Chapter 17

Highly Efficient Neural Differentiation of CD34-Positive Hair-Follicle-Associated Pluripotent Stem Cells Induced by Retinoic Acid and Serum-Free Medium

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Abstract

Neural differentiation of hair-follicle-associated pluripotent (HAP) stem cells residing in the bulge area is a promising autologous source for stem cell therapy. In the present chapter, we describe the identification and enrichment of $CD34^+$ HAP stem cells by magnetic-activated cell sorting (MACS), and induce them to differentiate into neuronal and glial cells using defined neural-induction media. The different neural cell populations arising during in vitro differentiation from HAP stem cells are characterized by reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemistry assay.

Key words Hair follicle, Stem cells, Neural differentiation, Magnetic-activated cell sorting, $CD34^+$ hair-follicle-associated-pluripotent (HAP) stem cell

1 Introduction

Hair follicle stem cells were first identified by Cotsarelis et al. [1] in the bulge area of the hair follicles. These cells are slow cycling and retain label for a relatively long time [2]. They are an easily accessible source of stem cells and play a critical role in the replenishment of hair follicles [3], epidermis [4], and sebaceous glands [5].

In 2003, Li et al. [6] discovered stem cells in the bulge are of hair follicles that express the neural stem cells marker, nestin, and suggested the relationship of the nestin-expressing stem cells of the hair follicles and the brain. The nestin-expressing stem cells of the hair follicle were termed hair-follicle-associated pluripotent (HAP) stem cells [7].

Subsequent studies have shown that HAP stem cells can differentiate into keratinocytes, neural cells [8, 9], melanocytes, Schwann cells [10], and endothelial cells [11]. The different markers expressed in HAP stem cells include nestin [9], Lgr6 [12], CD34 [13], CD200, K19 [14], as well as CD34 [2]. The neural potential of HAP stem cells was first described by Li et al. [6] and

Robert M. Hoffman (ed.), *Multipotent Stem Cells of the Hair Follicle: Methods and Protocols*, Methods in Molecular Biology, vol. 1453, DOI 10.1007/978-1-4939-3786-8_17,

subsequently by Sieber-Blum [15] and Amoh [16]. Various neural inducers such as neuregulin 1 [10], NT-3 [17, 18], glial cell-derived neurotrophic factor, brain-derived neurotrophic factor [19], and RPMI-1640 containing 10% fetal bovine serum (FBS) [20] have been used. In the present chapter, we demonstrate that CD34-expressing HAP stem cells can be efficiently isolated from mouse vibrissae follicles by high-gradient magnetic-activated cell sorting (MACS).

Recent studies have shown that using of all trans-retinoic acid (RA) [21], serum-free medium [22], and chemical treatment such as with β-mercaptoethanol (BME), butylated hydroxyanisole (BHA), and dimethyl sulfoxide (DMSO) [23] can induce neural differentiation of embryonic stem cells and bone marrow stem cells. Recently, RA was found to enhance HAP stem cells to differentiate to motor neurons [24].

Thus, we have purified $CD34^+$ HAP stem cells from the mouse hair follicle, and then the neural differentiation potential of these cells was evaluated in the presence of RA, serum-free medium, and neural differentiation medium (NDM).

We found that the serum-free medium and 1 μmol/L RA accelerate neurogenesis in the HAP stem cells and NDM induces rapid changes in cell morphology that leads to cell death.

2 Materials

2.1 Hair Follicle Stem Cells Isolation and Cultivation

1. Female mice (3–5 weeks) (Razi Institute, Karaj, Iran).
2. Trypsin-EDTA (0.05%, Invitrogen, Carlsbad, CA)
3. Dulbecco's modified Eagle's medium: Nutrient mixture F-12 1: 1 (Sigma, St. Louis, MO).
4. Fetal bovine serum (FBS, Invitrogen).
5. Growth media: 81.863 ml DMEM/F12, 10 ml FBS, 1 ml penicillin/streptomycin, 1 ml MEM non-essential amino acids, 2 ml bFGF (1000 ng), 2 ml EGF (2000 ng), 1 ml hydrocortisone (100×), 137 μl insulin, 1 ml (100×) cholera toxin.
6. Ethanol (70%).
7. Betadine.
8. Dispase (Sigma): Dissolve 10 mg dispase II in 10 PBS to prepare 1 mg/ml dispase and use freshly.
9. Stereo microscope (SZX12, Olympus, Tokyo, Japan).
10. Collagen coating: Dilute sterilely collagen type 1 solution (Sigma) to 50 μg/ml in distilled water. Add 4 ml diluted collagen to a T25 flask or 2 ml to six-well plates. Allow the collagen to bind for 2 h at room temperature or overnight at 2–8 °C. Remove excess fluid from the coated surface and allow it to dry for 2 h (*see* **Note 1**).

2.2 Magnetic-Activated Cell Sorting Components

1. Wash buffer: 1000 ml PBS containing 2 mM EDTA, pH 7.2. Add 800 ml PBS to 1-L in graduated cylinder. Weigh 292.24 g EDTA, and transfer to the cylinder. Add 200 ml of wash buffer to bring the volume up to 1 L. Store it at 4 °C (*see* **Note 2**).
2. Separation buffer: Prepare a solution containing phosphate buffer saline (PBS) supplemented with 0.5% bovine serum albumin (BSA) and 2 mM EDTA, pH 7.2. Add approximately 1000 ml wash buffer to 1-L graduated cylinder. Weigh 0.5 g BSA and transfer to a cylinder or dilute MACS BSA stock solution (130-091-376) 1:20 with wash buffer. Filter the solution with a 0.2 pore size filter. Keep buffer cold (2–8 °C).
3. PE-conjugated rat anti-mouse CD34 monoclonal antibody (Invitrogen).
4. Anti-PE Micro Bead (Miltenyi Biotec, San Diego, CA).
5. FcR Blocking reagent to avoid Fc receptor-mediated antibody labeling.
6. MiniMACS™ Separator (Miltenyi Biotec).
7. MultiStand (Miltenyi Biotec).
8. MS columns plus tubes (Miltenyi Biotec).
9. Hemocytometer.

2.3 Neural Induction Components

1. Non-essential amino acids (100×) (Invitrogen).
2. Penicillin/streptomycin (10,000 U/ml) (Invitrogen).
3. Fibroblast growth factor-b (bFGF) (Bio Vision, Milpitas, CA, 4037-50 μg): Dissolve 50 μg of bFGF in 10 ml sterilized distilled water, store at −80 °C in 500 ng/ml aliquots.
4. Epidermal growth factor (EGF) (Bio Vision, 4022-100 μg): Dissolve 100 μg of the growth factor in 10 ml sterilized distilled water, store at −80 °C in 1000 ng/ml aliquots.
5. β-Mercaptoethanol (BME, Sigma) (14.3 M). To prepare 100 mM BME: combine 61 μl BME with 9.939 ml of sterile distilled water and store at −30 °C in 1 ml aliquots. To prepare 1 mM BME add 1 mM of 100 mM BME to 99 ml DMEM/F12 and store at 4 °C up to 2 weeks.
6. Butylated hydroxyanisole (BHA, B1253): dissolve 180.24 mg BHA in 100 ml distilled water to make a 100× stock and store at 4 °C.
7. Dimethyl sulfoxide (DMSO) (Merck).
8. KCl (Merck): dissolve 1.864 g KCl in 10 ml distilled water to prepare 2500 mM/ml.
9. Hydrocortisone (Sigma): dissolve 1 mg hydrocortisone in 1 ml ethanol, then increase its volume to 10 ml with distilled water to prepare a 100× stock solution.

10. Heparin (1 mg/ml): Dissolve 27.778 mg heparin in 10 ml PBS medium to prepare 500 U/ml, aliquot and store at −80 °C.
11. N2 supplement (Invitrogen).
12. Valproic acid (Sigma): dissolve 332.38 mg in 10 ml distilled water to prepare a 200 mM stock solution.
13. Insulin (Invitrogen): add 137 µl stock solution to 100 ml DMEM/F12 to prepare 5 µg/ml.
14. Cholera toxin (Sigma): Dissolve 10 ng/ml, then add 10 µl in 10 ml sterile distilled water to dilute the solution to 100× and store at 4 °C (*see* **Note 3**).
15. Pre-induction medium: Sterilely combine: 72 ml DMEM/F12, 20 ml FBS, 1 ml penicillin/streptomycin, 1 ml cholera toxin (100×), 1 ml MEM non-essential amino acids, 1 mM 100 mM BME, 2 ml bFGF, and 2 ml EGF. Medium can be stored at 4 °C for up to 2 weeks.
16. Neural differentiation media (NDM): sterilely combine: 82 ml DMEM/F12, 10 ml FBS, 1 ml penicillin/streptomycin, 2 ml DMSO, 1 ml BHA (100× stock solution), 1 ml heparin (500 U), 1 ml KCl (2500 µg), 1 ml valproic acid, and 1 ml N2 supplement. Medium can be stored at 4 °C for up to 2 weeks.
17. Retinoic acid (RA) (Sigma): Dissolve 3 mg RA in 1 ml DMSO (0.01 M). Aliquot 50 µl into light protected vials and store at −20 °C. Dilute each 50 µl with 4.95 ml ethanol and make subsequent dilutions in growth medium as a working solution. The working solution may be stored up to 2 weeks (*see* **Note 4**).
18. RA neural induction medium: Sterilely combine 100 µl RA working solution (100 µg), 89.9 ml DMEM/F12, and 10 ml FBS. Medium can be stored at 4 °C for up to 2 weeks.
19. Serum-free neural induction medium: Sterilely combine 84 ml DMEM/F12, 1 ml penicillin/streptomycin, 1 ml MEM non-essential amino acids, 2 ml bFGF, and 2 ml EGF. Medium can be stored at 4 °C for up to 2 weeks.

2.4 RT-PCR

1. Trizol reagent (Invitrogen).
2. RNA isolation: For extraction of RNA, use molecular-biology grade chloroform (Merck), isopropanol (Merck), DNase and RNase-free ethanol (Fluka) and diethyl pyrocarbonate (DEPC) (Sigma)-treated water.
3. DEPC-treated water (0.1%): add 1 ml DEPC to liter of the autoclaved water in a fume-hood, and shake vigorously for at least 1 h at 100 rpm to ensure that it is completely dissolved. Finally, autoclave DEPC-treated water and store at 4 °C for further use.

4. cDNA synthesis: To produce complementary DNA (cDNA) from RNA template, use the RevertAid™ Minus First-Strand cDNA Synthesis Kit (Fermentase, Burlington, ON, Canada) containing the Moloney Murine Leukemia Virus (MMlV) Reverse Transcriptase and its buffer, ribonuclease inhibitor (40 U/IU) and random hexanucleotide primers.
5. PCR: 10 mM deoxynucleotide 5′ triphosphates (dNTPs) stocks, SmarTaq DNA polymerase, and its buffer as well as $MgCl_2$ (50 mM) (CinnaGen, Tehran, Iran).
6. Electrophoresis: electrophoresis system and UV transilluminator (UVIdoc, Uvitec), molecular-biology-grade agarose (Cinnagen); ethidium bromide solution (Merck), 6× loading Dye solution (Cinnagen), DNA size marker (Genedirex, SinaClon, Tehran, Iran), $Na_2EDTA{\cdot}2H_2O$ (Sigma), NaOH (Merck), boric acid (Cinnagen) and Tris-base (Sigma).
7. EDTA solution (0.5 M) : Add 1.58 g $Na_2EDTA{\cdot}2H_2O$ and 200 mg NaOH to 8 ml ddH_2O, mix well and increase the solution volume up to 10 ml with ddH_2O and adjust to pH 8.
8. Tris-base EDTA (TBE) solution (0.5×): Add 1 ml 0.5 M EDTA, 1.375 g boric acid and 2.7 g Tris-base to 40 ml ddH_2O. Mix well and adjust the solution volume up to 50 ml.
9. Agarose gel (1.2%): add 480 mg agarose powder to 45 ml 0.5× TBE and warm in microwave to ensure that it is dissolved. Add 5 μl ethydium bromide (10 μg/ml) to the agarose solution and cool at RT.
10. DNase I (Fermentas).
11. Use the Primer3 plus program design-specific primers with 17–21 bp nucleotides long and 50–60% GC content and synthesized with Gene Ziest (Gene Ziest Yakhteh Co., Tehran, Iran). All primer sequences used are shown in Table 1.

Table 1
Primer sequences, annealing temperature (AT), length of products (bp), and number of cycles for different genes

Gene	Primer sequences (5′ → 3′)	Annealing temperature	Bp length	Cycle
β-Tubulin	F: TCACTGTGCCTGAACTTACC R:GGAACATAGCCGTAAACTGC	47	318	30
Nestin	F:TCGAGCAGGAAGTGGTAGG R: TTGGGACCAGGGACTGTTA	49	352	30
MAP2	F:CCGGAAAACCACAGCAGCAAG R:TTGGAGGAGTGCGGATGATGG	57	434	30
GFAP	F: TCCGAGACGGTGGTCA R: GTCCCTCTCCACCTCCA	49.5	375	30

2.5 Immunocytochemistry Components

1. Paraformaldehyde (4%).
2. Blocking buffer: sterilely combine: 8.100 ml PBS, 0.5 ml goat serum (Invitrogen), or 0.5 ml FcR blocking reagent, 300 μl Triton X-100 (Sigma), 100 μl BSA, and 1 ml FBS.
3. Primary antibodies: rabbit anti-MAP2 polyclonal antibody (Sigma, 1:500) and rabbit anti-GFAP polyclonal antibody (Sigma, 1:500) (*see* **Note 5**).
4. Secondary antibody: Alexa Fluor® donkey anti-rabbit IgG (H + L) (Invitrogen, 0.5 ml).
5. 4,6-Diamidino-2-phenylindole (DAPI, GERBU, Heidelberg, Germany): add 1 ml of deionized water (diH_2O) to 1 mg DAPI to make a stock solution (*see* **Note 6**). Add 100 μl of the stock solution to 100 ml PBS to make a 1 μg DAPI stain solution.

3 Methods

3.1 Cell Culture

1. Sterilize the whisker pads with 70% alcohol and betadine.
2. Cut the upper lip and divide into small pieces, then place in 10 ml DMEM/F12 containing 10% penicillin/streptomycin and 0.5 μg/ml amphotericin B.
3. Incubate the tissue pieces in 1 mg/ml dispase II at room temperature for 30 min.
4. After two rinses in DMEM/F12, remove connective tissues around the follicles.
5. Lift out the vibrissa follicles under a stereo microscope.
6. Isolate the bulge region from the hair follicle by making two transverse sections above and below the region. Cut bulge regions into small pieces and add 2 ml trypsin/EDTA, incubate for 10 min.
7. After two washes with PBS, cultivate the bulge pieces in growth medium on collagen type 1-coated plates.

3.2 Separation of CD34-Positive Cells by Magnetic-Activated Cell Sorting (MACS)

Work rapidly, keep cells cold, and use pre-cooled solutions. Wash cells in the culture flask with PBS (*see* **Note 7**).

1. Isolate monolayer cells with 1.5 ml pre-warmed trypsin/EDTA until cells are detached.
2. Pass cells through 30 μm sterile CellTrics® filters in order to remove bulge pieces.
3. Centrifuge the cell suspension at $300 \times g$ for 10 min. Aspirate supernatant and determine the cell number, then wash cells with 10 ml wash buffer.
4. Resuspend 1×10^6 cell pellet in a final volume of 100 μl separation buffer containing 10 μl anti-CD34 primary antibody and

incubate for 20 min at 4–8 °C; then wash cells by adding 10 ml wash buffer and centrifuge at 300×*g* for 10 min (*see* **Note 8**).

5. Resuspend the cell pellet in 80 µl separation buffer and 20 µl anti-PE Micro Beads per 10^6 total cells. Mix well and incubate for 20 min at 4–8 °C.
6. Wash cells by adding 10 ml wash buffer, resuspend in 500 µl separation buffer and proceed to magnetic separation.
7. Place the column in the magnetic field of a mini MACS Separator. Prepare the column by rinsing with 500 µl wash buffer.
8. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells. Wash column with 1.5 ml wash buffer (*see* **Note 9**).
9. Remove MS column from the mini MACS separator and place it on a 15 ml conical tube.
10. Add 1 ml wash buffer onto the MS column and count the cell number. Immediately flush out the $CD34^+$ cells by pushing the plunger into the column (*see* **Note 10**).

3.3 Neural Induction Protocols

A different method was used for differentiating neural cells from HAP stem cells. Mouse HAP stem cells were maintained in growth medium.

3.3.1 NDM

1. Check that the cells have reached 70% confluence; wash the cells once with PBS.
2. Replace the medium with pre-induction medium consisting of DMEM/20% FBS/1 mM β-mercaptoethanol (BME), 10 ng/ml bFGF, 20 ng/ml EGF, and 10^{-9} M cholera toxin for 24 h.
3. Remove the pre-induction medium and wash the cells with PBS.
4. Use neuronal differentiation medium containing DMEM/F12, 10% FBS, 2% DMSO, 100 µM BHA, 5 U/ml heparin, 25 mM KCl, 2 mM valproic acid, and 1× N2 supplement, for 14 days.
5. Change neural induction medium every 3 days and allow the cells to differentiate for 14 days and process for immunocytochemistry.

3.3.2 RA Neural Induction

1. Wash the cells with PBS.
2. Replace the growth medium with neural induction medium containing DMEM/F12, 10% FBS, and 1 µM RA. Medium should be changed every 48 h.

3.3.3 Serum-Free Neural Induction

1. Wash the cells with PBS.
2. Replace the growth medium with serum-free medium containing 20 ng EGF, 10 ng bFGF, 1% N2 supplement, and 1% penicillin streptomycin. Medium should be changed every 48 h.

3.4 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

3.4.1 RNA Isolation

Since RNA is very sensitive to degradation by RNases, it is necessary that all the following steps for RNA extraction be performed with sterile filter tips.

1. Remove medium and wash the cultured cells with sterile PBS twice.
2. Add 500 µl Trizol to the cells and pass the resulting cell lysate several times through a pipette and collect the lysate into a 1.5 ml Eppendorf tube; store at −80 °C for further use.
3. Vortex tubes containing samples gently for 30 s and put the samples on ice for 5 min.
4. Add 100 µl chloroform to each tube and shake tubes gently by hand for 20 s. Then, incubate the tubes at room temperature for 2–3 min.
5. Centrifuge the samples at 12,000 × *g* for 15 min at 4 °C.
6. Take colorless upper aqueous supernatant and transfer it to a fresh Eppendorf tube.
7. Add one volume chloroform, vortex for 30 s and centrifuge at 4 °C for 5 min at 12,000 × *g*.
8. Transfer the supernatant into a new Eppendorf tube and add one volume isopropanol and mix well by hand.
9. Precipitate RNA at −20 °C for 30 min to 33 h and centrifuge at 12,000 × *g* for 30 min at 4 °C.
10. Discard supernatant and save the pellet (*see* **Note 11**).
11. Add at least 400 µl 75% Ethanol (dissolved in RNase-free water) to the RNA pellet and mix well, then centrifuge at 7500 × *g* for 5 min at 4 °C.
12. Remove the ethanol, and dry the pellet at 37 °C (*see* **Note 12**).
13. Resuspend the pellet in 20–30 µl DEPC-treated water and warm to 55–60 °C for 10 min.
14. Calculate the RNA concentration with Nanodrop kit, dissolve the RNA to obtain a ratio of $A_{260/280}$ ratio > 1.6. Use it for DNase treatment or store at −80 °C for further use (Table 1).

3.4.2 DNase Treatment

To ensure that the extracted RNA is free from any DNA contamination, the following steps are suggested:

1. Mix 1 µg mRNA, 1 µl DNase I enzyme (1 U/µl), 1 µl $MgCl_2$ in DEPC-treated water (to 10 µl) in 0.5 ml PCR tubes (*see* **Note 13**).
2. Incubate the tubes at 37 °C for 30 min.
3. Add 1 µl 50 mM EDTA and heat-inactivate at 65 °C for 10 min.

3.4.3 cDNA Synthesis

To generate complementary DNA, the following steps should be performed for the reverse transcription of DNase-treated RNA in a final 40 µl reaction solution.

1. Prepare a 20 µl RNA-primer mixture in a 0.2 ml microcentrifuge tube. Mix 1 µg RNA, 2 µl random hexamer primer, and 2 µl dNTP mix (10 mM) in sterile water. Spin tube by hand and incubate at 65 °C for 5 min and then chill on ice for 2 min.
2. Prepare a cDNA-synthesis mixture containing 4 µl RT-buffer (5×), 1 µl reverse transcriptase (RT) in 15 µl sterile water.
3. Add a cDNA-synthesis mix into the RNA-primer mixture and incubate at 25 °C for 10 min and then at 42 °C for 60 min to perform the reverse-transcription reaction.
4. Incubate the tube at 85 °C for 5 min to neutralize the RT enzyme reaction. Use cDNA in the PCR reaction mixture or store at −20 °C for further use (*see* **Note 14**).

3.4.4 PCR Reaction

For PCR of different genes, dilute all forward and reverse primers up to 10 pmol/L.

1. Add 30–50 ng template cDNA to the PCR solution containing ddH_2O, 10× PCR buffer, $MgCl_2$ (50 mM), dNTP mix (10 mM), primers (F + R), and SmarTaq DNA polymerase (5 U/µl) in a final volume of 25 µl. Perform PCR according to the following program: initial denaturation (94 °C for 5 min); denaturation in each cycle (94 °C for 45 s); annealing temperature (*see* Table 1), extension (72 °C for 45 s), and final extension (72 °C for 10 min). In the negative control, add ddH_2O instead of cDNA.
2. Add 1 µl loading dye to 10 µl sample and place in an electrophoresis system. Visualize and photograph the resultant bands with a UVIdoc system (*see* Fig. 1a).

3.5 Immunocytochemistry

1. Grow $CD34^+$ cells on collagen-coated six-well plates with growth medium. Replace the medium with fresh neural-induction medium.
2. Remove the culture medium and carefully wash cultures 3× with PBS.
3. Fix cells in 4% paraformaldehyde (pH 7.4) for 15 min at room temperature, wash three times with PBS, and incubate with blocking buffer for 30 min at room temperature.
4. Incubate cells overnight at 4 °C with primary antibodies (*see* **Note 15**).
5. Rinse the cells for 3 × 5 min in PBS to remove unbound primary antibodies. Dilute the secondary antibody in blocking buffer and apply for 2 h at room temperature.

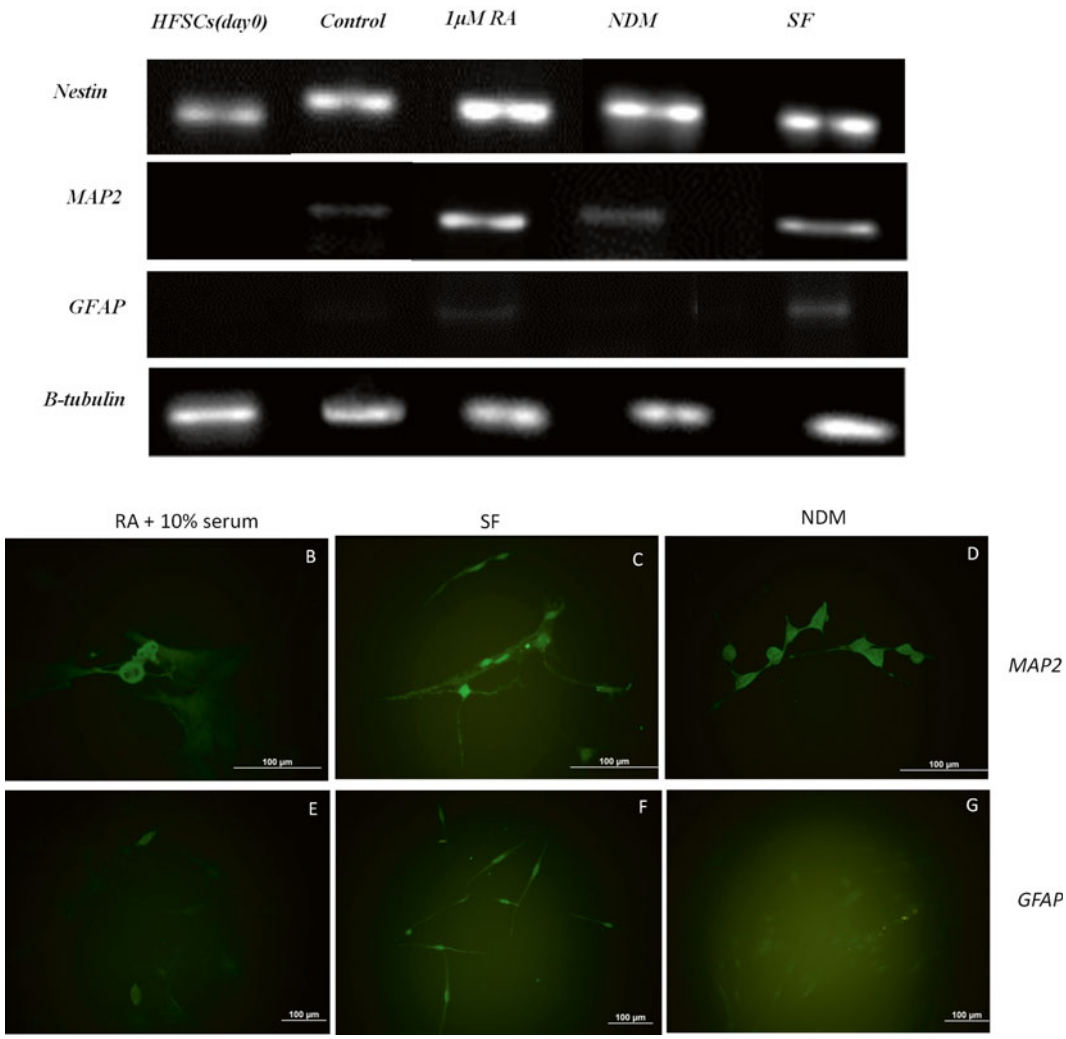

Fig. 1 Expression of neural (nestin and MAP2) genes increased in cells treated with 1 μM RA + 10 % serum, serum-free protocols, and NDM. Moreover, astrocyte differentiation was increased in serum-free medium (**a**). Images (**b**)–(**d**) showing increased MAP2 immunostaining in a week after neural induction in all the treated groups. Images (**e**)–(**g**) showing GFAP expression in the differentiated cells. Intriguingly, more GFAP positive glial cells were seen in the serum free-treated group (**f**). SF = serum free

6. After two washes, stain the cells with DAPI and incubate for 5 min in dark.
7. Wash two times with PBS and observe the cells with a fluorescence microscope (*see* Fig. 1b–g).

4 Notes

1. The coated plates may be stored at 4 °C for up to a month. Rinse the coated plates with sterile PBS or DMEM before cell culture, since the pH of coated surfaces is acidic, which is not suitable for the cell culture.

2. EDTA does not readily dissolve unless the pH is approximately 8. Diluted NaOH (1 N) can be used to adjust the pH to approximately 8 and then turn on a stir plate to mix the EDTA into solution. Adjust pH with HCl and sterilize by filtering through a 0.2 μm pore-size filtration unit.
3. In our experience, cholera toxin is useful in stimulating colony growth, which increases cyclic AMP in cultured keratinocytes.
4. RA solution is sensitive to light, air, and oxidizing agents. It is recommended to use all the stock solution immediately after opening the vials.
5. Primary antibodies are diluted in blocking buffer and secondary antibody is prepared in PBS containing 1 % BSA.
6. To dissolve DAPI sonicate for 1 min. The 1 mg/ml DAPI stock solution is divided in 100 μl aliquots and can be stored at −20 °C for up to 6 months.
7. All buffers and media used contain 1 % antibiotic-antimycotic solution (100 U/ml penicillin and 100 μg/ml streptomycin; 0.5 μg/ml amphotericin B) to avoid possible contamination from dissection. To prevent capping of antibodies on the cell surface and nonspecific cell labeling, carry out all procedures rapidly and keep cells cold.
8. For less than 1×10^6 total cells, use 90 μl separation buffer and 10 μl primary antibody. For 1×10^6–1×10^8 cells use 300 μl separation buffer.
9. Perform washing steps by adding 3×500 μl wash buffer only when the column reservoir is empty.
10. To increase the purity of CD 34^+ cells, repeat the magnetic separation procedure as described in Subheading 3.2, **steps 7–10** with a new MS column.
11. RNA precipitate is sometimes observed in the side or bottom of the tube. If so, it is better to remove the supernatant from the opposite wall.
12. It should be noted that the pellet should not be dried completely, since the extracted RNA may be degraded or its solubility will greatly decrease.
13. DNase I is sensitive to physical denaturing. Therefore, do not vortex the DNase.
14. To ensure that reverse transcription has been carried out, one group should be considered as a negative control (no RT enzyme).
15. Longer incubations at 4 °C may be better to increase specific binding and may be useful for very dilute primary antibodies.

Acknowledgments

This work was supported by grants from the Ardabil University of Medical Sciences.

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