-4, 20ng/mL HGF, 40nmol/mL dexamethasone and 10ng/mL ITS while CAFSCs of control group are remained in complete medium. Medium is refreshed every three days. Glycogen staining is carried out after 12 d of induction. Gene alb and AFP are detected by RT-PCR. Control groups are used for comparison.

RESULTS

CAFSCs morphology comparison cultured by complete medium with and without chicken serum

We compare the Cell morphological differences of CAFSCs with and without chicken serum culture in 24 h and 72 h after inoculation, respectively. Few differences have been found between them in 24 h after inoculation (Fig. 1A, B). It is because cells can survive depending on nutritional ingredients that matrix remains. CAFSCs without adding chicken serum culture in 72 h after inoculation are still

sparse, in poor growth state and differentiation appear (Fig. 1C), CAFSCs with adding chicken serum (5%) cultured in 72 h after inoculation come into large densities and less apoptotic cells have been found in medium (Fig. 1D). Amniotic fluid cells culture methods in the literature that we can access to now are unable to make CAFSCs grow and proliferate in vitro normally in this experiment. So we make a bold speculation that conventional amniotic fluid stem cells culture methods are unfit for bird AFSCs culture in vitro nowadays. Compared with FBS, chicken serum is different in amino acid and other nutritional ingredients. We simulate poultry growing environment of the body by adding chicken serum of certain proportion, which perfect result is gotten.

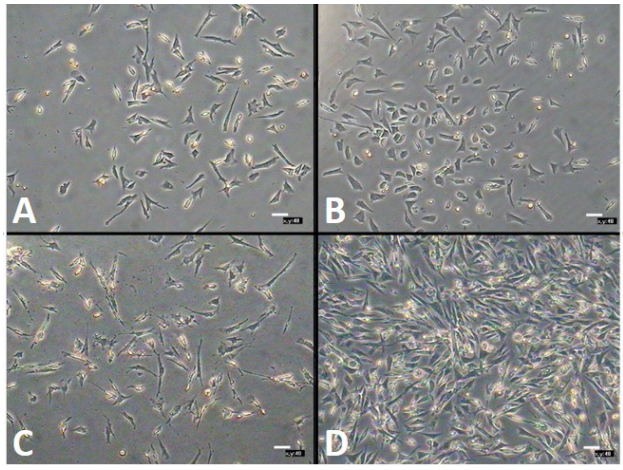

Fig. 1. CAFSCs morphology comparison of complete medium with and without adding chicken serum. A, cell morphological of CAFSCs without adding chicken serum culture in 24 h; B, cell morphological of CAFSCs with adding chicken serum (5%) culture in 24 h. Few differences have been found between A and B; C, cell morphological of CAFSCs without adding chicken serum culture in 72 h; D, cell morphological of CAFSCs with adding chicken serum (5%) culture in 72 h,cell number and morphology are better compared with C. Sparklets are hemocytes which come from matrix and will disappear after passage.

Morphology observation of CAFSCs subculture

After vaccination of primary cells, single cell migrates, attracts each other and gathers to form small cell group gradually. A typical nest clone emerges after cultured for 24 h (Fig. 2A). Passage after 2 d and cells evenly distribute in a petri dish, cell morphology come to the development of fibrosis, strong refraction, full cells, stronger stereo sense (Fig. 2B). Cells proliferate slowly before passage 10 and every generation for about 3-4 d. Then cells come into rapid proliferation and every generation for about 1-2 d (Fig. 2C). Cells proliferation rate begin to reduce after passage 26. Cells are cultured up to passage 34 with most cells showing signs of senescence such as slow cell proliferation, vacuolization, size and flat and stereo feeling disappear.

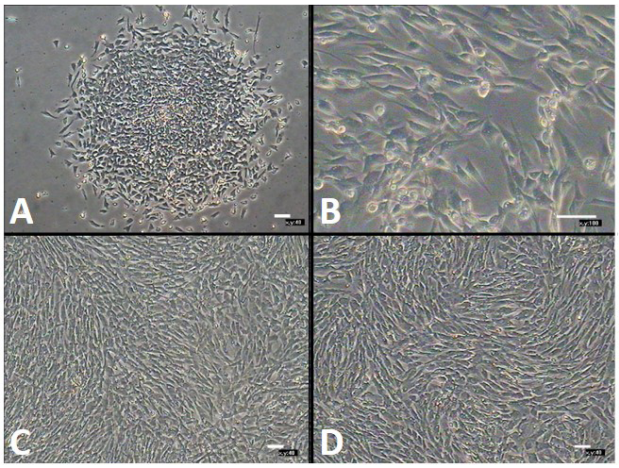

Fig. 2. Morphology observation of CAFSCs subculture. A, nest clone that cultured for 24 h, cells are more toward the center more densely populated, which show a strong proliferation ability; B, nest clone has been passaged and cells evenly distribute, cell morphology comes to the development of fibrosis, strong refraction, full cells, and stronger stereo sense; C, P8 of CAFSCs before passage, which cells grew densely; D, P20 of CAFSCs after passage for 1 d.

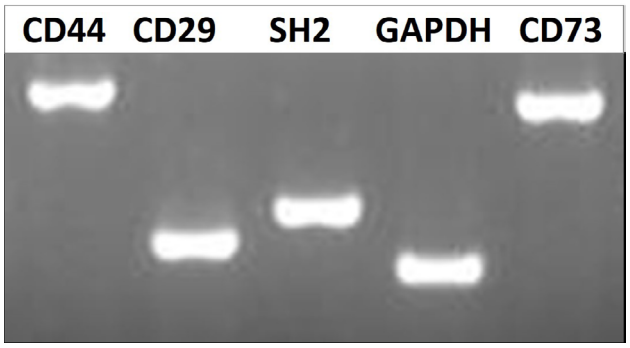

Fig. 3. RT-PCR detection of CAFSCs. Genes CD44, CD29, CD73, SH2 are detected positive with RT-PCR. We use GAPDH as internal control.

Gene and cell surface markers

Genes CD44, CD29, CD73, SH2 are detected positive with RT-PCR (Fig. 3). Cell surface markers Oct-4, CD105, nanog, CD73 and SSEA-4 are detected positive with immunofluorescence histochemistry on LSCM (laser scanning confocal microscope) (Fig. 4).

Amniotic fluid stem cells express a variety of markers,

amniotic fluid stem cells Tsai De and Coppi groups isolate express Nanog, SH2, SH3, SH4, OCT4, CD29, CD44, CD73, CD90, SSEA4 and HLA-ABC, express CD90, CD105, Tra-1-60, HLA-ABC and HLA-DR lowly, 90 proportion of CAFSCs express Oct4 (Zhang et al., 2009). Markers that we detect are included in them, which proves that cells isolated are amniotic fluid stem cells. Some of them are markers that mesenchymal stem cells and embryonic stem cells also express, which can prove the diverse sources of CAFSCs.

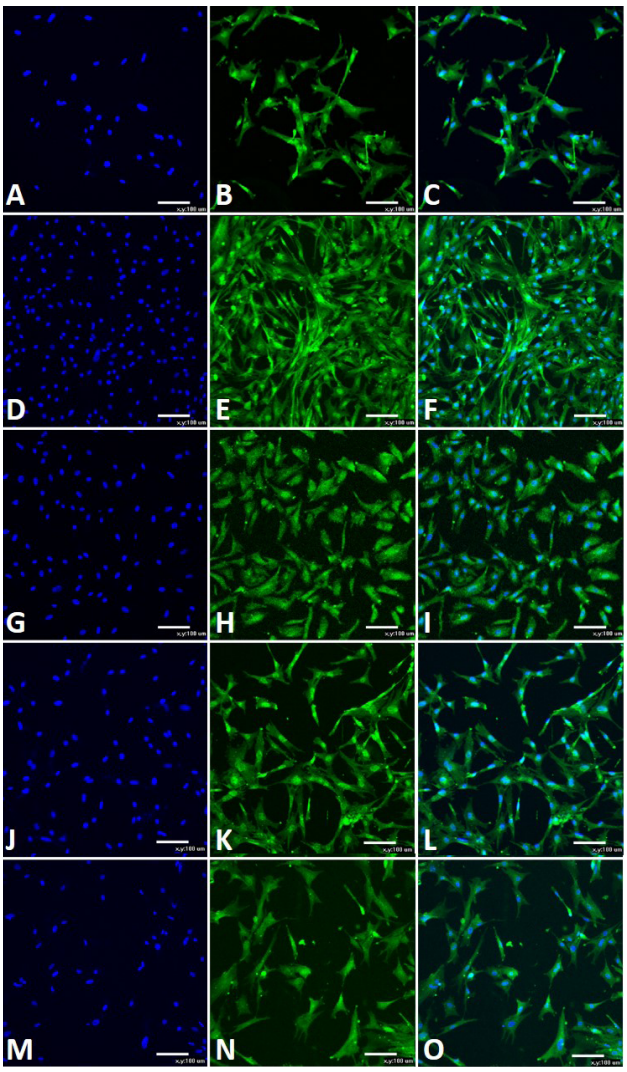

Fig. 4. Immunofluorescence histochemical detection of CAFSCs. Cell surface markers Oct-4, CD105, nanog, CD73 and SSEA-4 are detected positive with immunofluorescence histochemistry on LSCM. A, D, G, J and M, nuclei is stained by DAPI; B, Oct-4^{+}; E, CD105^{+}; H, nanog^{+}; K, CD73^{+}; N, SSEA-4^{+}; C, F, I, L and O, Merged.

Karyotype analysis

We carry out karyotype analysis, then find that chromosome is 2n=78, which is accord with the chicken chromosome number of branches (Fig. 5). CAFSCs have a normal karyotype and retain long telomeres. Giemsa band karyogram shows chromosomes of late passage (>250 p.d.) cells.

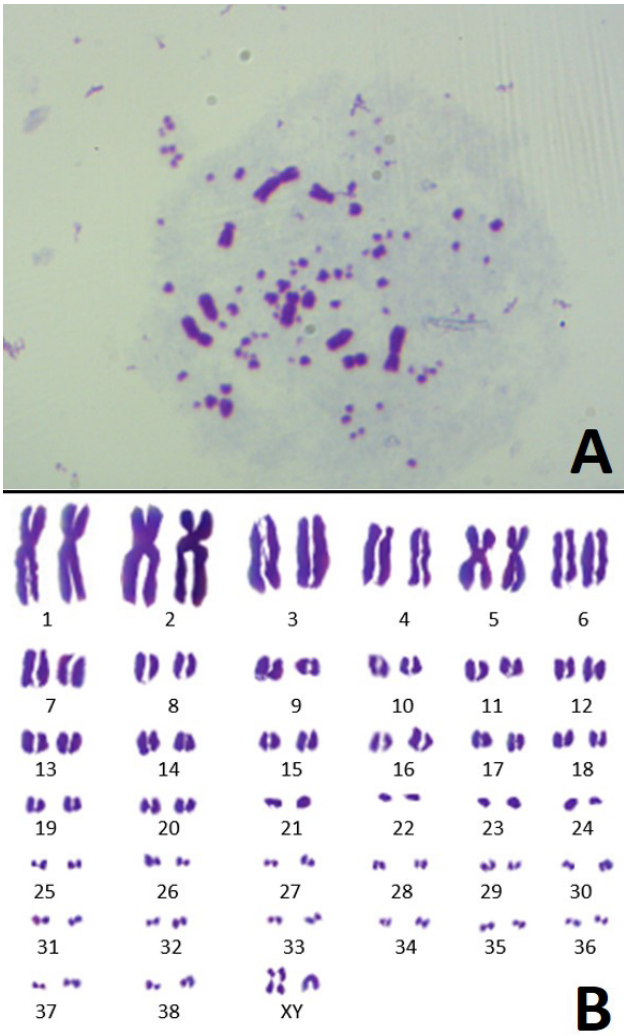

Fig. 5. Karyotype analysis of CAFSCs. A, chromosome doubling in mitosis anaphase under microscope; B, each pair of chromosomes after arrangement, a total of 38 to autosomal and a pair of sex chromosomes.

Self-renewal and proliferation of CAFSCs

Applying ELIASA, OD value is getted with CCK-8 kit. The growth curve of the CAFSCs appears as a typical "S" shape (Fig. 6). Incubation phase (0-48 h), exponential phase (48-96 h), stationary phase (96-120 h) and apoptosis phase (120-144 h) are displayed clearly. Nevertheless, P16

of CAFSCs proliferation capability is stronger than P6. P26 of CAFSCs proliferation capability is significantly lower than P6 and P16. Then along with passage numbers increasing, CAFSCs proliferation capability decline.

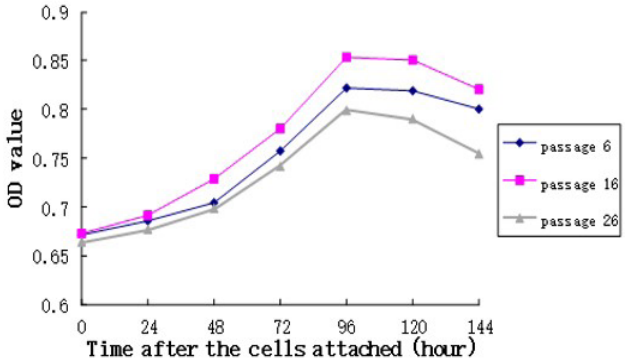

Fig. 6. Growth curve of CAFSCs. Incubation phase (0-48 h), exponental phase (48-96 h), stationary phase (96-120 h) and apoptosis phase (120-144 h) are displayed clearly. P16 CAFSCs proliferation capability is stronger than P6 and P26.

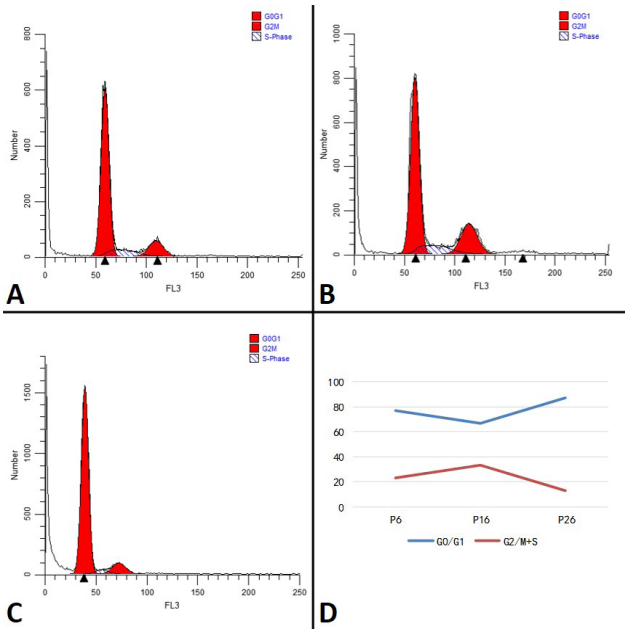

Fig. 7. Examination of cell cycle. A, cell quantities that lie in G0/G1, S and G2/M of P6 CAFSCs, respectively; B, cell quantities that lie in G0/G1, S and G2/M of P16 CAFSCs, respectively; C, cell quantities that lie in G0/G1, S and G2/M of P26 CAFSCs, respectively; D, line chart of G0/G1 and G2/M+S statistics. S phase represent DNA replication stage, G2/M represent cell division stage, G2/M+S can reflect cell proliferation vigor.

Examination of cell cycle

Cell cycle examination has been carried out on Flow Cytometer. We chose P6, P16, P26 of CAFSCs to detect their different cell cycle phases (Fig. 7). Formula G2/M+S reflect cell proliferation vigor of different passages (Table II). We find that proportion G2/M+S of P16 is the highest (33.25%), P6 take second place (23.25%) and P26 is the lowest (12.69%). It proves that P16 of CAFSCs proliferation capability is stronger than other two passages too.

Table II.- Percent counting for every stage of cell cycle.

	G0/G1	G2/M	S	G2/M+S
P6	76.75%	13.01%	10.24%	23.25%
P16	66.75%	21.28%	11.97%	33.25%
P26	87.31%	8.39%	3.30%	12.69%

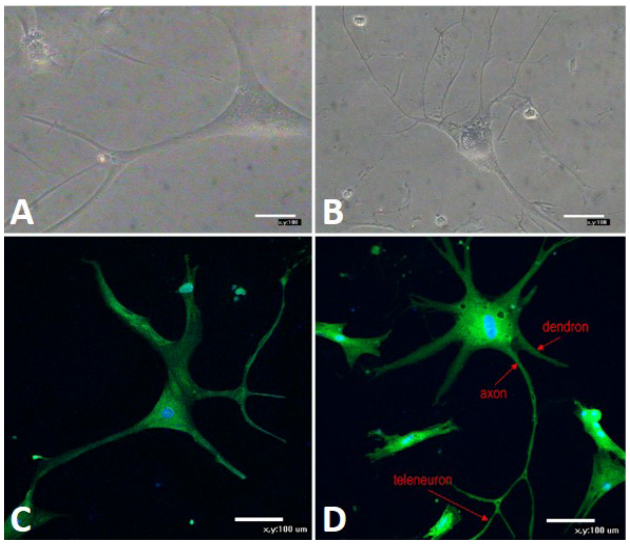

Fig. 8. Neuron induced differentiation of CAFSCs *in vitro*. A and B, morphology observation of induced cells under inverted microscope, dendron and axon emerge and grow mature after culture for 15 days; C, neuron specific marker NSE is detected positive by immunofluorescence histochemistry; D, neuron specific marker nestin is detected positive too. Red arrow stamp axon, dendron and teleneuron, respectively which are typical morphological markers of neuron.

Induced differentiation of CAFSCs in vitro

Neuron induced differentiation

The differentiation capacity of CAFSCs to neuron is researched and then detected by subsequent morphological and phenotypic analysis. Marked apoptosis of cells occur after adding up neuron inductive medium for 24 h. The cell survival become longer and reductive gradually along with

induced time prolonging. Dendron and axon emerge and grow mature after culture for 15 days (Fig. 8A, B). There are no morphological changes appearing in control group. Neuron specific markers NSE and nestin are detected positive by immunofluorescence histochemistry (Fig. 8C, D), while MAP2 and NFKB1 are detected positive by RT-PCR (Fig. 12).

Epithelium induced differentiation

The differentiation capacity of CAFSCs to epithelium is researched and then detected by subsequent morphological and phenotypic analysis. Not too many cells come to apoptosis after adding up epithelium inductive medium. Cell morphology changes from spindle to typically round or irregularly shaped after 12 days (Fig. 9A, B). There are no morphological changes appearing in control group. Epithelium specific markers aquaporin-5 and CK18 are detected positive by immunofluorescence histochemistry (Fig. 9C, D), while aquaporin-5 and CK1 are detected positive by RT-PCR (Fig. 12).

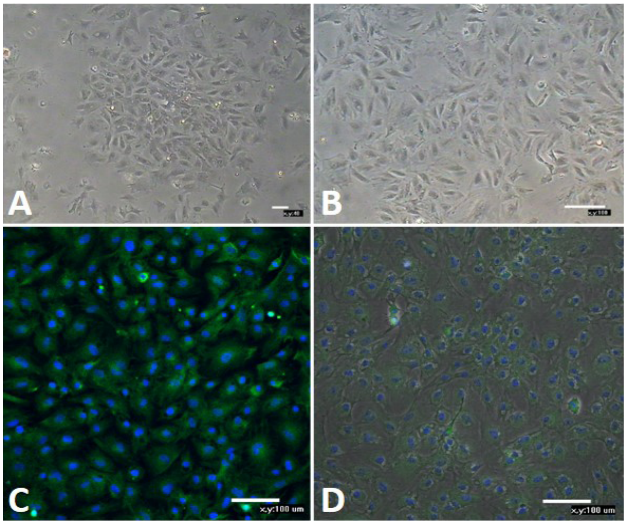

Fig. 9. Epithelium induced differentiation of CAFSCs *in vitro*. A and B, morphology observation of induced cells under inverted microscope, cell morphology change from spindle to typically round or irregularly shaped after 12 days; C, Epithelium specific marker aquaporin-5 is detected positive by immunofluorescence histochemistry; D, Epithelium specific marker CK18 is detected positive too.

Adipocyte induced differentiation

The differentiation capacity of CAFSCs to adipocyte is researched and then detected by subsequent morphological and phenotypic analysis. Cells grow slowly but less apoptosis exist using mixed medium containing CAFSCs medium and adipocyte inductive medium in early days. Cell morphology become bigger, Lipid droplets appear after culture for 8 d and become more and bigger with culture time prolonging. Lipid droplets become brighter after culture for 20 d (Fig. 10A, B) and present orange red after oil red O staining (Fig. 10C, D). There are no morphological changes appearing in control groups. Adipocyte specific markers LPL and PPARG are detected positive by RT-PCR (Fig. 12).

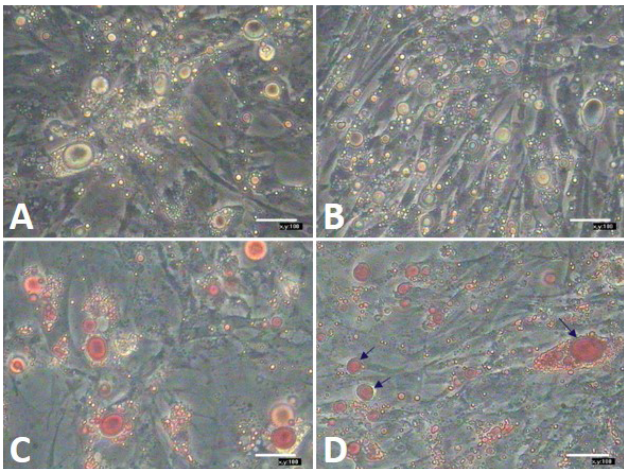

Fig. 10. Adipocyte induced differentiation of CAFSCs *in vitro*. A and B, morphology observation of induced cells under inverted microscope, lipid droplet become brighter after culture for 20 d; C and D, lipid droplet present orange red after oil red O staining. Blue arrow stamp lipid droplet, which is typical morphological marker of adipocyte.

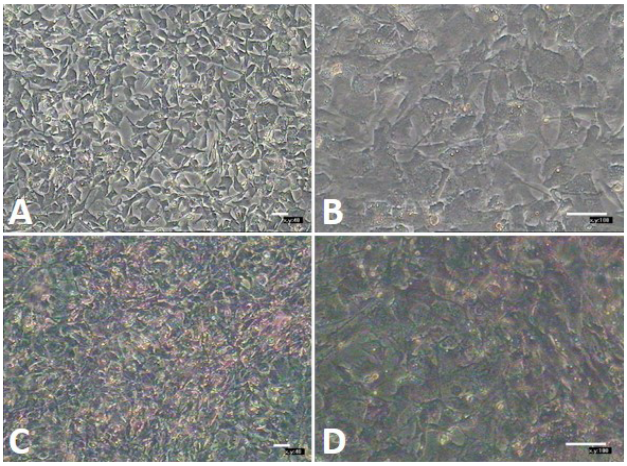

Fig. 11. Hepatocyte induced differentiation of CAFSCs *in vitro*. A and B, morphology observation of induced cells under inverted microscope, cells present polygonal and foliated after culture for 12 d; C and D, Glycogenosome present amaranth by glycogen staining. This can prove that high-passaged CAFSCs (P25) still maintain induced differentiation potential.

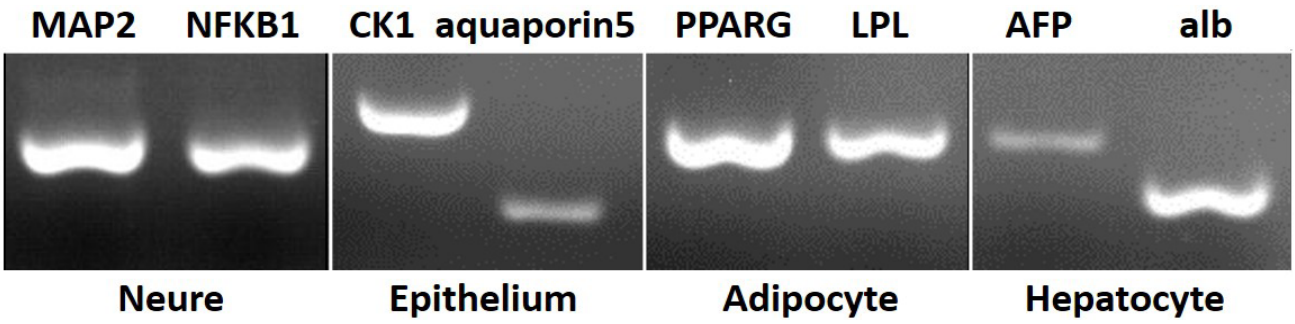

Fig. 12. RT-PCR detection of induced cells *in vitro*. Respective specific markers of neure (MAP2 ,NFKB1), epithelium (CK1, aquaporin5), adipocyte (PPARG, LPL) and hepatocyte(AFP, alb) are detected positive, which can further confirms the success of induction for CAFSCs *in vitro*.

Hepatocyte induced differentiation

The differentiation capacity of CAFSCs to hepatocyte is researched and then detected by subsequent morphological and phenotypic analysis. Cells become round gradually in early days and present polygonal and foliated later (Fig. 11A, B). Glycogen staining is carried out after 12 d and Glycogenosome present amaranth (Fig. 11C, D). Gene alb and AFP are detected positive by RT-PCR (Fig. 12). It is proved that high-passaged CAFSCs still maintain characteristics of stem cells and ability of induced differentiation *in vitro*.

DISCUSSION

Thermoelectric method is used to isolate CAFSCs, which let cells suspend adequately in amniotic fluid. This is a new attempt to isolate CAFSCs. These cells are gained hard in this research currently, although there are many perfect and efficient 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 and Gautam, 2012). They bring about little effects in this test, which are not suitable for proliferating CAFSCs extensively *in vitro* and its application in regenerative medicine potentially. A Thailand scientific research group declare that they find a new method to get high-concentration AFSCs as report goes in 2014, but they have not made this method public yet now. Thermoelectric method we attempt in this research has not be ripe now, though more cells getted, there exist a lot of problem such as containing major kinds of other cells; furthermore, it is hard to get exact figure of needed cells that isolated now. We will combine magnetic activated cell sorting in order to compare thermoelectric method and other isolation method in the future. More efforts will be given to this method in the following research.

Proliferation rate in early days is slower than that in culture metaphase. Refer to the culture experience of human AFSCs (Mosquera *et al*., 1999; Kaviani *et al*., 2003; Tsai *et al*., 2004; de Coppi, *et al*., 2007). We can realize that the constitution of poultry amniotic fluid is not simple too. It is possible that CAFSCs extractive contain major kinds of cells and their different proliferation rate slow the whole speed. Then cells are purified along with culture hour extending, proliferation rate is promoted. The isolation method of CAFSCs still need improvement.

Groping and optimizing culture system abiding by the idea of simulating the chicken body growth environment, involving adding proportion of chicken serum and some growth factors. We find that culture methods of amniotic fluid stem cells (AFSCs) in the literature we can refer to nowadays are not capable to let CAFSCs proliferate normally *in vitro* via plentiful failing exploration in the phase of preliminary experiment, so we infer that conventional methods at present is not suitable for culture of CAFSCs *in vitro*. CAFSCs will differentiate into epithelium automatically if EGF is added in extra proportion in medium, cell proliferation decline widely. CAFSCs will go to excessive fibrosis if bFGF is added in extra proportion in medium. Chicken serum and some growth factors are added in appropriate proportion to medium to simulate chicken body growth environment, which get better effects. There are many differences between chicken serum and fetal bovine serum (FBS), for instance, chicken serum contain arginine and histidine alone. Nevertheless, which nutrition constituents meet the need of CAFSCs is unknown yet. Mechanism of action of chicken serum for CAFSCs multiplication will be needed for deep investigation in the future.

CONCLUSION

More CAFSCs are gained via thermoelectric method with lower cost and simple operation, which is appropriate

for mass augmentation *in vitro*.

Genes are detected CD44, CD29, CD73, SH2 positive with RT-PCR. Cell surface markers Oct-4, CD105, nanog, CD73 and SSEA-4 are detected positive with immunofluorescence histochemistry on LSCM, which prove that cells isolated are amniotic fluid stem cells. Chromosome of CAFSCs is 2n=78 and normal, which is accord with the chicken chromosome number of branches.

The growth curve of the CAFSCs appears as a typical "S" shape. Cell cycle examination has been carried out on Flow Cytometer. Self-renewal and proliferation trait of CAFSCs presents that subculture in mid period propagate fastest.

Groping and optimizing culture system abiding by the idea of simulating the chicken body growth environment, involving adding appropriate proportion of chicken serum and some growth factors, CAFSCs can be transferred to 34 passages nowadays. CAFSCs are induced to neuron and epithelium (epiblast), adipocyte (mesoblast), hepatocyte (entoblast). These demonstrate that CAFSCs have strong capacity of self-renewal and induced differentiation, which meet the initial requirement of application to regenerative medicine.

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Statement of conflicts of interest

The authors declare no conflict about interest.

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