PLUCKED HUMAN SCALP HAIR FOLLICLES MAY SERVE INNER EAR CELL-BASED THERAPY

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Introduction

There is an increasing interest in the therapeutic potential of autologous stem cells. Advantages include minimizing the need for systemic immunosuppression while reducing ethical and regulatory issues[1]. Neural crest stem cells (NCSC) are considered highly suitable for autologous stem cells therapy, for they persist during adulthood and can be harvested from easily accessible sources. NCSCs have great potential in the development of a cell-based therapy to treat deafness, for the neural crest and the otic placode share similar molecular events during neurogenesis[2]. Moreover, NCSCs have recently been identified in the developing otic vesicle, contributing not only to the glia lineages but also to sensory and neuronal progenies[3]. It has been shown that stem cells from the neural crest are located in the adult hair follicle (HF) bulge[4]. These cells are multipotent and can differentiate into neurons, melanocytes and glia[4]. HF stem cells, due to their NC-origin and accessibility, may be of great use in the development of an autologous cell-based therapy to treat deafness. In that perspective, the use of plucked HFs will increase the practical application of HF stem cells.

Experimental Setup

Specimens: The specimens were obtained from the retro-auricular side of the scalp (n = 6).These specimens were left-overs from hair implantations (Fig.1A-D). The Medical Ethical Committee on research involving human tissue of the Netherlands approved the protocol.

Cultures: The bulb from the hair follicles was removed and a longitudinal incision was made (Fig.1E-F). Immediately thereafter, the capsules were plated in a PDL precoated 12-well dish and cultured in basic growth medium (Table1). To prevent outgrowth of other cells, the follicle was removed on the 3rd day after outgrowth of cells. At 70-80% confluence, the cells were passaged. For expansion, cells were seeded at expansion density (2.5x 10^3 cells per cm^2) of the respective tissue culture dish (TPP; 10 cm). After reaching 80% confluency, the cells were seeded at the same density in a 15 cm culture dish(TPP).

Cryopreservation: 1x 10^6 HF stem cells in 1 mL 10% DMSO in FBS.

Neural Differentiation : 3.5x 10^5 cells in basic growth medium were seeded per well of a 12-well plate on cover glasses. Before usage, cover glasses were etched, rinsed, air-dried and subsequently coated with PDL. Medium change was every other day. At 90%-100% confluency, (approximately 2-4 days after seeding) half of the supernatant was replaced by neural induction medium (NIM, table 1). After 7 days 0,5 µM retinoic acid and 1% N2 supplement were added to the cell culture upon appearance of neuron-like cells. Otherwise, half of the medium was replaced by fresh NIM. Cultures were monitored every other day; morphologies were categorized according to Fig 7.

Shear stress: Cells were resuspended at a density of ~4.0 x 10^6 cells/ml in medium and carefully triturated. For each sample 10µl of cell suspension was loaded into a 400µl syringe with 30 gauge needle. The sample then was ejected from the needle into a 1ml Eppendorf tube using a ProSense 17498 syringe pump (Fig.3.,settings: diameter 4.699 mm - rate 0.5ml/min).

Results

The yield of stem cells is, on average, 3x 10^4 cells/follicle 1 month after the start of the culture. In theory, the cellular yield of 6-10 HFs would be enough for inner ear transplantation purposes. However, often outgrowth of keratinocytes occurs (Fig.2, table2). These cultures were discarded. Cell viability after syringe-mediated disaggregation (30 Gauge, 0.5µl/min. Fig.3) and cryopreservation was 82.2%±2.3% (Table3). The NCSC protein profile was similar to previous results from HF obtained from skin biopsies[5] i.e., cells were Nestin^{+ve}, SOX9^{+ve},SLUG^{+ve}, AP-2a^{+ve}, SOX10^{-ve} (Fig.4). Neural differentiation (B3Tub^{+ve}, NeuF^{+ve} cells), with appropriate cellular morphology, is achieved within 4 weeks after neural induction (Figs 5-8).

Conclusions

Stem cells from plucked HF can easily be cultivated, expanded and kept frozen until needed, while keeping NCSC characteristics. This allows practical application of HF stem cells for inner ear cell based therapy.

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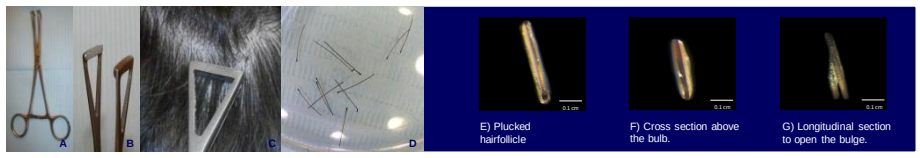


Fig.1A-C. Forceps for taking out hair follicles. D. Hair follicles have to remain soaked in buffer or medium.

Basic growth medium

DMEM/F12	Biochrom AG	
Glutamax	Gibco	1%
Antibiotic-antimycotic solution	Sigma-Aldrich	5%
Fetal calf serum	PAA	10%
B27 (without vitamin A)	Gibco	2%
N2max	R&D Systems	1%
Fibroblast Growth Factor	R&D Systems	20ng/ml
Epidermal Growth Factor	R&D Systems	20ng/ml

Table 1. Specific information composition media

Neuronal Induction Medium (NIM)

DMEM/F12	Biochrom AG	
Fetal calf serum	PAA	10%
Dexamethasone (DEX)	Sigma-Aldrich	1µM
Indomethacin	Sigma-Aldrich	200 µM
3-isobutyl-1-methylxanthine (IBMX)	Sigma-Aldrich	500 µM
ITS+3	Sigma-Aldrich	0,1%
Retinoic acid (R-A)	Invitrogen	0,5 µM

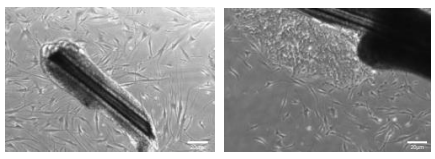


Fig.2A-B. Different cells grow out from hair follicles. A. Spindle-like cells. **B**. Keratinocytes close to the hair follicle

Different cellular outgrowth

Culture	HF (n)	HF with spindle cells	HF with KC outgrowth	% HF with spindle cells
905E1P0	12	7	4	42.1%
906E1P0	12	7	3	57.2%
916E1P0	13	8	5	37.5%
932E1P0	12	8	0	0%
934E1P0	30	20	1	95%

Table 2. variation in outgrowth : cultures with spindle-like cells and keratinocytes

Fig 3. Ejection with syringe pump

Type	Culture number	% living cells before	% living cells after ejection
HFSC	910E1P1	95.3	93.1
	916E1P2	96.0	93.4
	934E2P1	90.0	90.3
	Average	93.8	92.3
	St. deviation	3.28	1.71
HFSC cryo	909E1P3	82.6	83.1
	910E1P2	84.3	82.9
	916E1P9	79.7	82.8
	Average	82.2	82.9
	St. deviation	2.33	0.15

Table 3. Viability assay

Fig.4. HF-bulge-derived cells at day 2 of outgrowth: the neural crest markers Nestin, Slug , AP-2 and SOX9 are positive. SOX10 is negative.

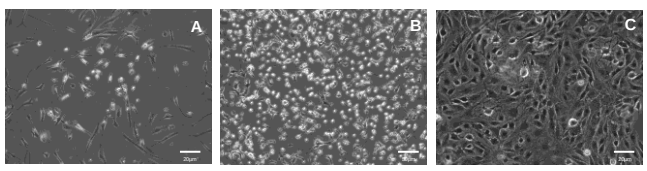
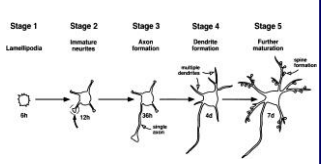


Fig.5. Neural differentiation of HF-bulge derived cells. **A**. eight days post explantation showing two population of cells, the spindle like cells with shining soma and the flattened cells. **B**. Neuron like morphologies appear 7 days after neural induction medium. **C**. Five days after addition of RA and N2. **D**. 2 days after addition of R-A and N2 supplement, neuronal morphologies stage 4 appeared.



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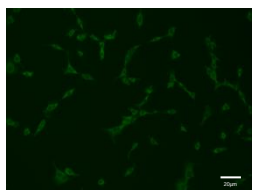

Fig.7. B3Tubulin^{+ve} cells from HF bulge cells before neural maturation.

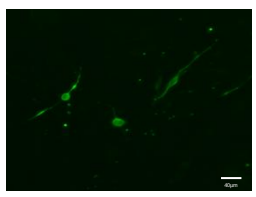

Fig.8. Neurofilament^{+ve} cells after 3 weeks of differentiation

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