Preparation and Cryopreserved of Fibroblasts from Frozen Embryos of (Arbor Acre) Chicken

Ahmed Khalid[1,2], Nagam khudhair[3,4],Yu Na[1], Shi Yu[1], Li Yulong[1], Zhang Guixue[1,*]

[1]*College of Animal Science and Technology, Northeast Agricultural University, Harbin 150030, China*
[2]*Department of Animal Production, College of Agriculture, University of Tikrit, Iraq.*
[3]*Key Laboratory of Agricultural Biological Functional Genes, Northeast Agricultural University, Harbin 150030, China.*
[4]*Biology Department, Education College for Women, Al-Anbar University, Ramadi, 31001, Iraq.*
[*]*Correspondence:Dr. Gui-xue Zhang*

ABSTRACT

This study is to establish fibroblast line of AA broiler chicken from frozen embryos. According to the requirement of (ATCC) tests, the study depended on testing several points such as growth curve, cryopreservation, Karyotyping of chromosomes and protein translation of transfected vector. The results showed that the cells were morphologically consistent with fibroblasts and the growth curve was sigmoidal with a population doubling time (PDT) was(41.6h FE , 43.2h AFE). Cell viability was 91.8% before cryopreservation and 82.2% after thawing. Karyotyping for chromosome number was of 2n=78 and the rate of cellular diploid was 92.20±0.56% for Fresh Embryos (FE) and 91.42±0.23% After Freezing Embryos (AFE). The fluorescent protein was translated at 24h, 48 h and 72h after transfection and the colonies of fluorescent fibroblast cells were formed one months later. In conclusion, the procedure for cryopreservation and thawing of embryos as well as preparation of fibroblast cells were proven to be practicable. The fibroblast line of AA broiler from frozen embryos has been established and the valuable genetic resource of AA broiler chicken was well protected at the cellular level.

Keyword: *-AA Broiler Chicken, Fibroblast cells, Froze Embryos, Fluorescent proteins and Karyotype.*

1. INTRODUCTION

Cryopreserving cell bank is an important tool for the preservation of genetic information. All genetic information of a species is stored in one cell because of the entire genome presence in the nucleus of somatic cells. Also, it can provide a precious experimental material for research in the life sciences of these species [1,2]. Arbor Acre (AA) broiler is among the most recognized and respected names in poultry industry. Even though some researches about establishing the fibroblast cell bank of poultry have been announced [2,3]), but there is not any report about producing AA broiler fibroblast bank,especially from frozen chicken embryos. Therefore, the aim of this study is to establish fibroblast line of AA broiler chicken from frozen embryos according to the specifications American type culture collection (ATCC).

2. MATERIALS AND METHODS

2.1 Materials

The embryos of Arbor Acre, AA broiler chicken were provided by the chicken Hatchery, college of Animal Science, Northeast Agriculture University, Harbin, China.

2.2 Preperation ofFibroblast cells From Fresh Embryos

The embryos were from stage 26 between 8-10 days of incubation eggs. After removing the head, limbs and the internal organs of the embryo, the samples were divided in half. The one half was used for culture fibroblast cells and the other was cryopreserved. The procedure for the isolation of fibroblast cells from fresh embryos was based on a previous description by Su et al [4] with some modifications. Briefly, the Fresh embryos were cut into small pieces of about 1 mm3. The samples were washed three times with (PBS), transferred to a new Petri dish containing PBS and incubated with trypsin at room temperature for 5-7 min with gentle oscillation then incubated with Ⅳ collagenase at room temperature for 5-7 min with gentle oscillation. Then mixture tissue and cell medium was filtered through 40 mm copper meshes. The cell suspension was collected and centrifuged at 300g for 5 minutes. The sediment was resuspended with DMEM (Invitrogen, USA). the cells were were adjusted to the intensity 5-10× 106 cells/ml in DMEM containing 10% FBS and cultured at 37°C in a humidified atmosphere with 5% CO2. The medium was changed after 12 h. After 3 to 5 passages, fibroblast cells were haversted and crypryserved as description below.

2.3 Cryopreservation, Thawingof Embryosand Preperation ofFibroblast cells

The freezing medium consisted of 80% DMEM, 10% FBS and 10% dimethyl sulfoxide. The half embryo was cut into small pieces of about 1 mm3, put into the freezing medium and cooled down slowly using a controlled-rate freezing assembly. They were cooled sequentially from room temperature (24°C) to 4ºC at the rate of 1ºC/min for 20 minutes, then temperature of vials were dropped to -20ºC at the rate of 0.4ºC/min for 60 minutes and at -80ºC for 12h, finally transferred to the liquid nitrogen (LN2) for a long term preservation. The vials were taken out from the liquid nitrogen and quickly thawed in 37-40^{o}C water bath with gentle shaking until the sample melt completely. Then they were centrifuged to remove the DMSO from the recovered embryo.

2.4 CryopreservationandThawing of Fibroblat cells

The procedure of temperature for cryopreservation and thawing of fibroblast cells (intensity 5-10 × 106) was the same as cryopreservation and thawing of embryos above.

2.5 Cell Viability and Cell Growth Curve

Cell viability was estimated by trypan blue dye test which used to determine viability before freezing and after recovery. We used the first passage of cells produced from frozen embryos and the first and second passage of fresh embryos, the cells were seeded in 24 well plates at 10^4/well and counted with an Invitrogen [Countess™ Automated Cell Counter]. Cells were seeded in 24 well plates and assessed by their population doubling time (PDT). The cells of density about 1.5×10^5 were cultured for 8 days and then counted every 24h, each time for three wells. Also, we use the third passage s after freezing second passage of fresh embryos to draw the growth curve and to calculate the population doubling time (PDT) which was determined from this curve of Suemori et al [5].

2.6 Karyotyping and Chromosome

The cell Chromosomes spread was prepared, fixed and stained following methods for Sun et al [6] . The modal AA broiler chicken cell karyotype was prepared by the method of Ford et al [7]. After 3 passages, the chromosomes from Fresh Embryos (FE) and After Freezing Embryos (AFE) were counted. Ten photographs of metaphase chromosomes were selected and the long and short arms of 10 pairs of macrochromosomes measured. According to Levan et al [8], the parameters of relative length, kinetochore type and centromere index were calculated.

2.7 Transfection of pEGFP-N3

After third passage the cells from both kinds of embryos were seeded in each well of the 24 well plate and transfected with 2µg plasmid DNA, pEGFP-N3 using the lipofectamine mediated 6µl (lipofectamine 2000) (Invitrogen, Carlsbad, CA, USA) [9].The cultured cells were observed at 24h,48h,72h,1 week, 1 month and 2 months respectively after transfection. The medium was renewed after 6h from transfection. The expression of the fluorescent protein was observed by digital inverted (fluorescence) microscope (EVOS) with excitation wavelengths of 470-531 nm, to determine the transfection efficiency. The morphology of positive cells in each experimental group, images were captured from 10 visual fields to calculate the transfection efficiencies which were formulated as the relationship between the positive cell number of total cell numbers [2].

C=V total for positive cell /V total cell number × 100

2.8 RNA Extraction and Reverse-Transcription PCR

Total RNA was extracted from cells, at 48h after transfection with Plasmid pEGFP-N3 by using Trizol reagent. Following RT-PCR and PCR, to prepared cDNA from this procedure was used in the following PCR reactions performed with 0.24 µmol/l of each sense and antisense primers, 0.06 mmol/l of rTaq polymerase, 0.8mmol/l deoxynucleotide mix, 10X PCR buffer and (β-actin) (No.NM-205518) as a template to amplify a 331-bp product with the following primer sequences: forward 5'-TCTTGGGTATGGAGTCCTG-3' and reverse 5'-TAGAAGCATT TGCGGTGG-3' was used to check the RT-PCR results, as a positive control. The results were analysed on a 1.5% agarose electrophoresis gels.

2.9 Microbial Detection

After third passage, fibroblast cells from both kinds of embryos were cultured in medium without antibiotics and observed for the presence of bacteria and fungi contamination was assessed within three days after subculturing according to the method described by Doyle et al[10]. While, detection of mycoplasma according to the American Type Culture Collection (ATCC), the cells were cultured in antibiotic-free medium at least 1 week, then fixed and stained with (DAPI).

3. RESULTS

3.1 Cell Morphology

Fibroblast cells from both kinds of embryos were initially mingled with epithelial cell, but Fibroblasts grew rapidly and replaced the epithelial cells gradually after 2-3 passages. The culture cells were examined by phase-contrast microscopy were fibroblasts identified by their flat, elongated spindle-shaped process possessing morphology, which same in both kinds . Also, the cells displayed typical fibrous characteristics with vigorous vitals cytoplasm, and during growth they showed morphological fibroblast-like radiating, flame-like or whirlpool-like shapes, our results are compatible with Wu et al and Bai et al[2,3], and increased the ratio of the number of cells increasingly regular shaped cells, and then a relatively pure fibroblast line was obtained as indicated in (Fig. 1. A). After the fifth passage appears the colonies cells of polygonal shapes (Fig. 1. B).

3.2 Microorganism Detection

The medium was clear for the whole time from both kinds of cells, showing no turbidity or other abnormal changes could be observed under the microscope. The results indicated that the chicken fibroblasts were negative of bacterial contamination and apparently free of Mycoplasma where the cells showed smooth surfaces, round nuclei with blue fluorescence, and no filamentous blue fluorescence around the nuclei when observed under a fluorescence microscope as shown in (Fig.1. C)

3.3 Cell Viability and Growth Curve

The viability of chicken fibroblast cells from fresh embryos was 91.8%, and viability of chicken fibroblast cells from embryos froze after recovery embryo was 82.2% with a significant differences ($P<0.05$). Whereas, for cell

passage 2 (Fresh embryos) before cryopreservation was 80.0%, and after recovery was 61.8%.The results show superiority of the Fresh Cells Passage 2 (FCP 2) without significant differences as well as the superiority of the After Freezing Cells Passage 2 (AFCP 2) with a significant difference of ($p<0.05$). Cells after recovery grew well and adhered to the flask well in 30~60 min, and had spread along the bottom wall by 48h later. As estimated by the trypan blue exclusion test (Fig.1. D). The growth curve of chicken fibroblast was appeared (S) shape (Fig.e1. E). After a latent period and an exponential growth period a stationary phase were observed. While, seeding the exponential growth began after 48h, cell densities were at its maximum after 4 days. From 6th day onwards, it had a significant difference than that of 5th days from seed ($P<0.05$), then cells entered the stationary phase, due to the fibroblast growth high density on the surface, preventing the arrival of nutrients to cells. The population doubling time (PDT) was about (41.6h FE , 43.2h AFE).

Table-1: Chromosome parameters of AA broiler chicken（♀).

Chromosome	Relative length (%)	Arm ratio	Centromere index (%)	Centromere type
1	25.64±0. 21	1.5	40	M
2	21.53±0. 23	1.35	42.5	M
3	19.48±0. 30	1.86	35	SM
4	15.38±0. 26	2.1	31.5	SM
5	14.35±0. 22	2.33	30	SM
6	14.30±0.48	2.08	32.5	SM
7	13.33±0.35	4	20	ST
8	12.30±0.42	1.9	34.5	SM
Z	11.79±0.44	1	50	M
W	5.12±0.38	∞	0	T

Ten photographs of metaphase chromosomes were selected of individual, and the long and short chromosome arms in nine pairs of macrochromosomes were measured. The parameters of relative length and centromere index were calculated. M = metacentric chromosome; SM = submetacentric chromosome; ST = subtelocentric chromosome. T, telocentric chromosomes.n=5, ($P<0.05$)

3.4 Karyotype and Chromosome Number

The chromosome number of AA broiler chicken was 2n=78, including 4 pairs of macrochromosomes (1-4), 5 intermediate chromosomes (5-9) and 30 pairs of microchromosomes (Fig. 1. F). The sex chromosome type was ZZ (♂) / ZW (♀) (Figure1I). The parameters including relative length, centromere index and kinetochore type were shown in (Table 1). In this experiment 100 representative spreads at metaphase of passage three was observed under the confocal microscope to count the chromosome numbers, and the mean proportion of diploid cells were 92.20±0.56% FE, 91.42±0.23% AFE respectively.

3.5 Transfection of Fluorescent Protein

The results showed that the highest number of cells with fluorescent signals appeared at 48h after transfection were (31.36% FE ,31.08 AFE %) with no significant differences ($P<0.05$)(Fig.2 A,B) which decreased gradually with the passage of time. While, some cells still showed fluorescent protein after 2 weeks and even after 1 months (Fig.2 A) by screening G418 resistance and monoclinic culture for 1 month, then we obtained colony with positive cell strains. At 48h after transfection, RNA was extracted from the fibroblast cells (Fig. 2C). RT-PCR and PCR showed that (β-actin) 331 bp fragment was gotten in cells with transfected plasmid pEGFP-N3 and in the control group cells. The distribution of the pEGFP-N3 fluorescence protein was showed in the nucleus and cytoplasm (Fig. 2D).

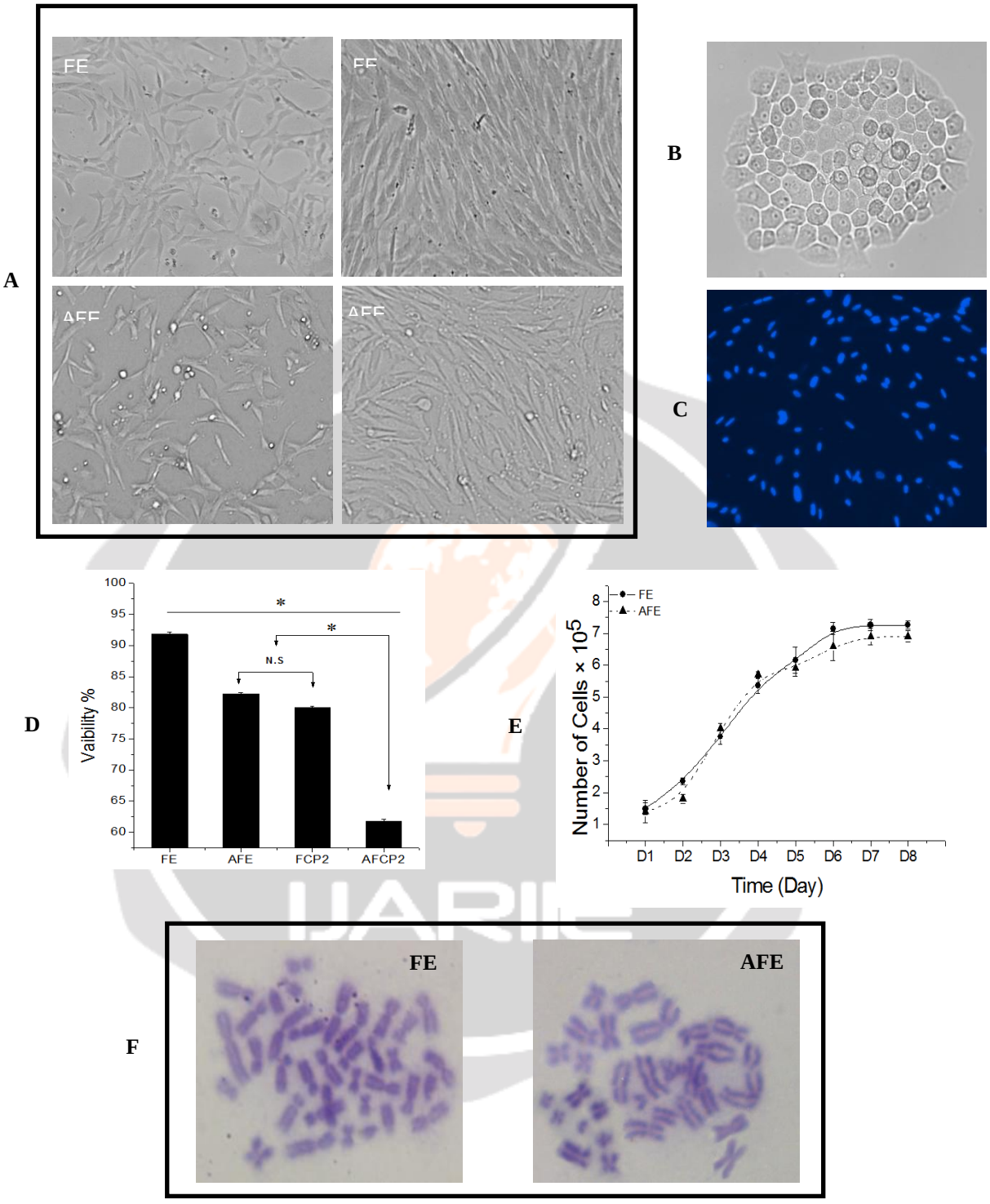

Fig -1: Morphology of a broiler chicken embryonic Fibroblasts. (**A**) Fibroblast cells after two passages (×10, ×10, ×4µm) forming the fibroblast-like shape. (**B**) Fibroblast cells after fifth passages (×20, ×4µm) appears the colonies cells of polygonal shapes and (**C**) fibroblasts stained with DAPI for mycoplasma contamination, negative (×10µm). (**D**)The viability of chicken fibroblast cells, fresh embryo chicken before freezing and after recovery with significant difference (*P<0.05). (**E**) The growth curve of AA broiler chicken embryonic fibroblasts after use (FBS) 10%.Cells were harvested and seeded in 24-well plate at 1.5×10^5/well and cultured for 8 days and then counted every 24 h. FE:

Fresh Embryos, AFE: After Freezing Embryos, FCP2: Fresh Cells Passage 2, AFCP 2: After Freezing Cells Passage 2..(**F**)Chromosome at metaphase and Karyotype in (FE and AFE) of AA broiler chicken.

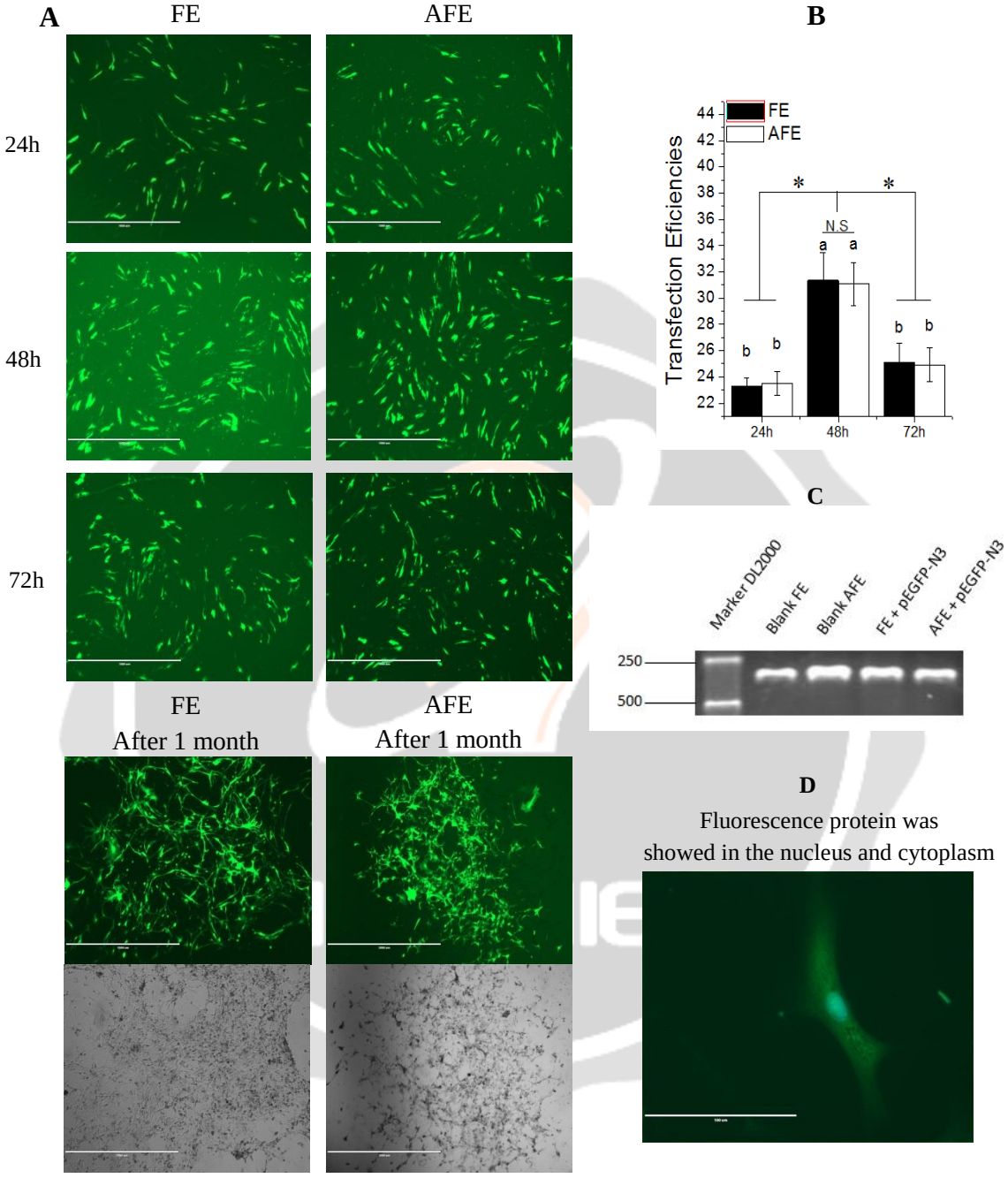

Fig -2: Expression of fluorescence protein in AA broiler chicken fibroblasts. (**A**) Comparative figure of (pEGFP-N3) fluorescent proteins at 24h, 48h, 72h and 1 month after transfection in AA broiler chicken embryonic fibroblasts using a digital inverted (fluorescence) microscope (EVOS) with excitation wavelengths of 470 and 531 nm to determine the transfection efficiency. (×4µm, Scale bar=1000µm).(**B**) Transfection efficiency of fluorescence protein in the AA broiler chicken embryonic fibroblasts. The strongest fluorescence intensity and the highest transfection efficiency of the fluorescent protein appeared 48h after transfection was (31.36% FE, 31.08 AFE %) there was a significant difference Compared with 24h and 72h (*P<0.05). **(C)** PCR Analysis of Positive, cDNA + Primer β-actin Clones (**D**) The distribution of the pEGFP-N3 fluorescence protein (green) was showed in the

nucleus (Blue) was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and cytoplasm (×40µm, Scale bar = 100 µm)

4. DISCUSSION

4.1 Cell Morphologyand Karyotype

Chicken embryos can be used as a potential model for better understanding the mechanisms of tissue-specific differentiation and regeneration that will help to devise strategies based on the transplantation of fibroblast cell-derived chicken tissues for restoring function to damaged or diseased tissues[11,12]. The morphological observation indicated that the primary cells usually coexist with epithelial cells, which may grow either in groups or sporadic [13]. Epithelial cells and fibroblasts have different tolerances to trypsin, We are aware of the fact that the used enzyme-based disaggregation of the tissue might be damaging and potentially alters the functional properties of the isolated cells. However, this issue is unavoidable in ex vivo cell cultures and well-established culture systems [14]. Also, we can pay special attention to the digestion time, so as to collect the cells needed at the appropriate time. The fibroblasts were first detached from the flask while the epithelial cells remained adherent when being subcultured[15], For this reason, a purified fibroblast line in both kinds of embryos could be obtained after 2-3 passages. It is believed the reason for the emergence of polygonal shaped cells after the fifth generation to the differentiation of epithelial cells.

Chromosome number and karyotype were the basis of Cytogenetics. They were trusty indices for identifying the taxonomic and sexual origin of a cell line, and also allow assessing whether the cell line is stable or variable. According to the international karyotype standard, poultry contains eight pairs of macro chromosomes, the sex chromosomes Z and W, and 30 pairs of microchromosomesLadjali-Mohammadi et al. (1999)[16]. The results indicate that no significant difference between both kinds of embryos ($p<0.05$). A small percentage of cells displayed abnormal chromosomes, presumably as a result of chromosome loss or overlap during preparation or chromosomal damage during culture and passage in-vitro. Because most chromosomes are microchromosomes, which were easily missed in the process of slide making, chromosome number and morphology of chicken karyotypes were difficult to determine.

4.2 The Viability of Fibroblast Cells from Freshand frozen Embryos

Embryo freezing opens the way for us to be able to get the cells with high activity and high density of fibroblast cells. In the freezing process when the temperature decreases to 0^{o}C cells suffer from physical and chemical injuries [17]. The addition of cryoprotectant during freezing can prevent ice crystals as well as injuries caused by these crystals [4]. This study indicates the possibility of freezing the embryos and then get on the line of fibroblasts and with good vitals. So not only should total cell number be considered but cell density may also very important [18]. Outperformed vital cells freeze embryos on embryonic cells fresh, if it has a comparison between it and the third passage s after freezing second passage of fresh embryos. Also, fresh chicken embryos can be stored for 6 to 8hr at about 24°C or up to 24 hr at 37°C in a special condition, whereas deep-frozen embryos can be stored for several months with no reduction in tissue embryonic survivability, and for several years with a small reduction in survivability. Thus, It is a good idea to routinely freeze all healthy fibroblast cells to maintain frozen stocks. The most successful thaws and later cultures were frozen as high-quality, undifferentiated, actively dividing cell colonies. Cells recover from a freeze more efficiently if handled gently as larger cell aggregates.

4.3 Expression of Exogenous Gene

The vector gene with stable structure, high - level expression and species - independent efficiency, [19] have been used as marker genes to observe the expression, contribution and function of target proteins in living cells and organisms [20]. They are characterized by brighter fluorescence, more efficient expression of the transcription than Lac Z, CAT and other common fluorescent markers in animal cells [21]. Concentration of DNA and lipofectamine, the DNA incubation time, lipofectamine- plasmid DNA complexes, and the presence of serum all can affect the efficiency of transfection, as shown by research on vero cells, Hela cells and various other cell lines as Shu et al. (2007)[22]. In this study, positive cells from both kinds of embryos were most numerous and the fluorescence signal was strong with the highest transfection efficiency at 48h after transfection were (31.36% FE, 31.08 AFE %) with no significant differences ($P<0.05$), which was slightly higher than that of 26.5% [4]. So, the results indicate that the

slow freeze did not affect the efficiency of transfection [23]. Also, the results show after 1 months that fluorescent protein expression did not affect on the growth and proliferation of the transfected cells, Where proteins and the clones were obtained one month later after selection with G418. Therefore, appeared new colonies of cells positive which indicating that the exogenous genes could be replicated, transcribed, translated and subsequently modified in the fibroblasts.

5. CONCLUSIONS

From the discussion above, it can be concluded that the fibroblasts from a fresh and frozen embryo can function normally. Therefore, The procedure for preparation of chicken fibroblast cells from frozen embryos was established. The valuable genetic resource of AA broiler chicken was well protected at the cellular level.

ACKNOWLEDGEMENT

This work was supported by the National International Scientific and Technological Cooperation Project (2011DFA30760-2-1) and This work was supported by Fund of Key Lab. NEAU. China (ARSAT-2014-01).

REFERENCES

[1] GAO, Y., BAI, C., XIONG, H., LI, Q., SHAN, Z., HUANG, L., ...& GUAN, W. (2013) Isolation and Characterization of Chicken Dermis-Derived Mesenchymal Stem/Progenitor Cells. *BioMed research international*, *2013*.

[2] WU, H., GUAN, W., LI, H., & MA, Y. (2008) Establishment and characteristics of white ear lobe chicken embryo fibroblast line and expression of six fluorescent proteins in the cells. *Cell biology international*, *32*(12), 1478-1485.

[3] Bai, C., Wang, D., Li, C., Jin, D., Li, C., Guan, W., & Ma, Y. (2011). Establishment and biological characteristics of a Jingning chicken embryonic fibroblast bank. *European journal of histochemistry: EJH*, 55(1).

[4] SU, X., JIN, D., LI, C., YUE, H., PU, Y., MA, Y., & GUAN, W. (2011) Establishment of Big Bone chicken fibroblast cell bank and study of its biological characteristics. *Belgian Journal of Zoology*, 141(1), 14-23.

[5] SUEMORI, H., YASUCHIKA, K., HASEGAWA, K., FUJIOKA, T., TSUNEYOSHI, N., & NAKATSUJI, N. (2006) Efficient establishment of human embryonic stem cell lines and long-term maintenance with stable karyotype by enzymatic bulk passage. *Biochemical and biophysical research communications*, 345(3), 926-932.

[6] SUN, Y. L., LIN, C. S., & CHOU, Y. C. (2006) Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. *Cell biology international*, *30*(12), 970-976.

[7] FORD, C. E., POLLOCK, D. L., & GUSTAVSSON, I. (1980) Proceedings of the First International Conference for the Standardisation of Banded Karyotypes of Domestic Animals University of Reading Reading, England 2nd-6th August 1976. *Hereditas*, *92*(1), 145-162.

[8] LEVAN, A., FREDGA, K., & SANDBERG, A. A. (1964) Nomenclature for centromeric position on chromosomes. *Hereditas*, *52*(2), 201-220.

[9] TSUCHIYA, R., YOSHIKI, F., KUDO, Y., & MORITA, M. (2002) Cell type-selective expression of green fluorescent protein and the calcium indicating protein, yellow cameleon, in rat cortical primary cultures. *Brain research*, *956*(2), 221-229.

[10] DOYLE, A., HAY, R., & KIRSOP, B. E. (1990) Animal cells, living resources for biotechnology. *Cambridge, New York*. 81-100.

[11] Wu, Y., Ge, C., Zeng, W., & Zhang, C. (2010). Induced multilineage differentiation of chicken embryonic germ cells via embryoid body formation.*Stem cells and development*, 19(2), 195-202.

[12] KYU CHOI, J., YEON AHN, J., IL AHN, J., HEE LEE, J., HYUN PARK, S., HEUM PARK, J., ... & MOOK LIM, J. (2012) Possibility to establish chicken stem cell from non-germline tissue; detection of colony-forming cells after chicken fibroblast culture and subsequent stem cell characterization. *The Journal of Poultry Science*, *49*(3), 196-204.

[13] Grout, B., Morris, J., & McLellan, M. (1990). Cryopreservation and the maintenance of cell lines. *Trends in biotechnology*, 8, 293-297.

[14] Willam, C., Koehne, P., Jürgensen, J. S., Gräfe, M., Wagner, K. D., Bachmann, S., ...&Eckardt, K. U. (2000). Tie2 receptor expression is stimulated by hypoxia and proinflammatory cytokines in human endothelial cells. *Circulation research*,87(5), 370-377.

[15] Hodges, J. (2006). Conservation of genes and culture: historical and contemporary issues. *Poultry science*, 85(2), 200-209.
[16] LADJALI-MOHAMMEDI, K., BITGOOD, J. J., TIXIER-BOICHARD, M., & PONCE DE LEON, F. A. (1999) International system for standardized avian karyotypes (ISSAK): standardized banded karyotypes of the domestic fowl (Gallus domesticus). *Cytogenetic and Genome Research*, *86*(3-4), 271-276.
[17] HAN, B., & BISCHOF, J. C. (2004) Direct cell injury associated with eutectic crystallization during freezing. *Cryobiology*, *48*(1), 8-21.
[18] Stephan, S., Johnson, W. E., & Roberts, S. (2011). The influence of nutrient supply and cell density on the growth and survival of intervertebral disc cells in 3D culture. *European cells and materials*, 22, 97-108.
[19] HENDRICKS, C. A., ALMEIDA, K. H., STITT, M. S., JONNALAGADDA, V. S., RUGO, R. E., KERRISON, G. F., & ENGELWARD, B. P. (2003) Spontaneous mitotic homologous recombination at an enhanced yellow fluorescent protein (EYFP) cDNA direct repeat in transgenic mice. *Proceedings of the National Academy of Sciences*, *100*(11), 6325-6330.
[20] CHENG, G., CHENG, X., & BAI, X. (2003) The gene construction and location in tubular epithelial cells of fused by green fluorescence protein and human kidney gene NaDC3. *JOURNAL OF CELL BIOLOGY*, *25*(3), 170-3.
[21] HEIM, R., CUBITT, A. B., & TSIEN, R. Y. (1995) Improved green fluorescence. *Nature*. 373: 663-664.
[22] SHU, J. H., ZHANG, Y., PAN, Z. F., PENG, S. Y., CAO, J. W., & LI, X. C. (2007) Construction of expression vector of human lactoferrin and its expression in bovine mammary epithelial cells. *Belgian journal of zoology*, *137*(2), 231.
[23] SASAKI, K., MIZUSAWA, H., ISHIDATE, M & TANAKA, N (1991) Gene Transfer into Mammalian Cells by Rapid Freezing. *In Vitro Cell*. 27A:86 88.