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Directional differentiation of chicken embryonic stem cells into osteoblasts, neuron-like cells and adipocytes

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Chicken embryonic stem (ES) cells are useful for producing transgenic chickens and preserving genetic material in avian species. In this study, the differentiation potential of chicken ES cells was investigated *in vitro*. Chicken ES cells were differentiated into osteoblasts cultured for 15 to 21 days in the induction media containing dexamethasone (DEX), β -glycerol phosphate (β -GP) and/or vitamin C (Vit C) respectively and differentiation rates ranging from 40 to 72% confirmed by Von Kossa, cytochemical and immunohistochemical staining. Chicken ES differentiated into neuron-like cells cultured for 3 to 7 days in the induction media containing retinoic acid (RA) and 3-isobutyl-1-methylxanthine (IBMX) and differentiation rates ranging from 70 to 84% were identified by toluidine blue staining and immunohistochemical staining. Also chicken ES was differentiated into adipocytes cultured for 21 days in the induction media containing DEX, insulin (Ins) and/or IBMX and differentiation rates ranging from 74 to 91% identified by oil red-O and reverse transcriptase-polymerase chain reaction (RT-PCR) for peroxisome proliferator activated receptor- γ (PPAR- γ) gene expression. These data suggest that like mammalian ES, chicken ES can differentiate into different cell types *in vitro*.

Key words: Chicken embryonic stem cells, *in vitro*, directional differentiation, osteoblasts, neuron-like cells, adipocytes.

INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass (ICM) of mammalian preimplantation blastula or primordial germ cells (PGCs) and cultured to establish cell clone lines *in vitro* (Xiao et al., 2000). Since the report of establishment of pluripotential cells from mouse embryos in 1981 (Evans and Kaufman, 1981), remarkable progress has been made in the isolation, cultivation and transgenic manipulation of mammalian ES cells. In addition, recent studies have shown that mammalian ES

cells can differentiate into multiple somatic cell types *in vitro*, showing a new avenue for cell-replacement therapy. Induction of hESCs into osteoblasts has been described (Sottile et al., 2003); a previous study has demonstrated that gastrin is a good chemical inducer in the differentiation of bone marrow mesenchymal stem cells (BMSCs) into neuron-like cells (Dong et al., 2004); it has been reported that ES cells could differentiate directly into nerve cells cultured in the culture system with RA, β -ME and conditioned medium of cerebral cortex and over 70% of them was NSE immunopositive (Guo et al., 2003); adipocytes could successfully be induced from rat bone mesenchymal stem cells (Naruse et al., 2004). Yagami et al. (2004) demonstrated that mesenchymal stem cells derived from human chondrogenic cell line could differentiate into adipocytes. However, as an optimal origin of stem cell, reports on *in vitro* differentiation of chicken ES cells are limited. In this study, we investigated the multipotential differentiation of chicken ES cells into different cell types *in vitro*. The experimental data showed that high percentages of chicken ES cells could differen-

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Abbreviations: ES, Embryonic stem; DEX, dexamethasone; β -GP, β -glycerol phosphate; IBMX, 3-isobutyl-1-methylxanthine; RA, retinoic acid; Ins, insulin; PPAR- γ , peroxisome proliferator activated receptor- γ ; ICM, inner cell mass; PGCs, primordial germ cells; BMSCs, bone marrow mesenchymal stem cells; CREB, cAMP response element binding; NSE, neuron specific enolase; NFM, neurofilament-M; GFAP, glia fiber acid protein.

Table 1. The compose of the induction media in orientation of osteoblasts.

Induction media	DMEM (%)	FBS (%)	β -ME (mol/l)	DEX (mol/l)	β -GP (mol/l)	Vit C (g/l)
I	90	10	5.5×10^{-5}	1×10^{-7}	0.01	0.05
II	90	10	5.5×10^{-5}	1×10^{-7}	1×10^{-4}	0.05
III	90	10	5.5×10^{-5}	1×10^{-8}	1×10^{-4}	0.05
IV	90	10	5.5×10^{-5}	1×10^{-8}	0.01	0.08
Control group	90	10	5.5×10^{-5}	/	/	/

DMEM, Dulbecco modified eagle medium; FBS, fetal bovine serum; β -ME, DEX, dexamethasone; β -GP, β -glycerol phosphate; Vit c, vitamin C.

tiate into osteoblasts, neuron-like cells or adipocytes after induction in different media for different days and will supply profound basis for further research.

MATERIALS AND METHODS

One-day-old fertilized eggs of Suqin chickens were obtained from the Institute of Poultry Science, Chinese Academy of Agriculture Sciences and incubated at 38.0°C with 60% relative humidity. The developmental stages of the embryos were determined in accordance with previously described methods (Hamburger and Hamilton, 1992; Li et al., 2003).

Isolation and cultivation of chicken ES cells

Isolation and cultivation of chicken ES cells were carried out following previously described method (Wu et al., 2008). In brief, blastoderm cells at stage X were collected by spoon method in tissue culture dishes and rinsed with Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) to remove the yolks and vitelline membrane. After washing with PBS, ES cells were transferred into fresh tissue culture dishes containing PBS plus 0.25% trypsin and 0.04% ethylenediaminetetraacetic acid (EDTA). Followed by digestion at 37.0°C for 5 to 8 min, the dissociated cells were collected by centrifugation at 1000 r/min for 8 to 10 min and suspended, then about $3 \sim 5 \times 10^4$ ES cells were maintained in a 5% CO_2 humidified atmosphere at 37.0°C with Dulbecco's modified eagle medium (DMEM, Gibco, Grand Island, N.Y., USA) and chicken embryonic fibroblast (CEF) cell monolayer, the medium was replaced daily with fresh medium. The cell colonies with undifferentiated morphology were rinsed three times with PBS and digested at 37.0°C for 2 to 3 min with 0.25% trypsin plus 0.04% EDTA. The dissociated ES clusters were suspended with pipette and sub-cultured in a 5% CO_2 humidified atmosphere at 37.0°C with gelatin-coated flasks containing feeder cell layer and DMEM medium. The medium was replaced daily with half fresh medium.

Identification of chicken ES cells

The morphology of the chicken ES cells was observed under inverted microscope. The histochemical staining for alkaline phosphatase (ALP) activity and immunocytochemistry staining were performed as previously described (Wu et al., 2008). Briefly, chicken ES cells from the 5th passage were diluted to 1×10^7 cells/ml with PBS for preparation of smear slides and fixed at 4°C for 10 min with 25% glutaraldehyde solution. After washing for two times with PBS, ALP staining solution was added and incubated at 4°C dark room. After 20 min staining, the slides were observed under microscopy for ALP-positive cells. Immunocytochemistry was used to detect

expression of stage-specific embryonic antigen-1 (SSEA-1, Genetimes Technology, Inc, Shanghai, China), ES cells were fixed with acetone/ethanol (3:2/v:v) at room temperature for 30 min after 5 times passage following washing three times with PBS and then blocked with 10% fetal calf serum (FCS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China) in PBS for 2 h, then washed with PBS, SSEA-1 antibody was added and the incubated at 37°C for 1 h. After washed three times with PBS, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse immunoglobulin G was added and incubated at 37°C for 45 min, then washed with PBS, cells were observed under an inverted fluorescence microscope for SSEA-1-positive cells. For immunocytochemistry staining of Hoechst 33342, chicken ES cells were fixed with acetone/ethanol (3:2/v:v) at room temperature for 30 min following washing two times with PBS. After washing three times with PBS, the slides were stained for 1 h with Hoechst33342 staining solution in dark room and observed under an inverted fluorescence microscope for Hoechst -positive cells.

Induction of chicken ES cells into osteoblasts

The chicken ES cells of the third passage were sub-cultured in DMEM medium for 48 h and then grown in different osteoblast induction media (Table 1), while the control group was fed on the complete medium. The induction medium was replaced at 3-day intervals with DMEM. After cultivation for 21 days, cells were examined with Von Kossa's or immunohistochemical staining (SABC, Wuhan Boster Biological Technology, Ltd, Wuhan, China) as previously described (Wei et al., 2007).

Induction of chicken ES cells into neuron-like cells

The ES cells from the third passage were sub-cultured with DMEM supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing). 48 h post-cultivation, the cells were separated for induction group and control group, incubated in neuron-like cell induction medium I, II, III (Table 2) or DMEM medium, respectively. One day after induction, the cells were observed daily under an inverted microscope and the medium was changed every three days. The derived neuron-like cells were identified first by toluidine blue staining as previously described (Qin, 2006) and then by immunohistochemical staining using the monoclonal antibody against neuron specific enolase (NSE), neurofilament-M (NFM) or glia fiber acid protein (GFAP) (NSE, NFM, GFAP, Fuzhou Maixin. Biological Engineering Technology Co., Ltd, Fuzhou, China) following the manufacturer's instructions 4 days post- induction.

Induction of chicken ES cells into adipocytes

Chicken ES cells of the third passage were sub-cultured in DMEM

Table 2. The compose of the induction media in orientation of neuron-like cells.

Induction media	DMEM (%)	FBS (%)	β -ME (mol/l)	RA (mol/l)	IBMX (mol/l)
I	90	10	5.5×10^{-5}	2×10^{-6}	/
II	90	10	5.5×10^{-5}	/	5×10^{-3}
III	90	10	5.5×10^{-5}	2×10^{-6}	5×10^{-3}
Control group	90	10	5.5×10^{-5}	/	/

RA, Retionic acid; IBMX, 3-isobutyl-1-methylxanthine.

medium with 10% FBS for 48 h. The cells were induced firstly in adipocyte induction medium with 10% FBS, 5.5×10^{-5} mol/l β -ME (Sigma-Aldrich, Inc., St. Louis, MO, USA), 1×10^{-6} mol/L DEX (Maixin), 10 mg/ml Ins (Maixin), and 5×10^{-4} mol/l IBMX (Sigma) for 3 days and then in DMEM medium supplemented with 10% FBS, 5.5×10^{-5} mol/L β -ME, 10 mg/ml Ins. After cultivation for additional 1 day, the medium was changed by induction solutions. Thereafter, the earlier step was repeated three times. 21 days post-induction, the derived adipocytes were identified by oil red O staining as previously described (Yu et al., 2008). To confirm the identity of adipocytes, PPAR- γ gene transcription was detected by RT-PCR. Total RNA of cells was extracted with Trizol Reagent (ChinaGen Biological Technology, Ltd, Shenzhen, China). The cDNA was synthesized by using the Revert Aid first strand cDNA synthesis kit (ChinaGen Biological Technology) according to the manufacturer's protocol. The PCR primers were designed according to the PPAR- γ mRNA sequence (GenBank accession number AF470456) with the following sequences: 5'-TCAGTTTCGCTCTGTGGA-3' (forward) and 5'-TGCCAGGTCATGTCATCTA-3' (reverse). PCR was carried out under the following conditions: initial denaturation for 5 min at 95°C, denaturation for 40 s at 95°C, primer annealing for 40 s at 55°C and elongation for 40 s at 72°C. After 32 cycles, the amplification was terminated with a final extension for 10 min at 72°C, followed by rapid cooling to 4°C. PCR products were then analyzed by polyacrylamide gel electrophoresis (PAGE).

Differentiation ratio of chicken ES-derived cells

The chicken ES-derived cells were counted by 10 fields chosen at random by an inverted microscope. Then the percentages of each differentiated cells were calculated.

Statistical analysis

All data were performed by using the Student's t-test in SPSS 11.5 software for significant difference ($P < 0.05$) and the results were presented as means \pm standard error (SD).

RESULTS

Identification of chicken ES cells

Under a light microscope, chicken ES cells were round in shape with large nucleus, high nuclear-cytoplasm ratio and one or several nucleoli. 24 h post-cultivation, small colonies were observed, appearing a nest, hill-like or sunflower-like, etc (Figure 1a) and the number of colonies greatly increased from 24 to 48 h. The cell colonies were alkaline phosphatase (ALP)-positive (Figure 1b). After

staining with Hoechst33342 solution and detecting SSEA-1 expression, there were strong blue fluorescence (Figure 1c) in the nuclei of ES cell and strong green fluorescent (Figure 1d) cell colonies observed under microscope.

Influence of different induction media on differentiation of chicken ES cells into osteoblasts

After 48 h incubation at different induction media for osteoblast, 40 to 72% of ES cells were differentiated into osteoblasts (Table 3). Among the four induction media, the induction media I and II had better induction effect than the induction media III and IV, showing significant difference ($P < 0.01$). In addition, the control group showed significantly lower than the groups using the induction media ($P < 0.01$). In the first 2 days after cultivation in the osteoblast induction medium, the morphology of chicken ES cells remained unchanged. However, the shape of the induced cells began to expand from day 3 and appeared to be fibroblast-like cells from day 3 to 6 after induction. Most of them had triangular, polygonal or irregular morphology from day 9. Within day 15 to 21, the derived osteoblasts had a more scattered distribution with dense granular mineralized nodules, the number of which increased gradually, forming white calcified nodules eventually. On day 21, the identity of the derived osteoblasts was confirmed by ALP and Von Kossa's staining (Figure 2a), while the control group and feeder layer failed to stain. Moreover, the differentiated cells reacted positively with the antibody against collagen I (Figure 2b).

Influence of different induction media on differentiation of chicken ES cells into neuron-like cells

After incubation in different neuron-like cell induction media for 48 h, 71 to 84% chicken ES cells were differentiated into neuron-like cells, among which the induction medium I or II had significantly higher ($P < 0.01$) induction effect than the induction medium III. No significant difference ($P < 0.05$) could be seen between the induction medium I and II (Table 4). From day 2 after induction, cells began to migrate from the cell colonies

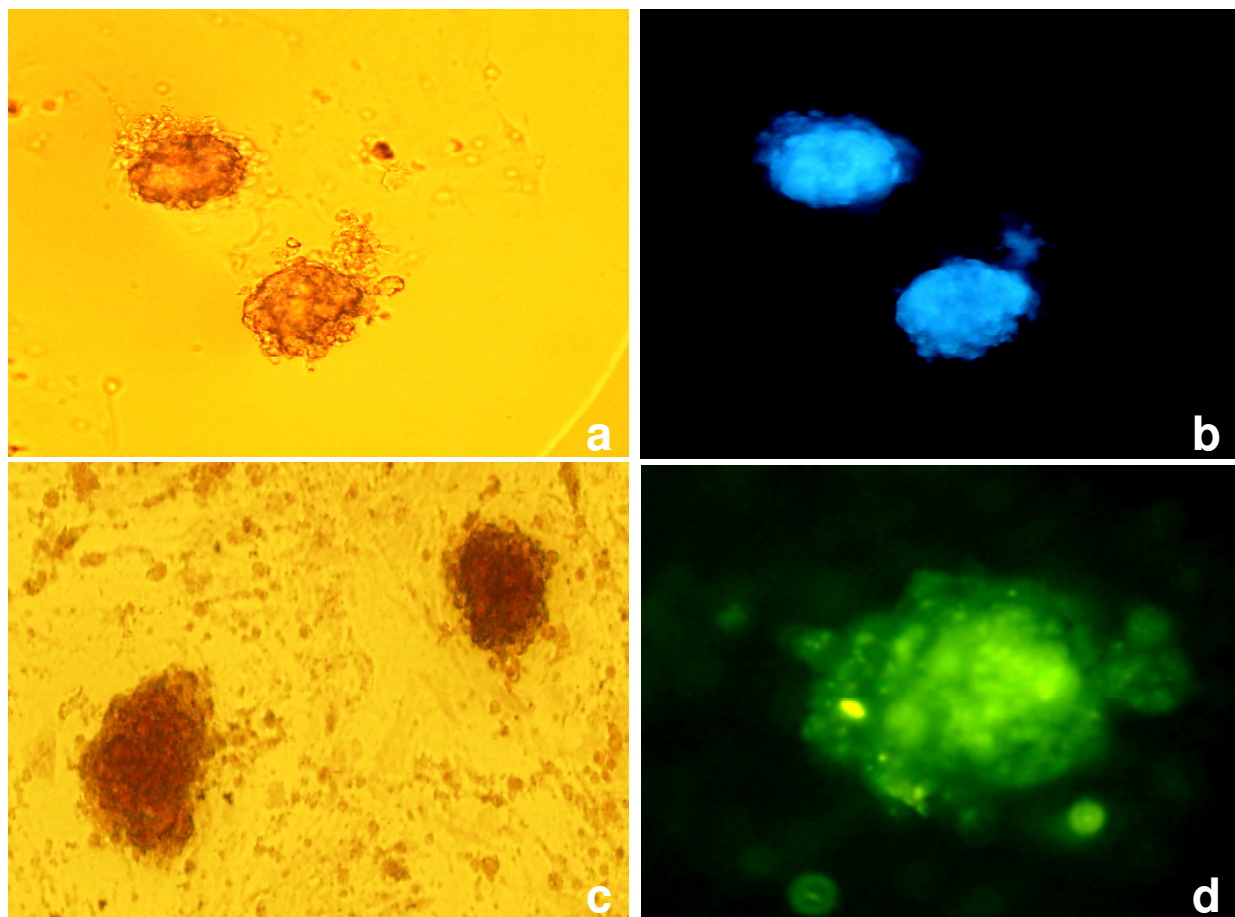


Figure 1. Identification of chicken ES cells. (A) The 5th generation ES cells were cultured for 5 days and the cell colonies were observed directly under microscope (200 x); (b) immunofluorescence analysis shows Hoechst -positive cells (200 x); (c) the cell colonies were positive for ALP staining (200 x); (d) the clones of chicken ES cell showed SSEA-1 positive (400 x).

Table 3. The differentiation rate of induction of osteoblast (n=10).

Induction media	Number of ES cell	Number of osteoblast	Differentiation rate
I	33.90±4.12	24.50±3.75	0.72±0.06 ^A
II	37.80±7.23	26.40±6.60	0.69±0.06 ^A
III	34.90±5.53	14.20±3.91	0.40±0.07 ^B
IV	35.10±5.63	20.10±2.56	0.58±0.08 ^C
Control group	35.00±8.01	0.50±0.53	0.01±0.01 ^D

In the same column, the numbers with different superscripts are significantly different ($P < 0.01$).

and to form a network structure gradually. On day 4 after induction, the derived neuron-like cells showed more typical morphology and approximately 70% of them had neuron phenotype based on toluidine blue staining with visible Nissl bodies in the cytoplasm (Figure 3a). From day 7 after induction, the derived neuronal-like cells were positive for NSE (Figure 3b) or NFM (Figure 3c), but not for GFAP (Figure 3d). At the same time, there was no neuron-like cell in the control group.

Influence of induction medium on differentiation of chicken ES cells into adipocytes

From day 2 to 3 after induction, small lipid granules were observable around the nuclei of the induced ES cells, the number of which increased as extension of culture time. 9 days post-induction, more and larger lipid granules were seen, forming a typical ring-like configuration on the 11 to 21th day after induction. 21 days post induction; all of ES

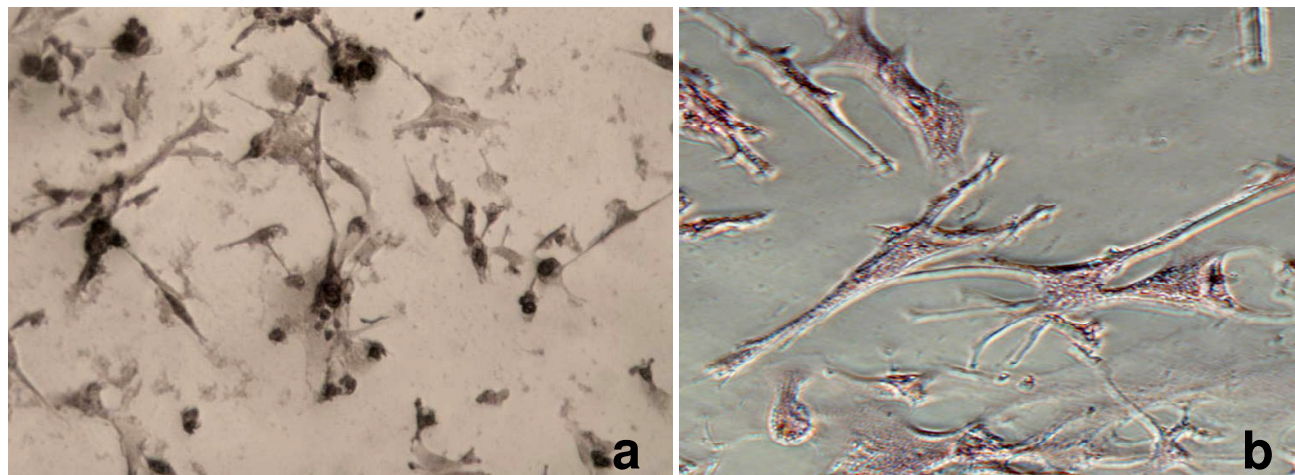


Figure 2. Identification of chicken ES-derived osteoblast cells. The derived adherent cells were examined on day 21 after induction. (A) Chicken ES cells had been induced for 21 days, the Von Kossa's stain was positive (200 x); (b) chicken ES cells had been induced for 21 days, immunohistochemistry stain showed the derived osteoblast to be Collagen I positive (400 x).

Table 4. The differentiation rate of induction of neuron-like cells (n=10).

Induction media	Number of ES cell	Number of neuron-like cell	Differentiation rate
I	36.10±4.68	30.30±3.37	0.84±0.05 ^A
II	35.90±4.70	29.00±3.33	0.81±0.05 ^A
III	35.80±4.08	25.30±3.23	0.71±0.06 ^B
Control group	36.40±5.42	0.80±0.42	0.02±0.01 ^C

In the same column, the numbers with different superscripts are significantly different ($P < 0.01$).

cells were differentiated into adipocytes (Figure 4a). RT-PCR amplified an expected 301 bp product in the differentiated adipocytes by using PPAR- γ -specific primers on day 21 after induction (Figure 4b).

DISCUSSION

ES cells from mammalian have been shown to be able to differentiate into various cell types, including sperm cells *in vitro*, which are valuable cell sources for regenerative medicine and transgenic studies. As the important animal model for developmental studies, the differentiation potential of chicken ES cells remains to be investigated *in vitro*. In this study, ES cells from the chicken embryos at stage X were induced under similar conditions for directional differentiation of mammalian ES cells into osteoblasts, neuron-like cells and adipocytes. After induction for different days, typical osteoblasts, neuron-like cells and adipocytes were observed by microscopy and their identities were confirmed by cytochemistry, immunohistochemistry and/or RT-PCR amplification for marker gene expression. These data suggest that chicken ES cells could be induced into various cell types under suitable conditions *in vitro*, with the possibility of

using these cells for chicken biotechnology.

DEX, β -GP and Vit C are the most common inducers for osteoblast differentiation. DEX can not only contribute to the proliferation and differentiation of mammalian ES cells into osteoblasts, but also promote ALP activity, expression of osteocalcin and increase type I collagen mRNA and extracellular matrix (ECM). β -GP can promote the calcium deposition in osteoblasts and thus, osteocalcin calcification by providing the phosphoric acid ions (Coelho and Fernandes, 2000). Vit C is involved in the terminal hydroxylation of proline and lysine. In addition, Vit C can regulate the activities of ALP and the synthesis of type I collagen (Otsuka et al., 1999). By using a combination of the three components in the induction media, 40 to 72% of chicken ES cells were induced into osteoblasts in this study. Although, the differentiation rate in the induction medium II had no significant difference from that in the induction medium I, the differentiation time was delayed by 2 to 3 days, which could be due to different concentrations of β -GP in the two media. In the induction medium III containing lower concentrations of DEX and β -GP, the differentiation rate was significantly lower ($P < 0.01$) than that in the induction medium I, indicating the significant role of the two chemicals in osteoblast proliferation and/or osteocalcin secretion.

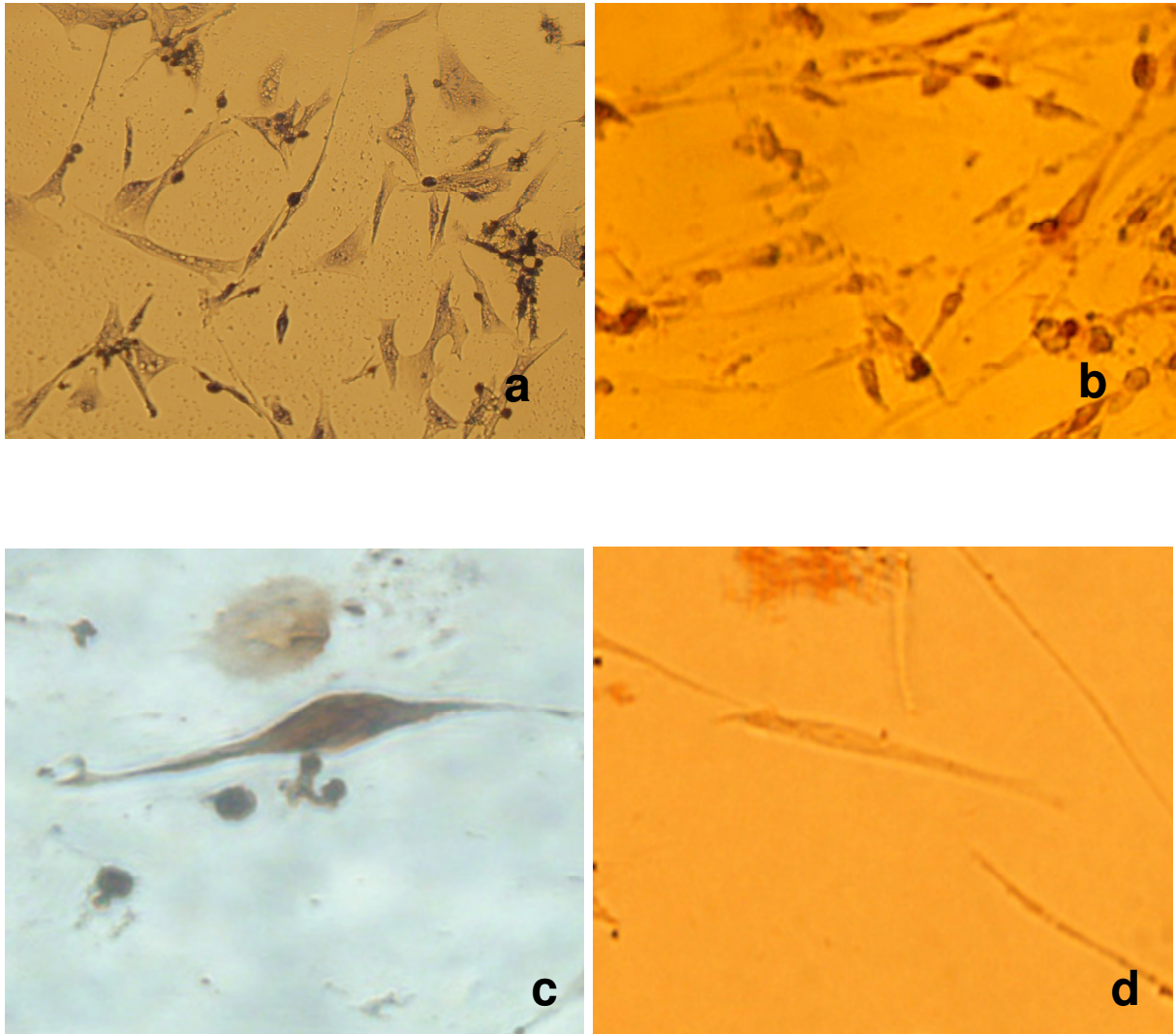


Figure 3. Identification of the chicken ES-derived neuron-like cells. The derived cells were examined on day 4 to day 7 after induction. (a) Chicken ES cells had been induced for 4d, toluidine blue stain showed the forming neuron to be nuclear blue positive (200 x); (b) after 6 days induction, differentiated cells derived in chicken ES cells with typical neuron morphology, possessed NSE positive (200 x); (c) after 6 days induction, differentiated cells derived in chicken ES cells with typical neuron morphology, showed NF positive (400 x); (d) chicken ES cells had been induced for 6 days, immunohistochemistry staining showed the forming neuronal-like cells to be GFAP negative (400 x).

Finally, the differentiation rate in the induction medium IV was significantly higher ($P < 0.01$) than that in the medium III, indicating that Vit C can promote the formation of calcified nodules.

RA is one of the most important extrinsic morphogens, which can promote neural differentiation (Ross et al., 2000) and induce mammalian ES cells into many cell types, including cardiac muscle cells, nerve cells and other mesoblast cells (Okada et al., 2004). In addition, RA can be used as the inducer of embryonic carcinoma cells (Bang et al., 1994). However, RA is also the inducer of cell apoptosis (Ross et al., 2000) and thus, the appropriate concentration must be used for *in vitro* differentiation studies (Fraichard et al., 1995). In addition,

IBMX is a phosphodiesterase inhibitor that can induce differentiation of stem cells into neuron-like cells by increasing the concentration of intracellular cAMP (Bang et al., 1994). In this study, the induction medium I or medium II had higher differentiation rates (81 to 84%) than medium III plus 2×10^{-6} mol/l RA and 5×10^{-3} mol/l IBMX, suggesting that a combination of RA and IBMX has no synergism on the induction of chicken ES cells into neuron-like cells.

For efficient derivation of adipocytes, ES cells were cultured in the induction medium supplemented with 1×10^{-6} mol/l DEX, 5×10^{-4} mol/l IBMX and 10 mg/l insulin and the culture medium plus 10 mg/l insulin only. It has been reported that DEX can activate the glucocorticoid

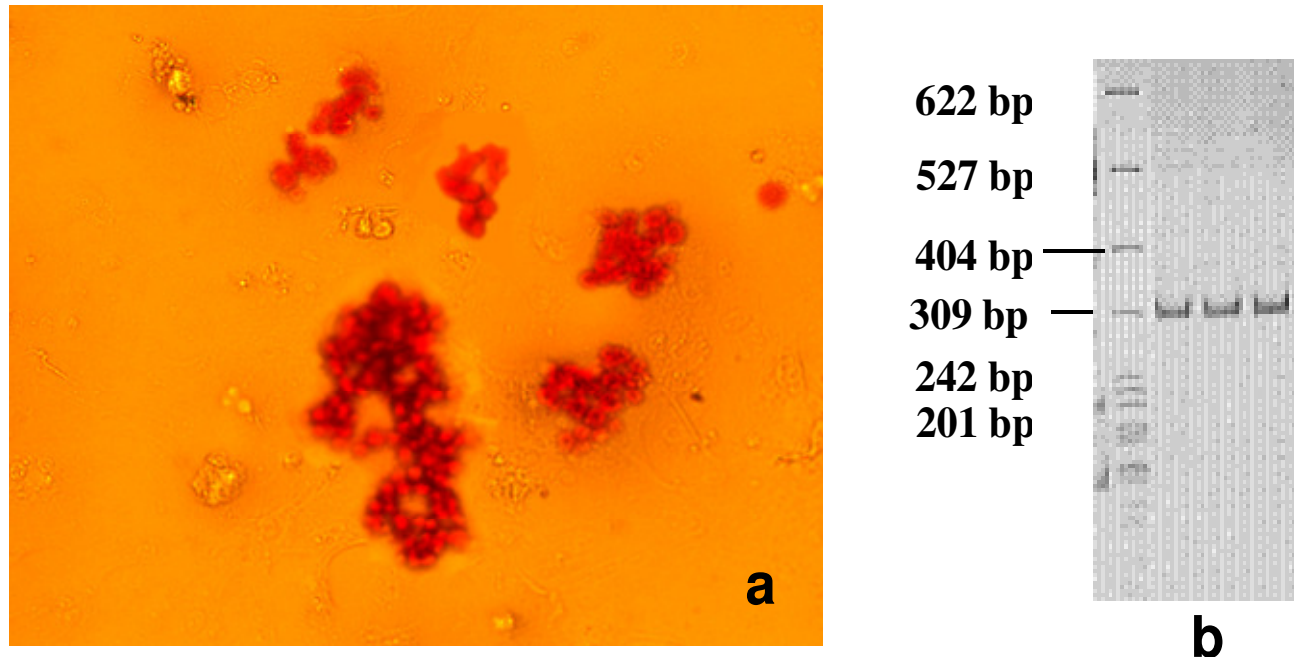


Figure 4. Identification of chicken ES-derived adipocytes. (A) The derived cells were examined directly for lipid granules by oil red O staining on day 21 (200 x); (b) expression of PPAR- γ mRNA in the cells was detected by RT-PCT on day 21 after induction.

receptor of the nuclear hormone receptor superfamily on the cell surface (Yamaguchi et al., 1998) and the transcription factor CCAAT/enhancer-binding protein b (C/EBPb). IBMX can activate the related transcription factor C/EBPd. C/EBPb and C/EBPd in turn induce transcription of C/EBPa and PPAR. C/EBPa and PPAR direct the final phase of adipogenesis by activating expression of adipocyte-specific genes, such as fatty acid synthetase, fatty acid-binding protein, leptin and adiponectin (Yamaguchi et al., 1998; Tang et al., 1999). Insulin can control phosphorylation and transcription of cAMP response element binding (CREB), while CREB can enhance expression of C/EBP and PPAR- γ (Klemm et al., 1998). In addition, insulin may be involved in regulation of adipocyte differentiation by activating the phosphoinositide 3-kinase signaling pathway (Gagnon et al., 2001).

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