

Investigation of Characteristics of Feather Follicle Stem Cells and Their Regeneration Potential

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Feather follicles have the extraordinary ability to regenerate and undergo molting cycles. Being tissue-specific stem cells, feather follicle stem cells (FFSCs) have a strong capacity for proliferation and are presumed to be progenitor cells for various epidermal organs. In order to characterize FFSCs and to understand how the feather epidermis and FFSCs produce such a reliable differentiation program resulting in the formation of complex feathers, We developed a culture scheme to select and expand FFSCs from chick feather follicles. FFSCs were examined with cell profiles, multipotential differentiation and immunocytochemical staining. FFSCs from a single clone were capable of self-renewal and proliferation. These cells expressed integrin β 1, CD49c, cytokeratin 15 (K15), cytokeratin 19 (K19) and a neural-genic cell marker, nestin, but not a terminal differentiation-related keratinocyte marker, cytokeratin 10 (K10). FFSCs could trans-differentiate into adipocytes, neurocytes and keratinocytes. The formation of micro-feather like structures *ex-vivo* also revealed the potential of regeneration. These results demonstrate that FFSCs possess the properties of stem/progenitor cells and may therefore serve as a useful model for studying mechanisms of stem cell differentiation and their involvement in organ regeneration.

Keywords: Feather follicle stem cells, trans-differentiation, regeneration

Introduction

In comparison to mammalian hair, feather regeneration involves more complex physiological and cellular metabolic mechanisms in regard to many aspects such as growth rate, expansion ability, keratinization, and the formation of diverse patterns, colors and shapes. The prominence and relative ease of access of feather follicles make them clear targets for the study of stem cells and the investigation of regeneration mechanisms [1-3]. In the past decade, a large amount of phenomenological information has been gathered through electron microscopy studies on the ultrastructure of embryonic down feathers including many details of cell organization and keratinization [4-7]. Feather follicles have been used as a model system for *Evo-Devo* research in recent years, in which five successive morphological changes have been defined to occur in feather recycling [8-10].

Feather follicle stem cells (FFSCs) are presumed to be mainly responsible for the rebuilding of epithelial tissues[11]. Once activated, they can generate proliferating progenies, from which required cell lineages are differentiated and particular tissues are constructed.

By mapping follicle stem cells *in vivo*, FFSCs are defined as the label-retaining cells (LRCs) locating in anatomically distinct regions of feather follicles and maintaining homeostasis between the collar and the ectoderm of the dermal papilla during the feather regeneration cycle. LRCs possess multi-potentiality and can be molded into different feather forms [12-13]. Our previous studies on FFSCs demonstrated that feather keratinocytes appeared as colony-forming cells and were capable of delivering exogenous genes[2].

In order to define FFSCs and to clarify the transition forms of stem cells involved in the successive morphological changes. We developed a novel technique for the isolation and long-term culture of FFSCs. We demonstrated that FFSCs were very flexible cell types, from which multilineage cells could derive, in a manner beyond the principle of general plasticity of adult stem cells. This study advances our understanding of feather regeneration, also adding to our knowledge of more general epithelial regeneration for other taxa.

Material and methods

Chemicals

Epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-I (IGF-I), dexamethasone, indomethacin, 3-isobutyl-1-methylxanthine, hydrocortisone, collagenase II, collagen IV, 100× penicillin–streptomycin solution (PS), Oil Red O, bovine serum albumin (BSA), and RNase were purchased from Sigma (Hangzhou, China). DMEM, trypsin/EDTA, and propidium iodide (PI) were from GIBCO (Hangzhou, China). Fetal calf serum (FCS) was from PAA (Hangzhou, China). Anti-chicken integrin β 1 was from Chemicon International Inc. (Temecula, CA, USA). Anti-mouse CD49c was from YLEM s.r.l. (Rome, Italy). Mouse anti-cytokeratin 15 and mouse anti-cytokeratin 19 were from BD Biosciences (Beijing, China). Mouse anti-cytokeratin 10, anti-nestin, anti-neuron specific enolase, anti-glial fibrillary acidic protein, Cy3-, FITC-conjugated secondary antibody and DAB kit were from Boster (Wuhan, China), and the AP-BCIP/NBT system was from Genmed Scientifics Inc. (Shanghai, China).

Isolation and culture of FFSCs

Feather follicles were carefully dissected from wings as Xi *et al* described [2]. Fragments containing the pulp and sheath were removed, leaving tissue mainly comprising of collars. Analysis of paraffin sections was performed to verify the identity of the collars as described previously [14]. Samples were washed three times in Hanks balanced salt solution. The tissue was then sequentially digested with 0.125% collagenase type II for 12 h at 4°C and 0.25% trypsin&0.02% EDTA for another 20 min at 37°C. Collected cells were suspended in the growth medium comprised of DMEM, 10% FCS, 100 U/ml penicillin/streptomycin, 4 ng/ml EGF and 4 ng/ml bFGF, and seeded onto a collagen IV-coated plate. Cell cultures were treated with two schemes. In an early selection scheme, non-attached cells were discarded after 30 min of initial incubation. In a later selection scheme, seeding cells were cultured for 2 days before any non-attached cells were discarded. Cells at 90% confluence were harvested using 0.25% trypsin&0.02% EDTA and cloned using the limited dilution method for cell line establishment [15]. To determine cell growth, cells at passage 1, 3 and 10 from the early selection scheme were seeded at a density of 3×10^4 cells/ml. Cells in each of the three wells were collected for counting at each 24 h interval.

The experimental research on animals followed the recognized guidelines approved by Laboratory Animal Management Committee of Zhejiang University.

Flow cytometry

Cells ($\times 10^6$) at passage 3 were treated with 100 μ g/ml RNase and then incubated with PI. Flow cytometric analysis was performed using a BD FACScalibur instrument (Becton Dickinson, Shanghai, China).

Multilineage differentiation of FFSCs

FFSCs at passage 3 were analyzed for their differentiation potential into adipocytic, neurocytic and keratinocytic lineages for at least three replicates. Non-induced FFSCs at passage 3 served as a control group. For neurogenic differentiation, cells at 70–80% confluence were exposed to a pre-induction medium containing DMEM with 10 ng/ml bFGF for 24 h and were then transferred to a complete induction medium containing DMEM, 100 ng/ml bFGF and 100 ng/ml EGF. Cells in each of three wells were analyzed by immunoassay and RT-PCR at given intervals. For adipogenic differentiation, FFSCs at 80–90% confluence were exposed to an induction medium containing DMEM, 10% FCS, 10 ng/ml IGF-I, 100 μ M indomethacin, 1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 100 ng/ml bFGF and 100 ng/ml EGF. Cells in each of three wells, collected at 24 h intervals, were examined for triglyceride production using Oil Red O staining. The efficiency of transformation was determined from the absorbance of Oil Red O at 510 nm. Cells induced for 5 days were analyzed with RT-PCR to verify the expression of peroxisome proliferator-activated receptor gamma (PPAR γ) by adipocytes and were subjected to double staining with Oil Red O and anti-cytokeratin 15. For keratinogenic differentiation, FFSCs at 90% confluence were cultured in DMEM with 10% FCS, 100 U/ml penicillin, 100 U/ml streptomycin, 4 ng/ml EGF, 4 ng/ml bFGF, together with the addition of 1.5 mM Ca^{2+} . Cells induced for 5 days were used for immunocytochemical analysis.

Immunoassays

The keratinocyte cellular markers, integrin β 1, CD49c, cytokeratin (K15) and cytokeratin (K19) were selected to define FFSCs according to their specific expression between the putative FFSCs isolated from the early selection scheme and the cells obtained from the later selection scheme. Cells at passage 3 were subjected to immunocytochemical staining under the manufacturers' instruction with minor modifications. Briefly, cells were fixed with 4% paraformaldehyde for 60 min, rinsed with PBS, soaked in the mixture of 30% hydrogen peroxide (Sinopharm Chemical Reagent Co., Ltd) and 99% methanol (1:50) for 30 min to deactivate endogenous peroxidase, and then washed with distilled water twice and blocked with 10% BSA for 30 min. Following incubation with the primary antibody for 1 h at 37°C, the cells were treated with an ELIVisionTM plus kit and colored with DAB kit per manufacturers' protocols and visualized by the reverted light microscope (Nikon, Shanghai, China). The following primary antibodies were used: anti-integrin β 1, anti-CD49c, anti-K15 (1:100), anti-K19 (1:100) and anti-K10 (1:100).

For immunoassays of cells induced by neurogenic culture media, we performed fluorescence double staining at representative periods to detect the expression of nestin and K15 in FFSCs. Cells were treated with both primary

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antibodies. Secondary antibodies conjugated to FITC and Cy3 were used for fluorescence detection followed by counterstaining with DAPI to reveal nuclei.

In order to further track the neural lineage differentiation cells, cells were fixed and incubated with anti-nestin (1:100), anti-neuron specific endolase (NSE) (1:100), anti-glial fibrillary acidic protein (GFAP) (1:100). Anti-K10 (1:100) antibody was used to verify the keratinocyte lineage differentiation. All samples were treated with an ELIVision™plus kit and colored with DAB kit as above. To measure adipogenesis, cells were immunostained with anti-cytokeratin 15 (1:100) after Oil Red O staining.

RT-PCR

The expression levels of nestin, PPAR γ and β -actin (as an internal control) were examined by semiquantitative RT-PCR. Total RNA was extracted using TRIZOL according to the manufacturer's instructions, and then used for cDNA synthesis. The RNA concentration was determined using a Quanta plate reader. The following primers were used: nestin (GenBank ID 395890) (213 bp), 5'-CTG GCA GGG ACA ACC TGT AT-3' and 5'-CTG TTT CTC CTC ATG C-3'; PPAR γ (GenBank ID 373928) (169 bp), 5'- GAG ATC GCC CAG GTT TGT TA-3' and 5'-TGC ACG TGT TCC GTT ACA AT-3'; β -actin (GenBank ID 396526) (239 bp), 5'-GCT GTG TTC CCT TCC ATT GT-3' and 5'-GCC TCA GTA AGC AGG ACA GG-3'. PCR was performed in a 20 μ l mixture containing 1 \times PCR buffer, 0.5 U of AmpliTaq-Gold enzyme, 0.2 mM of each dNTP, 1.5 mM MgCl $_2$, 0.2 μ M of each primer and 2 μ l of each RT product as a template. PCR products were then loaded on a 2% agarose gel and subjected to electrophoresis. The relationship between the nestin fluorescence intensity and induction time was also analyzed using Image-Pro Plus software (version 5.0.2).

Observation of 3-dimension structures in FFSCs cultures

Cells collected from feather follicles were seeded into a collagen IV-coated 6-well plate at a density 1 \times 10 6 cells/ml and incubated for 2 days at 37°C in 5% CO $_2$. Then non-attached cells were discarded by replacement with fresh medium. Cells at sub-confluence were harvested and seeded into a 6-well plate at a density 1 \times 10 5 cells/ml for ex vivo expansion until passage 2. Cells at passage 3 were immunostained with anti-integrin β 1, CD49c, K15, K19 and K10 antibodies as above. Then the harvested cells were seeded in a 6-well plate at a density 5 \times 10 4 cells/ml and cultured for a further 3 weeks. Observation of the structural morphology was carried out under an inverted microscope (Nikon, Shanghai, China).

Statistical analysis

SPSS software (version 13.0) was used for statistical analysis. A *P* value less than 0.05 was accepted as statistically significant.

Results

Cellular characterizations

Paraffin sections of collars of feather follicles provided for primary materials in this study were showed as Figure.1A. Cells isolated from the collars were round or ovate with strong refractive properties. In the early selection scheme, non-attached cells were discarded 30 min after initial seeding and then attached cells incubated for a further 150 min, at which time some of the cells were triangular or polygonal in shape (Figure. 1B). A number of colonies formed and satellite cells associated with each colony grew radially after 3 days of culture. Growing colonies had a smooth, round perimeter with smaller satellite cells (Figure. 1C). The cells gradually adopted a cobblestone appearance after 7 days (Figure. 1D). Cells from a single colony showed a strong capacity for self-renewal and could be expanded successively for more than 3 months. In the control culture groups, cells selected from the later selection system showed a multiplicity of morphological cell types, abortive colonies for further passages. The two growth trends are similar but the growth potential is significantly different (*P*<0.05) (Figure. 1I-J).

Figure 1

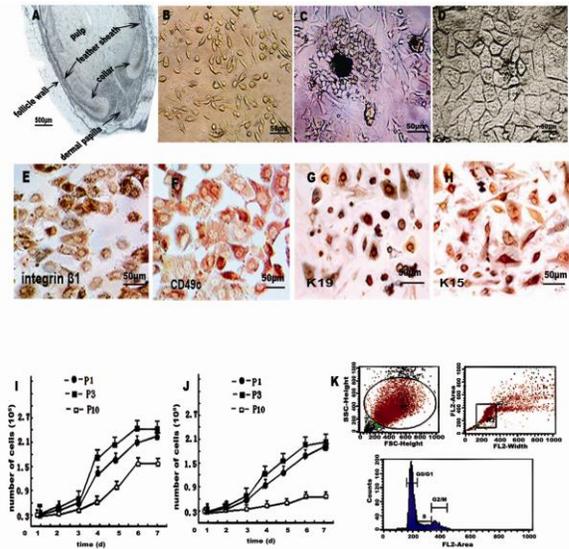


Figure 1. Cellular characteristics of FFSCs. Paraffin sections of feather follicle showed collar and dermal papilla, from which the putative FFSCs were isolated (A). Cells attached to the plate appear triangular or polygonal in morphology after culture for 150 min in the early selection scheme (B). Colonies with passage potential were intensively observed during the culture (C). Later, the cells at subconfluence showed a typical cobblestone shape (D). The growth curves for cells selected in the early and later selection schemes at passages 1, 3 and 10 are showing in (I) and (J). The two growth trends are similar but the growth potential is significantly different (*P*<0.05). Cell cycle analysis showed that in the early scheme, cells in G0/G1 phases were more numerous than cells in other phases (K). FFSCs were confirmed by the immunocytochemical staining with such cellular markers as integrin β 1 (E), CD49c (F), K19 (G) and K15 (H).

The cell cycle profile showed that $89.0 \pm 0.8\%$ of the cells from the early selection scheme existed in the G0/G1 phase, with $7.5 \pm 0.5\%$ of cells in the G2 phase, and $3.5 \pm 0.5\%$ of cells in the S phase (Figure. 1K). The result suggests that the high percentage of cells in the G0/G1 phase were in a relatively undifferentiated state.

The putative FFSCs obtained from the early selection scheme showed an intensive and higher levels expression of integrin $\beta 1$ and CD49c and the cellular proteins K15 and K19 as expected (Figure1. E-H). Cells collected with the late selection scheme were also immunocytochemically stained with anti-integrin $\beta 1$, CD49c, K15 and K19 antibodies. We found a few cells are positive to above examined markers, indicating the culture was a vigorous mixture, not only included a small number of FFSCs but also a major contamination of non-specific cells (data not shown).

Multiple lineage differentiation

Cells at passage 3 were used for examining their neurogenesis, adipogenesis and keratinogenesis potential.

In the neurogenesis culture, cells were treated with the complete neurogenic media for 5 h after pre-induction for 24 h. The morphology of cells appeared a polar, bipolar or multipolar in shape and interlinked to form network. Fluorescence double staining showed that a strong expression of K15 was found in FFSCs at the initial culture (Figure. 2A1) and during pre-trans-differentiation into neural cells (Figure.2A2). However, no such fluorescence was detected during trans-differentiation into neurons after induction for 5 h (Figure.2A3). On the other hand, the expression of nestin was detected either in some initial culture of FFSCs (Figure. 2B1), or during pre-trans-differentiation into neural cells (Figure. 2 B2) and after induction for 5 h (Figure. 2 B3). The nuclei revealed by DAPI were showed in the images (Figure. 2 C1-C3).

Figure 2

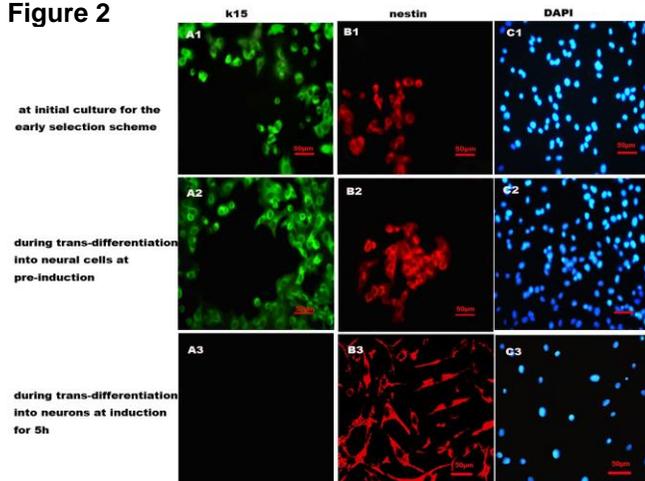


Figure 2. Fluorescence double staining for K15 and nestin at the different stage of neurogenesis culture. Both K15-positive and nesting positive cells appeared in the initial FFSCs culture (A1-B1). The similar situation was detected after pre-induction for 24 hr. When the induction lasted for 5 h, cells expressed no K15 (A5) but express nestin (B5). Images of C1-C3 show DAPI staining for the nuclei.

The DAB colorization assays showed that the expression of nestin in the cells were barely detectable after induction for 24 hr (Figure.3 A). However the cells were immunohistochemically positive to NSE and GFAP at this stage (Figure.3 B-C). The nestin expression in cells was examined by semi-quantitative RT-PCR in a time-dependent manner up to 24 hr. The result showed that the expression of nestin was undetectable beyond 18hr (Figure. 3I). The relationship between the fluorescence intensity of nestin expression and time of induction was analyzed (Figure. 3J). This showed that nestin expression gradually decreased as induction progressed.

In the adipogenesis culture, cells began to produce small, translucent intracellular vacuoles and then gradually filled the cytoplasm along the cell membrane after 16hr incubation with adipogenic medium. FFSCs then transformed from a cobblestone shape to a round or oval morphology with increasing refraction. Double staining for K15 and Oil Red O demonstrated that cells did not express K15 but were positive to Oil Red O, indicating that cells were differentiated into adipocytes (Figure. 3D).

Figure 3

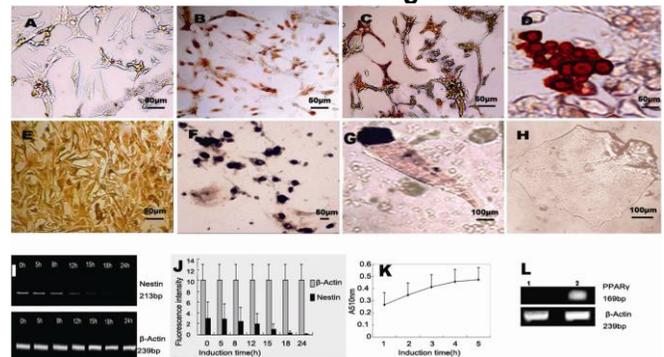


Figure 3. Multilineage differentiation of FFSCs and 3-dimension potential. During neurogenic differentiation, FFSCs derived cells appeared as progenitor cells with neural-like characteristics and neurocytes gradually. The cells appeared negative to nestin (A), but positive to GFAP (B) and NSE (C) at the stage of 24 hr induction. Induced cells were analyzed by RT-PCR for nestin mRNA expression during induction for 0–24 h; β -actin served as an internal control (I). The relationship between fluorescence intensity and induction time showed that nestin expression decreased gradually as induction continued (J). In adipogenesis culture, cells were induced for 5 days and typical mature fat morphology and lipid vacuoles showing strong lipid refraction were observed. After a double staining with Oil Red O and K15, lipid vacuoles showed orange or red but no blue color for K15, indicating that cells were differentiated into adipocytes (D). Relationship between triglyceride content and days following initial adipogenic induction were showed in (K). By RT-PCR, the mRNA expression of PPAR γ was not detectable in initial culture of FFSCs (L, lane 1) but in FFSCs-derived adipocytes (L, lane 2). In the assay of FFSCs differentiated into keratinocytes, cells changed from cobblestone shape to ovate one after induction for 2 days. Stratified epithelium and desmosomes were observed after 5 days. Induced cells were intensively positive to K10 (E). The formation of micro-structures was observed in cells obtained from the later selection scheme, not the early selection scheme. These structures such as cell clusters (F), hierarchical branches and micro-feather like structures with hierarchical branches (G) and multi-layered transformations of a keratinocyte sheet appeared (H).

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Expression of the PPAR γ gene in adipocytes induced for 5 days was detected by RT-PCR, as shown in Figure. 3L. The efficiency of adipogenesis was evaluated using the absorbance of Oil Red O at 510 nm. A positive relationship with cell density was clearly evident. In addition, the efficiency increased as induction progressed (Figure. 3K). In the keratinogenesis assay, cell morphology appeared a changing process from a cobblestone to oval or square shapes after induction for 2 days. A stratified epithelium, with dark keratin particles were observed after 5 days. A strong expression of K10 was obviously detectable (Figure. 3E).

Three -dimention potential

We approached both cultures in the early selection scheme and in the later selection scheme to analyze the potential of FFSC involvement in the formation of epidermal appendages. The attached cells expanded after 2 passages were then seeded at a density of 1×10^4 cells/ml and cultured for more than 3 weeks. We found that populations of organized cells could form, sometimes resembling asymmetrical downy-like feathers in the later selection scheme (Figure. 3F). These structures represent the hierarchical branches or multi-layered transformations of a keratinocyte sheet (Figure. 3 G-H). However, no such structures were observed in the cells selected from the early scheme. The cells from dermal papilla included in the later selection system might play a critical role in activating FFSCs to differentiate and form the organized structures.

Discussion

Stem cells in the epidermis can produce daughter cells to form the interfollicular epidermis and follicles. In rats, stem cells in follicles were able to generate all of the lineages of hair follicles and sebaceous glands [16]. Epidermal stem cells have the potential to differentiate into multi-lineage cell populations [17-21]. In avian species, an *in vitro* reconstitution system has been used to form feather buds or follicles and has revealed that the formation of individual feather is a self-organizing process which depends on the properties of the cells and the environment [22]. The whole feather is regenerated from a few stem cells [23]. The stem cells must involve in a multiple transition in different cell types.

We harvested FFSCs by postponing the selection of attached cells from feather follicles. Compared to the *ex vivo* expansion of keratinocyte stem cells in mammals, irradiated fibroblasts were required as a feeder layer [17-18, 24]. However, in our experiments, FFSCs collected in the early selection showed high potential for expansion *ex vivo* without any feeder layer and efficiently avoided being contaminated from fibroblasts, which happened previously [2]. The cells obtained from the early selection scheme showed cobble-like in morphology and were positive to integrin $\beta 1$, CD49c, K15, and K19 as the results of immunoasay demonstrated. The culture scheme for selection and expansion FFSCs from chick feather follicles was much more efficient than previous one, in which the developed fibroblasts in the culture might serve as a feeder layer 2. Notably, even cells purified in the early selection scheme still belonged to a stem cell mixture, with some cells expressing K15 and others expressing nestin

at identical stages (Figure 2). The critical properties for stem cells are self-renew and multi-potential. Stem cells might either maintain the characteristics of renewing by simple proliferation or undergo dividing asymmetrically, which in the later case the mixture of different cell lineages appears. In the present study, we first develop the early selection system to get relative pure FFSCs, and then we investigated the cellular characteristics for FFSCs. The results showed that FFSCs held self-renew potential up to 15-17 passages and were differentiated to neural, adipogenic, and keratinogenic lineages, demonstrating the stem characteristics of FFSCs. Additionally, FFSCs were immunocytochemically positive to integrin $\beta 1$ and CD49c as the properties of the keratinocyte colony-forming cells (KFCs). The corresponding immunoreactivity for such cellular markers as K15 and K19 in FFSCs also coincides with the results for human epithelial hair follicle stem cells, in which K15 is expressed at earlier stage than K19 [25-26]. It would be deceive to screen and identify more specific molecular markers in order to pure the feather follicle stem cells and to conduct further studies involved in the application of the stem cells and the establishment of organoid *in vitro*. The present study confirmed that FFSCs have multipotential to differentiate into adipocytes, neurocytes and keratinocytes under special culture conditions. We found that FFSCs can differentiate spontaneously into adipose tissue in culture. However, the differentiation was rare and inadequately detectable. Under adipose induction, most of the cells showed considerable morphological change and produced lipid vacuoles after 16h. This is in direct contrast to other reports on the adipogenic differentiation of mesenchymal stem cells. These required induction for 21 days, whereas FFSCs required only 5-day induction to achieve adipogenic morphology [27]. This might be related to a species- and/or cell type-specific difference and a difference in induction medium.

In the neurogenesis culture, on the one hand, the proportion of nestin-positive cells before induction was similar to that for induction of FFSCs into neural cells. This might because nestin-positive FFSCs hold the potential to trans-differentiate into neural cells whereas nestin-negative FFSCs could not tolerate the change in environment and turned to apoptosis. Moreover, nerve cells appeared in feather follicles during feather morphogenesis and what relationships exist between nestin-positive FFSCs and such nerve cells might be worthy of further study.

On the other hand, the neurogenesis of FFSCs evidenced a process of gradual differentiation. In the early stage, sufficient amount of cells appeared positive to nestin, but negative to NSE and GFAP (markers of neurons and astrocytes, respectively) [28-29]. As induction continued, nestin-positive cells decreased, however, NSE- and GFAP-positive cells increased. After 24 h, most of the cells were positive to GFAP or NSE but negative to nestin. Fluctuating expression of nestin, NSE and GFAP indicated that FFSCs experienced rapid but transitory expression of progenitor cells with neural-like characteristics during the early stage of induction and were then transformed into neural cells.

Ca $^{2+}$ is a key requirement for cell differentiation, particularly for keratinocyte differentiation, through its regulation of transglutaminase activation of the epidermis [30-31]. We

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found that FFSCs are sensitive to Ca²⁺ and a low Ca²⁺ concentration could promote FFSCs proliferation. However, Ca²⁺ concentrations >1.5 mM facilitated keratinogenic FFSCs differentiation and the formation of desmosomes and stratified epithelium. This suggests that the Ca²⁺ concentration might regulate both proliferation and keratinogenic differentiation of FFSCs.

In conclusion, we have developed a novel culture scheme to isolate FFSCs, which were characterized by the specific expression of such cellular markers as integrin β 1, CD49c, cytokeratin (K15), cytokeratin (K19). FFSCs could initiate multiple differentiation and organize 3-dimensional structures under appropriate culture conditions. It was inferred that FFSCs should possess the ability to form epidermal appendages under the influence of the paracrine effects of differentiated cells and cell-cell contact in a mixed culture. With the continuing development of skin tissue engineering, organ regeneration, gene therapy and other such research areas, FFSCs may become a highly useful cell model for the study of regeneration mechanisms, vaccine exploitation and avian-specific protein expression.

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