

## Isolation and characterization of duck embryonic neural stem and progenitor cells

L. Hou,\*†<sup>1</sup> D. Jin,\*†<sup>1</sup> E. Gu,\* Y. Pu,\* F. Li,\* W. Guan,\*<sup>2</sup> and Y. Ma\*<sup>2</sup>

\**Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100193, P. R. China;*

†*College of Life Sciences and Bioengineering, Beijing Jiaotong University, Beijing 100044, P. R. China;*

‡*College of Biological Sciences, China Agricultural University (CAU), Beijing 100193, P. R. China*

**ABSTRACT** Both embryonic and adult neural stem cells (NSC) of rodents and humans have been isolated and cultured in vitro to date, and they are thought to have tremendous clinical promise in restoring the diseased or injured central nervous system. However, there are few counterpart reports on neural stem cells from birds. This study explored the isolation and culture system of duck neural stem and progenitor cells (NSPC) and investigated their major biological properties. Cells from the dorsal ventricular ridges of 10- to 13-d embryos were isolated, cultured, and purified by using a neurosphere assay. Growth kinetics and karyotype were analyzed. The differentiation potential of NSPC was detected by immunofluorescence. Apoptosis and acetyla-

tion level of histone 4 lysine 12 (H4K12) were assessed. Results indicated that the nestin-positive neurospheres derived from duck embryos were able to self-renew and differentiate into neurons, astrocytes, and oligodendrocytes, and were prone to be transfected with exogenous genes. Karyotype analysis showed that 95% (38 out of 40) of cells of the population were diploid. Apoptosis detection indicated that the apoptotic rate was elevated with increasing passage number and culture time. The cells were highly acetylated and exhibited typical NSPC properties. Efficiently transfected with fluorescent genes, they were available for gene therapy and suitable for research on intracellular distribution of proteins of interest.

**Key words:** duck, neural stem cell, isolation, characteristic

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### INTRODUCTION

The viewpoint that the brain did not have a pool of stem cells was held for decades until it was reported that some regions of the central nervous system exhibited self-renewal properties (Altman, 1969). Neural stem cells (NSC) were first isolated from mouse striatal primordia (Reynolds et al., 1992). Since then, important insights have been gained in further research.

Embryonic NSC are widely distributed in the brain and spinal cord, whereas adult NSC are mainly in the subventricular zone of the lateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus (Kuhn et al., 1996; Zhao et al., 2008; Ma et al., 2009). Many factors, including species, age of donor, the region dissected, cell concentration, ingredients of the media, and growth factors, all have critical influences on the successful in vitro culture of NSC (Kornblum, 2007).

According to discrepancies in their origins and stages, they respond to epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or both; it seems, however, that their combined use might be more efficacious (Minger et al., 1996; Represa et al., 2001; Gregg and Weiss, 2003). Evidence is accumulating that a wide variety of neurotrophic factors are closely linked with the survival and proliferative activities of central nervous system-derived cells (Arsenijevic et al., 2001; Shimazaki et al., 2001; Chojnacki et al., 2008). Other than direct administration, localized production of growth factors from both normal and genetically modified fibroblast grafts has been developed that has led to an outgrowth of the neural cells (Tuszynski et al., 1994; Chalmers et al., 1995). The disaggregated cells are typically cultured as suspended spheres or on a matrix as substrate for binding, and they can be induced to differentiate into neural cells in vitro by withdrawing the mitogens or by exposing them to other factors (Gage, 2000; Rietze and Reynolds, 2006; Chojnacki and Weiss, 2008; Deleyrolle et al., 2008; Deleyrolle and Reynolds, 2009).

Neural stem cells from the mouse, rat, human, and several other mammals have been isolated and cultured successfully (Shihabuddin et al., 1997; Wilson and Ed-

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<sup>1</sup>These authors contributed equally to this work.

<sup>2</sup>Corresponding authors: weijunguan301@gmail.com, Yuehui\_Ma@hotmail.com

lund, 2001), and studies using these models have given rise to many interesting findings (Chuang, 2010; Makri et al., 2010). A large body of evidence suggests that the NSC, even distal to the lesion epicenter, preferentially migrate to the damaged areas when grafted into the injured or maldeveloped cellular milieu of afflicted patients (Park et al., 2002; Nakamura et al., 2005; Okano et al., 2007).

Many factors, such as a lack of awareness or negligence regarding the therapeutic potential of NSC, such as unwillingness to investigate animals other than the model species and the difficulty of operating on the delicate bird cytoarchitecture, are all responsible for the lag in bird neurology of 2 decades or so compared with that of mammals. As a result, few studies, if any, of bird NSC have been reported heretofore. Hence, it is reasonable to anticipate that progress in birds, which are rarely involved, would explain the nature of their stem cells from a different perspective. This research explored the isolation and culture system of duck cortex-derived NSPC and profiled their distinctive biological features, thereby providing a theoretical and experimental basis for future investigations regarding avian neurogenesis.

## MATERIALS AND METHODS

### **Isolation, Purification, and In Vitro Expansion of Duck NSPC**

Bird experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School. Ten- to 13-d-old duck (*Anseriformes anatidae*) embryonic brains were isolated and rinsed 3 times and then placed in precooled normal saline water. The dorsal ventricular ridges of the brain were isolated, rinsed, and transferred to complete neural stem cell media [1:1 Dulbecco's modified Eagle medium:nutrient mixture F-12 (**DMEM/F12**, Gibco, Carlsbad, CA); 2% B27 supplement (Gibco); 20 ng/mL of EGF and bFGF (PeproTech, Rocky Hill, NJ); 100 U/mL of ampicillin (Harbin Pharmaceutical Group Holding Co. Ltd., Harbin, China); and 100 µg/mL of streptomycin (Harbin Pharmaceutical Group Holding Co. Ltd.)], cleaved into 1.0 mm<sup>3</sup> pieces, and pipetted repeatedly to prepare a homogeneous monoblast suspension, which was subsequently filtered through 400- and 800-mesh sieves, in that order. The entire operation was performed under a low temperature to protect the cortex tissues. The cells were plated in flasks at a concentration of  $2 \times 10^5$  cells/mL and were cultured in a humidified incubator with 5% CO<sub>2</sub> at 37°C. Media were half refreshed every 2 to 3 d.

The cells were subcultured when the neurosphere diameter was approximately 200 µm. After the culture was kept still for 10 min, the supernatant half media

were removed to eliminate monoblasts and dead cells. After the culture was centrifuged at  $219 \times g$  at room temperature for 5 min, 500 to 1,000 µL of trypsin solution (0.125% in PBS; Gibco) was added according to the size of the pellet. The mixture was pipetted repeatedly and incubated in a water bath at 37°C for 5 to 10 min, centrifuged at  $219 \times g$  at room temperature for 5 min, resuspended, and cultured in complete NSC media at a concentration of  $2 \times 10^5$  cells/mL.

### **Cryopreservation and Apoptosis Detection**

Cryopreservation, recovery, and viability assessment were performed as described previously (Wu et al., 2008). The cells were stained with an Annexin V-fluorescein isothiocyanate (**FITC**) Apoptosis Detection Kit I (BD, Franklin Lakes, NJ), and 5 visual fields were selected randomly to calculate the rates of apoptosis and necrosis.

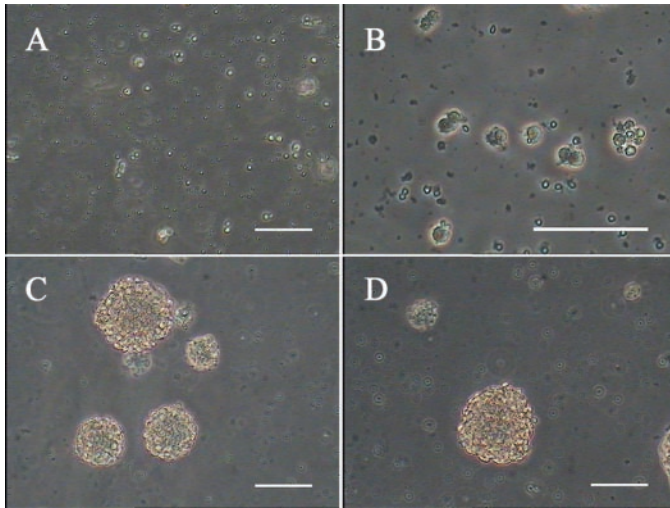
### **Neural Colony-Forming Cell Assay**

Primary and passage 2 neurospheres were harvested, dissociated, and subjected to serial dilution. A single cell in 0.5 mL of culture media was plated per well onto 96-well microplates. Colonies were counted 72 h later.

### **Immunofluorescence**

Indirect immunofluorescence was used to identify the NSPC by the specific marker nestin (Osborn, 2006). Neurospheres of passage 2 were dissociated and plated at a concentration of  $2 \times 10^5$  cells/mL onto poly-L-lysine-coated (Sigma, St. Louis, MO) 96-well microplates, and cultured in DMEM/F12 media supplemented with 10% fetal bovine serum (HyClone, South Logan, UT) to induce differentiation. After 7 d, specific markers of neural cells, including neuron-specific enolase (**NSE**), glial fibrillary acidic protein (**GFAP**), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (**CNP**) were detected by indirect immunofluorescence.

The media were removed, and the cells were fixed in 4% paraformaldehyde for 15 min and then washed in PBS 3 times. The cells were blocked by 10% normal goat serum (Zhongshan Goldenbridge, Beijing, China) for 30 min at room temperature. The cells were subsequently incubated in 3% BSA containing rabbit anti-nestin, NSE, GFAP, and CNP (1:200, Beijing B&M Biotech Co. Ltd., Beijing, China) for 1 h at room temperature and then washed 3 times (10 min each) with PBS. The cells were then incubated in PBS containing FITC-conjugated goat anti-rabbit secondary antibody (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA) for 1 h at 37°C and washed 3 times with PBS. 4',6-Diamidino-2-phenylindole was added as a counterstain, and staining was observed immediately with a confocal microscope (TE-2000-E, Nikon Inc., Tokyo, Japan).



**Figure 1.** Morphology of duck neural stem and progenitor cells (NSPC). (A) Primary NSPC, exhibiting suspended round shapes, clear boundaries, and high refractivity; (B) NSPC in the dividing phase; (C) neurospheres of passage 2; (D) neurospheres generated from resuscitated NSPC at d 7. Scale bars = 50  $\mu$ m.

### Karyotype Analysis

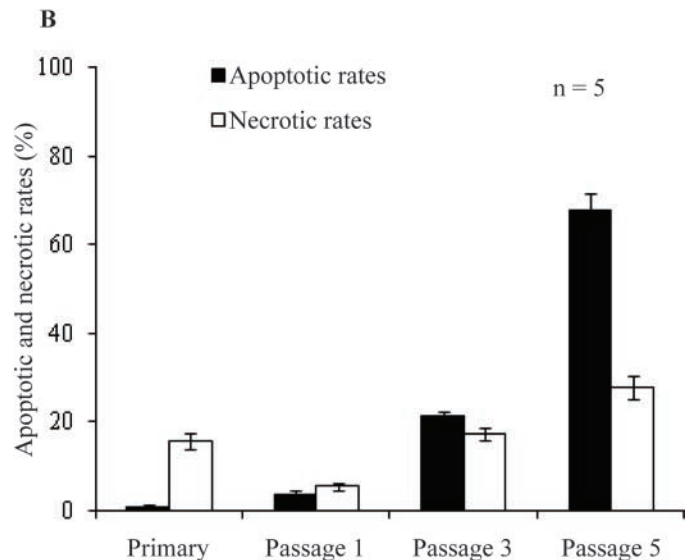
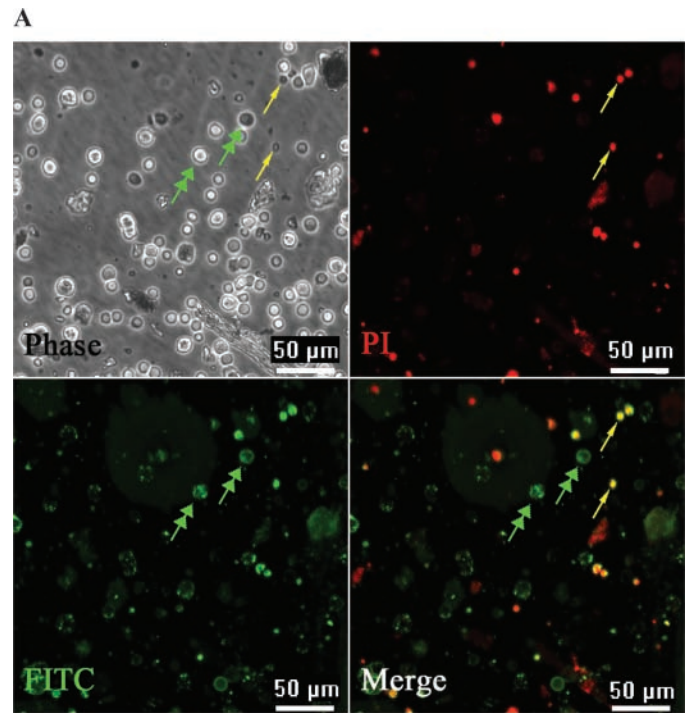
Karyotype and chromosome staining were performed as described previously (Suemori et al., 2006). The proportion of diploid cells was calculated according to results from 100 spreads. Chromosome parameters were calculated as described previously (Levan et al., 1964).

### Acetylation Analysis

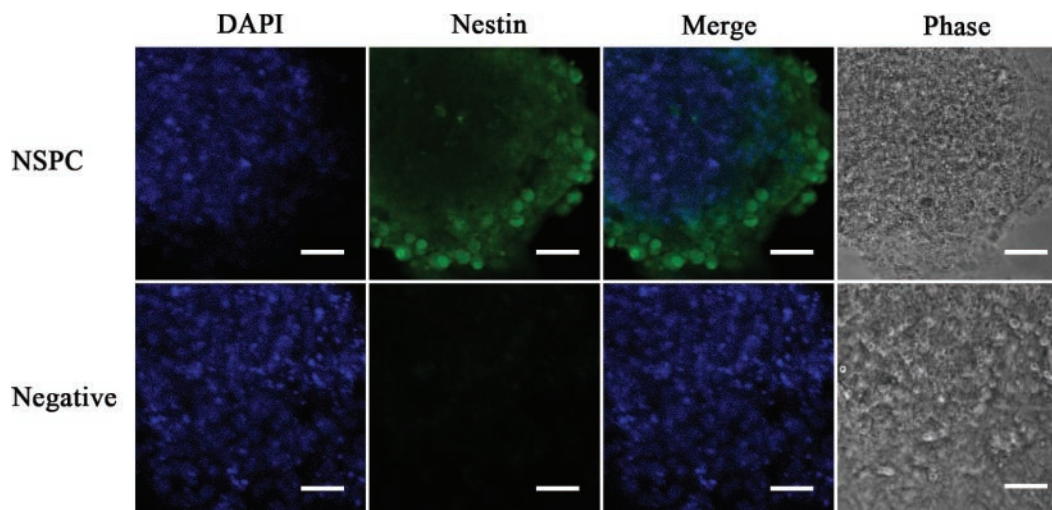
Anti-histone acetylated histone 4 lysine 12 (acH4K12; 1:200; Santa Cruz) and FITC-conjugated mouse-to-rabbit secondary antibody (1:100, Santa Cruz) were used to analyze the acetylation level. The immunofluorescence protocol was the same as that for identification. The software EZ-C1 3.70 FreeViewer (Nikon Inc.) was used to calculate the fluorescence intensities of acH4K12 and PI, and the ratio of the former to the latter was recorded and analyzed with SPSS 13.0 software (SPSS Inc., Chicago, IL) for paired *t*-test.

### Expression of Exogenous Genes

Duck NSC from passage 2 were plated on poly-L-lysine-coated 24-well microplates and transfected with plasmid DNA (pEGFP-N3, pEYFP-N1, and pDsRed1-N1, where pEGFP-N3, pEYFP-N1, and pDsRed1-N1 refer to plasmids encoding the red, green, and yellow fluorescent genes, respectively; Clontech, Mountain View, CA) by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as described previously (Escriviou et al., 2001; Tsuchiya et al., 2002). The cells, observed at 24, 48, and 72 h upon transfection, were detected for the expression of fluorescent proteins, with excitation wavelengths of 488 nm (pEGFP-N3), 488 and 543 nm (pEYFP-N1), and 543 nm (pDsRed1-N1), respectively.



**Figure 2.** Apoptosis detection of in vitro-cultured duck neural stem and progenitor cells (NSPC). (A) Apoptosis detection of duck NSPC in passage 3 (scale bars = 50  $\mu$ m); (B) apoptotic and necrotic rates of duck NSPC. Apoptotic cells have externalized phosphatidylserine (PS) and intact membrane so as to be dyed with Annexin V-fluorescein isothiocyanate (FITC; green fluorescent; BD, Franklin Lakes, NJ) and to exclude propidium iodide (PI; red fluorescent), whereas necrotic cells possess both externalized PS and permeable membrane and, consequently, could be stained with both Annexin V-FITC and PI and display a merged fluorescence (yellow). Double arrows (green) point to apoptotic cells, and single arrows (yellow) point to necrotic cells. The apoptosis analysis suggested there was a degenerative trend in the entire population as serial passage progressed further. Color version available in the online PDF.



**Figure 3.** Expression of nestin in duck neural stem and progenitor cells (NSPC). Because the neurospheres are heterogeneous clusters of neural stem and progenitor cells per se, the fluorescence intensity and positive cell distribution within them are not homogeneous. The results suggested that bona fide neural stem cells existed in the uncommitted neurospheres. The negative control was prepared in the same manner, except that the step of primary antibody incubation was omitted. DAPI = 4',6-diamidino-2-phenylindole. Scale bars = 20  $\mu$ m. Color version available in the online PDF.

Computer-aided design software (CAD 2009, ZWsoft Inc., Guangzhou, China) was used to determine the transfection efficiencies by calculating the fluorescent areas of positive cells.

## RESULTS

### *In Vitro* Culture of Duck NSPC

The primary duck NSPC grew in suspension and exhibited a round shape, with clear boundaries and high refractivity (Figure 1A). Four to 5 d later, a large number of neurospheres had diameters up to 80 to 100  $\mu$ m. The secondary neurospheres were subcultured. Some single NSPC were in the dividing phase after 24 h (Figure 1B) and then formed tertiary neurospheres, which grew to a size of 80 to 100  $\mu$ m after 7 to 9 d (Figure 1C). The duck NSPC could be subcultured to passage 5 at most.

Cryopreservation had a slight effect on the NSPC. The viabilities were  $87.57 \pm 0.08\%$  and  $53.38 \pm 0.07\%$  before and after cryopreservation, respectively. The resuscitated NSPC began to proliferate after 24 to 72 h and formed neurospheres of 80 to 100  $\mu$ m 7 to 10 d later (Figure 1D). Apoptosis detection showed that in vitro-cultured NSPC exhibited higher apoptotic and necrotic rates as the passage number and culture time increased (Figure 2).

### Neural Colony-Forming Cell Assay

The colony-forming rate of primary NSPC was lower than that of passage 2 NSPC (Table 1). We observed that some cells divided and formed small colonies after 3 d, and then formed large colonies 7 to 10 d later. The disaggregated cells were able to form secondary neurospheres.

### Identification and Induced Differentiation

The neurospheres of passage 2 were detected by immunofluorescence, and the results showed that the uncommitted cells were nestin positive (Figure 3). On induced differentiation in DMEM/F12 media supplemented with 10% fetal bovine serum on 96-well microplates coated with poly-L-lysine, the NSE, GFAP, and CNP were expressed in certain groups of the population at d 7 (Figure 4).

### Karyotype Analysis

The genome of duck NSC was composed of  $2n = 78$  chromosomes, including 8 pairs of macrochromosomes and 31 pairs of microchromosomes with sex chromosomes Z and W, with the ZZ type (Figure 5) for the male and the ZW type for the female. The chromosome parameters are listed in Table 2. Karyotype analysis indicated that 95% of the NSC were diploid.

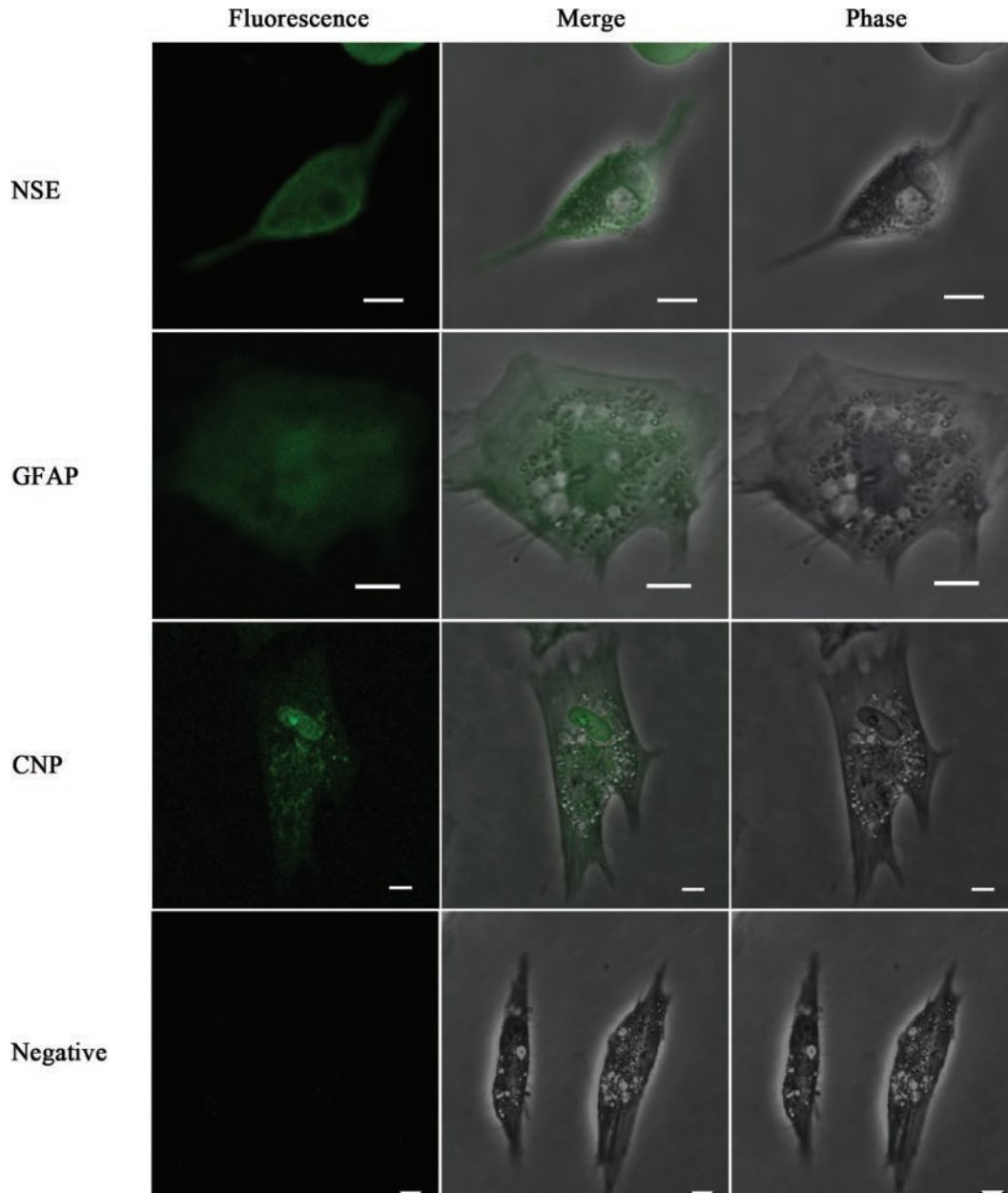
### Acetylation Level

Results suggested that the acetylation level of duck NSPC was significantly higher than that of the fibroblasts ( $P < 0.05$ ; Figure 6), implying that NSPC might be better candidates for donor cells in nuclear transfer.

**Table 1.** Neural colony-forming cell assay

Passage no.	Wells <sup>1</sup>	Colonies	Colony-forming rate (%)
Primary	459	4	0.87
2	421	39	9.26

<sup>1</sup>Well number represents the number of wells in which a single cell was plated.



**Figure 4.** Differentiation of the duck neural stem and progenitor cells (NSPC) in Dulbecco's modified Eagle medium:nutrient mixture F-12 (DMEM/F12, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum. Indirect immunofluorescence suggested that the NSPC had given rise to 3 kinds of differentiated progeny, namely, neurons, astrocytes, and oligodendrocytes. The negative control was prepared without incubation of primary antibodies. NSE = neuron-specific enolase; GFAP = glial fibrillary acidic protein; CNP = 2',3'-cyclic nucleotide 3'-phosphodiesterase. Scale bars = 10  $\mu$ m. Color version available in the online PDF.

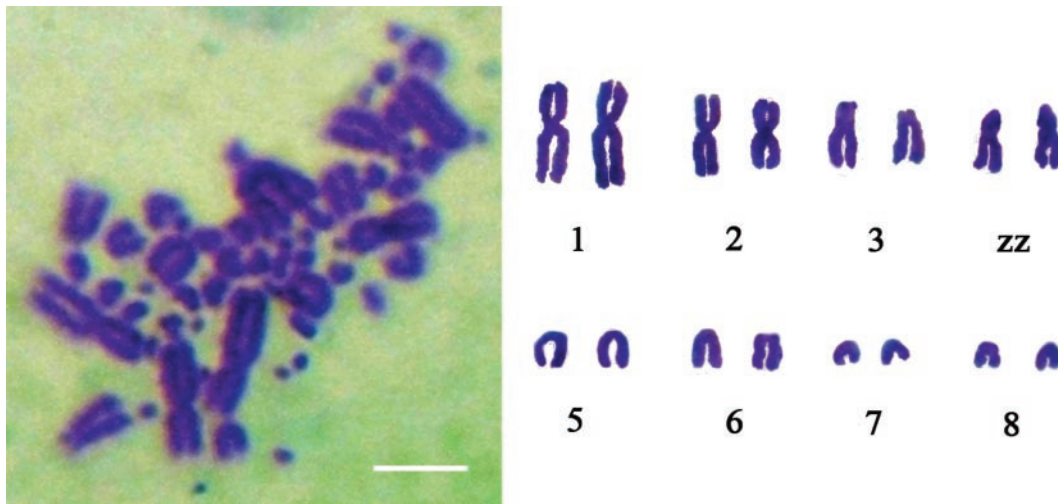
### Expression of Exogenous Genes

The expression levels of pEGFP-N3, pEYFP-N1, and pDsRed1-N1 were maximal at 48 h (Figure 7). In addition, the transfection efficiencies of the yellow (pEYFP-N1,  $16.45 \pm 1.45\%$ ) and red (pDsRed1-N1,  $12.74 \pm 1.72\%$ ) fluorescent proteins were significantly lower ( $P < 0.05$ ) than those of the green fluorescent proteins (pEGFP-N3,  $23.56 \pm 1.99\%$ ).

### DISCUSSION

In this research, trypsin disaggregation and mechanical isolation, both with innate drawbacks if applied

alone, were adopted in a synergic manner to obtain subcultured NSPC with higher viability and division potential. Apoptosis detection further illustrated a trend of degeneration in the process of serial passage. Evidence in a previous study showed that fibroblast growth factor-2 + EGF in adherent culture completely suppressed the differentiation of mouse NSC and efficiently sustained their long-term expansion (Conti et al., 2005). The system used here, supplemented with bFGF and EGF, supported ex vivo culture of duck NSPC with a limited number of passages and gave rise to some differentiated cells with neuron- or glia- like shapes. This might have resulted from a species differ-



**Figure 5.** A representative spread at metaphase (left) and the karyotype (right) of duck neural stem and progenitor cells (NSPC), ZZ type (♂). The genome of duck NSPC was composed of  $2n = 78$  chromosomes, including 8 pairs of macrochromosomes, as shown on the right, and 31 pairs of microchromosomes not detailed. The result that 95% of cells of the entire population were diploid potentially supported the genetic stability of the cultured cells. PI = propidium iodide; acH4K12 = acetylated histone 4 lysine 12 (H4K12). Scale bar = 5  $\mu\text{m}$ . Color version available in the online PDF.

ence or ineffective suppression of the lineage commitment and apoptosis within the neurospheres.

The neural colony-forming cell assay, a reliable protocol for the enumeration of NSC based on their proliferative potential (Louis et al., 2008; Marshall Li et al., 2007), was adopted, and the results suggested that the NSPC possessed the capacity for self-renewal in vitro. The frequency of NSC increased with serial passage processing until a balance between purification and differentiation was achieved (Deleyrolle et al., 2008), so passage 2 had a higher colony-forming rate.

In this research, the expression of NSE, GFAP, and CNP on the mitogens withdrawn implied that the NSPC had developed into neurons, astrocytes, and oligodendrocytes and, consequently, verified their multilineage differentiation capacity. Nonetheless, the molecular mechanisms underlying how the NSPC exit the stem cell state remain to be elucidated.

Ample evidence suggests that a high acetylation level and demethylation could dramatically improve the efficiency of reprogramming and significantly promote the developmental rates of embryos in vitro (Rybouchkin et al., 2006; Wee et al., 2007; Ma et al., 2008), and that

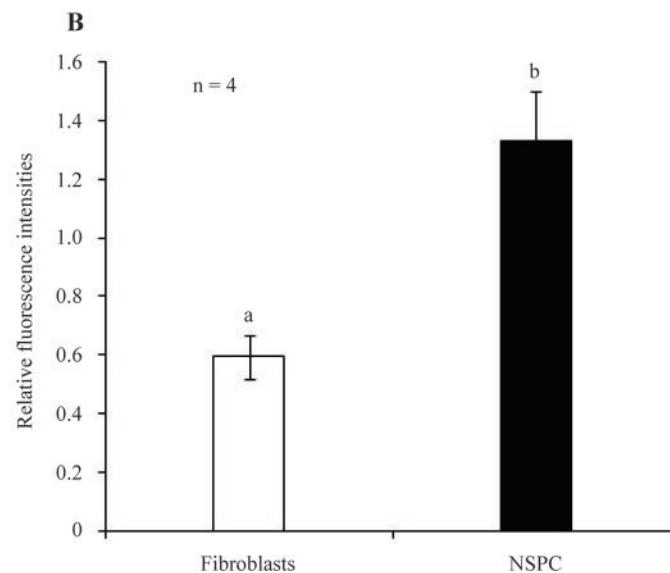
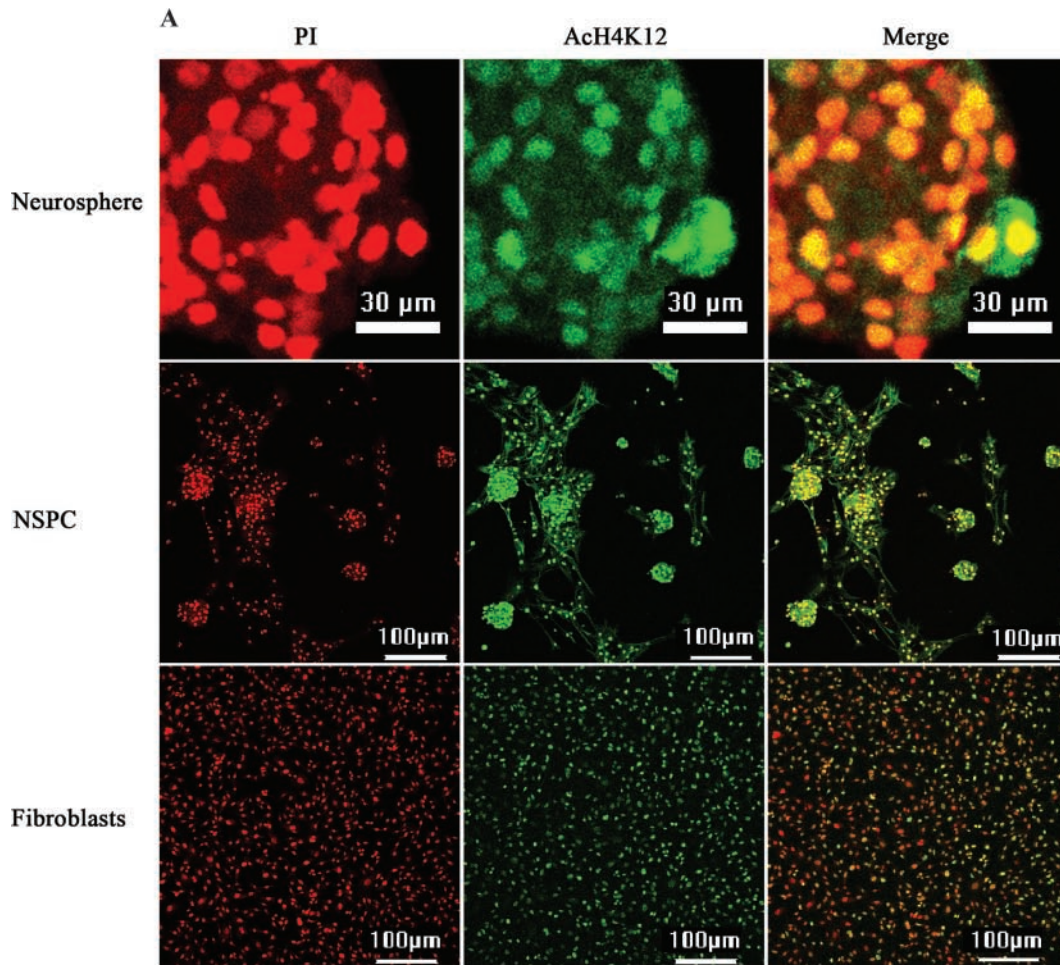
the differentiation plasticity of neural cells is in part regulated by epigenetic mechanisms intrinsic to the cells (Nakashima et al., 2009; Hsieh and Eisch, 2010). The acetylation level of duck NSC was significantly higher than that of the fibroblasts, suggesting that they are at an early stage of the differentiation repertoire.

Neural stem or progenitor cells from human, rodents, and other mammals could be transfected via electroporation, lipofection, or other nonviral delivery vehicles, or could be transduced with adenovirus vectors or lentiviral vectors, (Geraerts et al., 2006; Wang et al., 2007; Barnabé-Heider et al., 2008; Kim et al., 2009; Kwon et al., 2010), for the purposes of genetic modification and investigations of the state and fate commitment of stem cells. Consistent with this, the duck NSPC were efficiently transfected with 3 fluorescent and displayed intensified fluorescence, and the transfected cells showed regular morphology and turgor vitalis cytoplasm, with no perceivable difference in growth. Therefore, their isolation and culture provided novel opportunities for research on neurogenesis, tumorigenesis, genetic intervention, and transgenic therapeutics.

**Table 2.** Chromosome parameters of the duck neural stem and progenitor cells, ZZ type (♂)

Chromosome no.	Relative length (%)	Arm ratio	Centromere index (%)	Centromere morphology <sup>1</sup>
1	26.45 ± 0.63	1.78 ± 0.03	34.67 ± 0.51	SM
2	18.67 ± 0.96	1.54 ± 0.05	42.37 ± 0.22	M
3	11.95 ± 0.23	—	—	T
4 (Z)	10.42 ± 0.34	3.67 ± 0.04	21.57 ± 0.35	ST
5	8.67 ± 0.09	—	—	T
6	7.85 ± 0.14	—	—	T
7	5.83 ± 0.06	—	—	T
8	4.35 ± 0.03	—	—	T

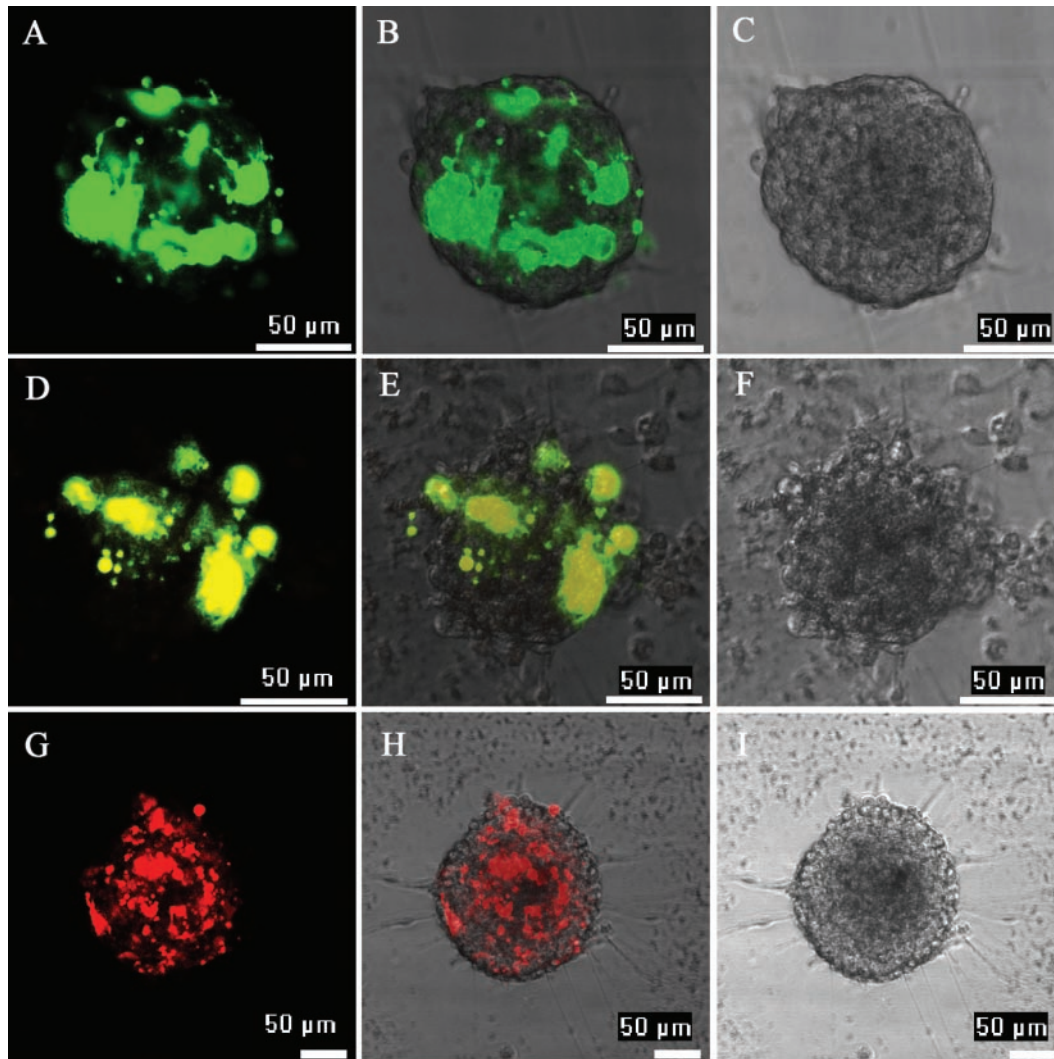
<sup>1</sup>M = metacentric chromosome; SM = submetacentric chromosome; ST = subtelocentric chromosome; T = telocentric chromosome.



**Figure 6.** Acetylation levels of the duck neural stem and progenitor cells (NSPC) and the fibroblasts. (A) Immunofluorescence for acetylated histone 4 lysine 12 (acH4K12) in NSPC and fibroblasts. Counterstaining with propidium iodide (PI) was performed to visualize the cellular DNA. Scale bars represent 30, 100, and 100  $\mu\text{m}$  in the figures showing neurospheres (floating aggregates), NSPC (monolayer cultured on microplates coated with poly-L-lysine, Sigma, St. Louis, MO), and duck fibroblasts, respectively. (B) Relative fluorescence intensities of the duck NSPC and the fibroblasts. Different letters (a, b) signify a statistically significant difference ( $P < 0.05$ ). Color version available in the online PDF.

In summary, duck embryonic NSPC were successfully isolated and purified through serial passage. The cells possess the capacity for self-renewal, are nestin positive, and gave rise to differentiated progeny, including neu-

rons, astrocytes, and oligodendrocytes, with mitogens withdrawn. As the passage number increased, a degenerative trend was observed, as reflected by increasing apoptotic ratios. Future work should focus on creating



**Figure 7.** The expression of pEGFP-N3, pEYFP-N1, and pDsRed-N1 (Clontech, Mountain View, CA) in duck neural stem and progenitor cells at 48 h posttransfection. (A to C) Cells transfected with pEGFP-N3; (D to F) cells transfected with pEYFP-N1; (G to I) cells transfected with pDsRed-N1. The expression of the 3 types of exogenous fluorescence genes was optimal at 48 h, and the transfected cells exhibited no obvious difference in morphology and proliferation compared with controls. The pEGFP-N3, pEYFP-N1, and pDsRed-N1 refer to plasmids encoding the red, green, and yellow fluorescent genes, respectively. Scale bars = 50  $\mu$ m. Color version available in the online PDF.

a better cellular niche to increase the passage numbers and reduce their apoptotic rate to realize the ultimate *in vitro* culture of avian NSC.

## ACKNOWLEDGMENTS

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