Original Article

Isolation and Characterization of Neural Crest Stem Cells from Adult Human Hair Follicles

(neural crest / stem cells / human hair follicle / expression profile)

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Abstract. Neural crest (NC) is a transient embryonic tissue, whose cells are motile and multipotent until they reach their destination and differentiate according to microenvironmental cues into a variety of cell types. However, a subpopulation of these cells remains multipotent. They were found, among other locations, in a bulge of adult murine whisker follicle and were designated epidermal neural crest stem cells (EPI-NCSCs). The aim of this work is to ascertain whether the EPI-NCSCs could be isolated from human hair follicles as well. Due to their exceptional properties, they could represent potential candidates for stem cell therapy. The presented work focuses on the isolation and characterization of EPI-NCSCs from human skin. We obtained a population of cells that expressed markers of NC, NC progeny and general stem cell markers. After prolonged cultivation, the subpopulation of cells spontaneously differentiated into some of NC derivatives, i.e. neurons, smooth muscle cells and Schwann cell progenitors. Targeted differentiation with neuregulin 1 highly increased the number of Schwann cells in the culture. Human EPI-NCSCs could also grow under non-adherent conditions and form 3-dimensional spheres. Microarray analysis was performed and gene profile of human EPI-NCSCs was compared with the list of key genes of murine EPI-NCSCs and the list of genes up-regulated in newly induced NC cells. This revealed 94% and 88% similarity, respectively. All presented results strongly support the NCSC identity and multipotency of isolated human cells. These cells could thus be used in regenerative medicine, especially because of the easy accessibility of donor tissue.

Received April 21, 2010. Accepted May 6, 2010.

This study was supported by MSMT CR projects VZ0021620806, M0538, and LC06061 and Charles University in Prague project No. 260510.

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Abbreviations: EPI-NCSCs – epidermal neural crest stem cells, GFAP – glial fibrillary acidic protein, hEPI-NCSCs – human EPI-NCSCs, NC – neural crest, NCCs – neural crest cells, NCSCs – neural crest stem cells, SMA – smooth muscle actin.

Introduction

Tissue-specific adult stem cells have long attracted attention, especially since they could be isolated from one individual, expanded and eventually differentiated *in vitro* and transplanted back into the same individual. Accessibility of donor tissue is of considerable importance in such case. Compared to embryonic stem cells, there is no need to use immunosupressive medication since they are the patient's own cells and there will therefore be no graft rejection and no problems of ethical nature. Compared to induced pluripotent stem cells (Takahashi and Yamanaka, 2006) or nuclear transfer, there is no need for genetic or mechanic manipulation.

Neural crest (NC) is a transient embryonic tissue that arises at the border between neural and non-neural ectoderm in early stages of development. Following neural tube closure, NC cells (NCCs) undergo epithelial-mesenchymal transition and migrate along defined pathways to populate various regions of the embryo (reviewed in Le Douarin and Kalcheim, 1999). NCCs contribute to a diverse array of cell types, including multiple skeletal cells (chondroblasts, osteoblasts and odontoblasts), pigment cells, smooth muscle cells, peripheral neurons (sensory and autonomic), glia and mechanoreceptors (Merkel cells) (Szeder et al., 2003).

NC induction involves a set of genes that are up-regulated during this process. Gammill and Bronner-Fraser (2002) and Adams et al. (2008) performed genomic analysis of NC induction and revealed 183 genes in total that were up-regulated during NC induction.

NC development involves a process of progressive fate restriction, whereby multipotent cells tend to become increasingly limited in their potencies over time (reviewed in Weston, 1991). The premigratory NCCs are diverse, some of them multipotent and others appar-

ently fate-restricted (Bronner-Fraser et al., 1980). Several multipotent or at least fate-restricted progenitor cells are resident in specific locations in later stages of development or even in adulthood. Neural crest stem cells (NCSCs) were isolated from rodent embryonic and postnatal tissues: sciatic nerve (Morrison et al., 1999), gut (Bixby et al., 2002; Kruger et al., 2002), dorsal root ganglion (Li et al., 2007), heart (Tomita et al., 2005) or cornea (Yoshida et al., 2006). NCSCs were also described in murine and human epidermis and/or dermis (Fernandes et al., 2004; Sieber-Blum et al., 2004; Amoh et al., 2005; Toma et al., 2005; Wong et al., 2006; Yu et al., 2006, 2010).

Sieber-Blum et al. (2004) and Sieber-Blum and Grim (2004) described NCSCs in the bulge region of adult murine whisker follicle and designated them epidermal NC stem cells (EPI-NCSCs). The method of isolation was based on the emigration of cells from explanted bulge region. Emigrated cells expressed Sox10, a marker of NC (Britsch et al., 2001; Rehberg et al., 2002), and intermediate filament protein nestin, a marker of immature and undifferentiated cells (Lendahl et al., 1990; Lothian and Lendahl, 1997; Mokrý et al., 2004). Nestin is also expressed in some cells in the bulge of hair follicle (Li et al., 2003). EPI-NCSCs were serially cultured and under conditions that favoured differentiation showed a broad potential for generating cells expressing markers appropriate for neurons, glia, smooth muscle cells, chondrocytes and melanocytes. EPI-NCSCs responded to neuregulin 1 by generating Schwann cells, and to BMP2 by generating chondrocytes (Sieber-Blum et al., 2004).

Murine EPI-NCSCs were characterized at the molecular level by Long Serial Analysis of Gene Expression (Hu et al., 2006). As a result, 62 genes were listed as EPI-NCSCs key genes; 19 of them were unique within the hair follicle milieu and were designated molecular signature of EPI-NCSCs.

The exceptional properties of EPI-NCSCs mentioned above predetermine them as potential candidates for regenerative medicine. In the presented study we thus focused on the isolation and cultivation of human EPI-NCSCs (hEPI-NCSCs) using the method introduced by Sieber-Blum et al. (2004) for murine cells. Their characterization comprised immunocytochemical detection of appropriate markers together with transcriptome analysis by RT-PCR and microarray. Our results showed that hEPI-NCSCs exhibited high similarity with EPI-NCSCs of the mouse.

Material and Methods

Isolation of follicles

We used skin biopsies obtained from healthy individuals undergoing skin surgery at the Department of Dermatology of Charles University in Prague, First Faculty of Medicine and General University Hospital with their informed consent and the approval of the Local Ethical Committee according to the principles of Helsinki Declaration. Skin biopsies were obtained from different parts of the body, namely from the face, back, forearm, thigh, calf, chest and pubic region. Depending on the type of skin as well as gender of donors we were able to obtain 5–15 follicles from approximately 1 cm^2 skin biopsies. The follicles were mechanically dissected and most of adipose and connective tissue was removed. As the bulge is not a clearly visible structure during preparation, we excised an area under the sebaceous gland (Fig. 1). The dissected piece was rinsed in PBS and explanted onto a culture dish.

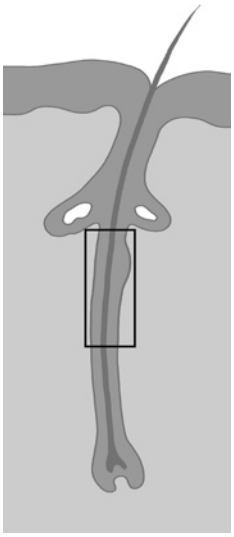

Fig. 1. Scheme of human hair follicle in anagen phase. Black box demarcates the dissected part of the follicle. The first transversal cut was made directly under the lower rim of the sebaceous gland attachment site, the other approximately in the second third of the follicle length.

Culture conditions

Tissue explants and cells were cultivated on a collagen (Inamed Biomaterials, Fremont, CA)-coated culture dish in α-MEM supplemented with ribonucleosides, deoxyribonucleosides and glutamine (Gibco, Carlsbad, CA), 10% fetal bovine serum (Biochrom, Cambridge, UK), 5% extract from 11-day-old chicken embryos, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, gentamycin 100 μg/ml, amphotericin 2.5 μg/ml – Sigma, St. Louis, MO) at 37 ° C and 5% CO_2. The explanted tissue was removed four days after the onset of emigration, and the cells were subcultured to reduce focal high cell to avoid high focal cell density and rapid differentiation. For the same reason, the confluence of cells was kept bellow 50 %. The subculture was performed using the mixture of 0.25% trypsin and 0.02% EDTA, 1 : 1 (Biochrom). Half of the medium was changed every two days. EPI-NCSCs could be cultured for at least four months, but our experiments were performed with cells during the first month after the onset of emigration.

Targeted differentiation into Schwann cells was achieved by supplementation of culture medium with

10 nM neuregulin 1 (PeproTech, Rocky Hill, NJ). For differentiation into chondrocytes, 10 ng/ml of BMP2 (PeproTech) was added to culture medium.

Spheres were cultivated in medium composed of DMEM : F12 (3 : 1) supplemented with B27 serum-free supplement (Sigma-Aldrich, St. Louis, MO), 40 ng/ml FGF2 (PeproTech) and 20 ng/ml EGF (PeproTech).

Tissue cultures were monitored under an Olympus IX71 microscope (Olympus Co., Tokyo, Japan) and documented by DP50 camera (Olympus).

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 30 min in 4 °C, then washed and permeabilized with 0.05% Tween 20 in PBS for 10 min. Blocking of non-specific binding was performed by 10% serum from the animal in which the secondary antibody was raised. The following primary antibodies were used for indirect immunocytochemistry: anti-nestin (1 : 200, Chemicon, Millipore, Billerica, MA), anti-SOX10 (1 : 20, CeMines Golden, CO), anti-β-III tubulin (clone TuJ-1, 1 : 100, Stem Cell, Vancouver, Canada), anti-smooth muscle actin (1 : 800, Sigma), anti-S100b (1 : 100, Sigma), anti-GFAP, (1 : 500, Chemicon), anti-collagen type II (1 : 10, Abcam, Cambridge, UK), anti-nanog (1 : 20, BD Biosciences, Mississauga, ON). Incubation was performed overnight at 4 °C. Corresponding secondary antibodies (conjugated with TRITC, FITC or Cy5, Jackson Immunoresearch Laboratories, West Grove, PA) were applied in dilution 1 : 200 for two hours at room temperature. Anti-SOX10 and anti-nanog immunoreactions were amplified using biotin-conjugated secondary antibodies and Cy5-conjugated streptavidine (1 : 150, Jackson Immunoresearch Laboratories). We used BX51 microscope (Olympus), DP71 camera (Olympus) and Cell P software (Olympus) for image acquisition.

RNA analysis

Total RNA for RT-PCR and microarray analysis was isolated from subconfluent secondary culture cells using RNeasy Micro Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. For RT-PCR, first strand cDNA synthesis was performed by AccuSript Reverse Transcriptase (Stratagene, Santa Clara, CA) and primed by a mixture of oligo(dT) primers and random hexamers (3 : 1, respectively). Red Taq Ready Mix (Sigma-Aldrich) and gene-specific primers were used for PCR amplification of target genes. Primers of NC and NC progeny were adopted from Yu et al. (2006); the others were designed using Primer3 software (Rozen and Skaletsky, 2000). Where it was possible, the primers were designed to span the intron region to avoid false amplification caused by any possible traces of genomic DNA. The list of primers is shown in supplementary Table S1. PCR cycling conditions were: 10 min 94 °C followed by 35 cycles of 94 °C, 30 s; 58 °C, 45 s; 72 °C, 45 s and final 72 °C, 5-min elongating step. The length of PCR fragments ranged between 100 kb and 600 kb.

RNA for gene expression-profiling analysis was isolated from 4th and 8th passage cells in two biological replicates. Quantity of the RNA was measured in ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). RNA integrity was tested in 2100 Bioanalyzer (Agilent, Houston, TX). Only samples with RIN $\geq$ 9.8 were used. The amount of 150 ng RNA was amplified with Illumina TotalPrep RNA Amplification Kit (Ambion Inc, Foster City, CA) and cRNA was hybridized in HumanRef-8 v3 Expression BeadChip (Illumina, San Diego, CA) according to manufacturer's instructions. Acquired data were analysed by using BeadStudio 3.3 software (Illumina). The data was background corrected and normalized by the quantile method. Only genes with P values lower than 0.05 were considered to be expressed significantly above the background. In case of multiple probes for a gene, the gene was considered as being expressed if at least one of the probes had the P value lower than 0.05.

Results

Emigrated cells express markers of NC and its derivatives

Five to eight days post-explantation cells with spindle or stellate morphology emigrated from the dissected part of the follicle with increasing numbers over time (Fig. 2A). Indirect immunocytochemistry demonstrated that emigrated cells expressed marker of early NCC transcription factor SOX10 (Fig. 2B, C). Its distribution was mainly nuclear, consistent with prevalent localization of this nucleocytoplasmic shuttle protein (Rehberg et al, 2002). Nevertheless, a subset of cells exhibited nuclear and cytoplasmic localization (Fig. 2C, arrowheads). Human EPI-NCSCs also expressed intermediate filament protein nestin (Fig. 2D). Figure 2E shows co-expression of both of these NCSC markers in single cells. Expression of embryonic stem cell marker nanog was also detected in most of emigrated cells (Fig. 2F).

Morphological differences appeared after approximately two weeks of cultivation with reduced subculture. Besides small spindle or stellate cells we could see large flattened cells. These cells expressed smooth muscle actin (SMA) (Fig. 3A), suggesting that they differentiated into smooth muscle cells/myofibroblasts. We also observed cells with long processes. These cells followed the neuronal pathway and expressed neuronal type β-III tubulin (Fig. 3B). Some of cells expressed S100b (Fig. 3C), a marker of Schwann cell precursors (Parkinson et al., 2001).

Targeted differentiation into one type of NC progeny – glial cells – was also tested. Consistent with results of Shah et al. (1994), addition of neuregulin 1 promoted differentiation into Schwann cells, and the number of glial fibrillary acidic protein (GFAP) positive cells was approximately six times higher in medium supplemented with neuregulin 1 after two weeks of cultivation (Fig. 3D).

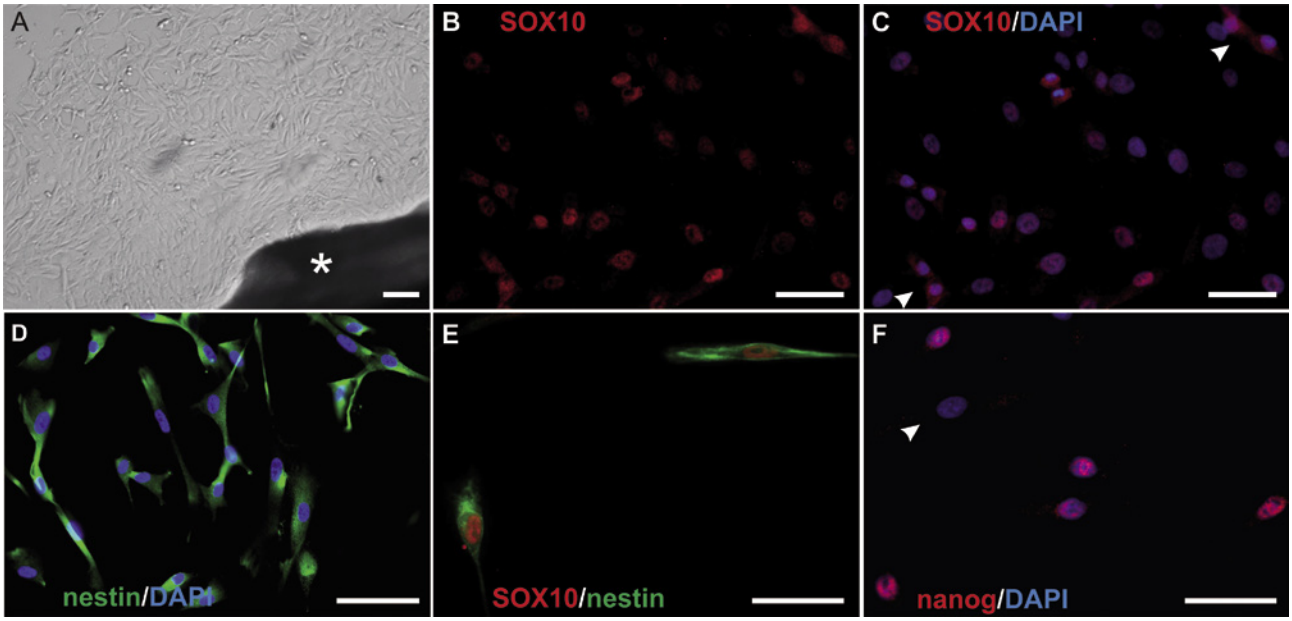

Fig. 2. Immunocytochemical characterization of hEPI-NCSCs. (A) hEPI-NCSCs emigrating from human hair follicle explant (asterisk) eight days post explantation. (B) Expression of NC marker SOX10. (C) Co-localization of nuclear stain DAPI and SOX10. Distribution of this nucleocytoplasmic shuttle protein is mainly nuclear, but in some cells it is also located in the cytoplasm (arrowheads). (D) Expression of marker of immature and undifferentiated cells nestin. (E) hEPI-NCSCs co-express both SOX10 (red) and nestin (green). (F) Most of emigrated cells express stem cell marker nanog. Nevertheless, some are negative (arrowhead). Scale bars = 100 μm

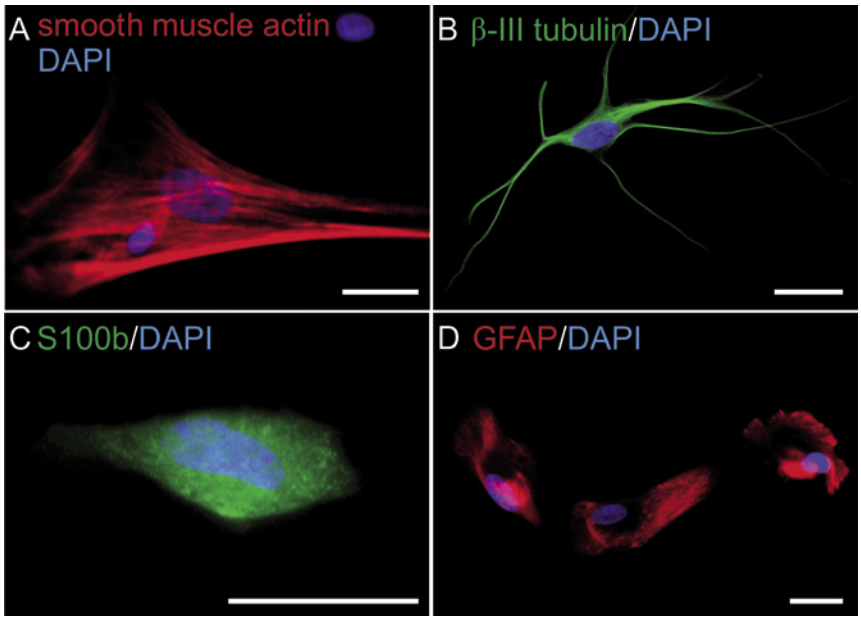

Fig. 3. Differentiation of hEPI-NCSCs into NC progeny. After prolonged cultivation some cells spontaneously differentiated into (A) large flattened smooth muscle cells/myofibroblasts expressing smooth muscle actin, (B) β-III tubulin-positive neuron with long processes and (C) S100b-positive Schwann cell progenitor. (D) The presence of neuregulin 1 highly increased the number of cells differentiating into Schwann cells. These cells then exhibited glial fibrillary acidic protein immunoreactivity. Scale bars = 50 μm

Differentiation into chondrocytes was also tested. Nevertheless, supplementation of medium with BMP2 did not result in collagen type II-positive chondrocytes in hEPI-NCSC culture (not shown).

Expression of other NC and NC progeny markers was further examined by RT-PCR. Lanes 1–5 in Fig. 4 show that mRNA of NC marker genes *BMP4, SNAIL, SLUG, SOX9* and *TWIST* was present in emigrated cells. Lanes 6–12 in Fig. 4 show expression of NC progeny

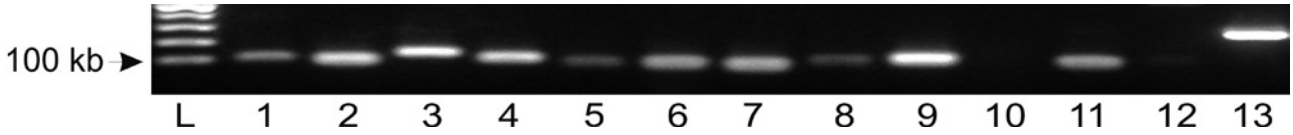

Fig. 4. RT-PCR detection of some NC and NC progeny markers. Lanes 1–5 show that hEPI-NCSCs express markers of NC (1 – *BMP4*, 2 – *SNAIL*, 3 – *SLUG*, 4 – *SOX9*, 5 – *TWIST*). Lanes 6–12 show expression of some NC progeny markers. The culture contained smooth muscle cells expressing desmin (6) and calponin (7) and neurons expressing *MAP2* (8) and β-III tubulin (9). There was no *CK20* positivity (10), suggesting no differentiation into Merkel cells. The culture contained melanocyte progenitors that expressed *MITF* (11), but not its downstream gene *TYRP1* (12). (13) Expression of housekeeping gene *GAPDH*. L = 100 kb ladder.

markers, i.e. desmin (6) and calponin (7) for smooth muscle cells, *MAP2* (8) and β-III tubulin (9) for neurons. There was no detectable expression of *CK20* (10), a marker of Merkel cells. The emigrated cells expressed melanocyte-specific gene *MITF* (11) but not its downstream target gene *TYRP1* (12) (reviewed in Goding, 2000), suggesting the progenitor character of cells, which was further supported by the absence of melanin granules in the cultured cells.

Cells form spheres

In order to further explore the "stem properties" of emigrated cells, they were cultivated under non-adherent conditions to find out whether they are able to form 3-dimensional spheres. Secondary culture cells were trypsinized and transferred to serum-free medium supplemented with growth factors. In five weeks 3-dimensional spheres were formed from single cells (Fig. 5A), which proved their clonogenic potential.

To test the viability of cells within the sphere, we transferred eight randomly chosen spheres onto collagen-coated culture dishes. Spindle-shaped cells appeared approx. 12 h after adhesion (Fig. 5B) in all cases. Furthermore, we trypsinized the spheres, seeded cells onto collagen-coated dishes and obtained culture of cells with the same immunocytochemical properties as had the original cells (data not shown).

Microarray analysis

In order to extend information obtained using methods described above, we applied whole-genome microarray analysis using HumanRef-8 v3 Expression BeadChip (Illumina). This BeadChip targets approximately 24,500 well-annotated transcripts with up-to-date content derived from the National Center for Biotechnology Information Reference Sequence database. Two different passages in two independent biological replicates were tested on four chips. The number of genes detected with P value ≤ 0.05 ranged between 9811 and 9960.

EPI-NCSC molecular signature genes

Our hEPI-NCSC microarray results were screened for the expression of genes that were identified as the key genes of murine EPI-NCSCs by Hu et al. (2006). Of the 62 genes listed in their original paper, 51 had probes present on the BeadChip and 48 of them, i.e. approxi-

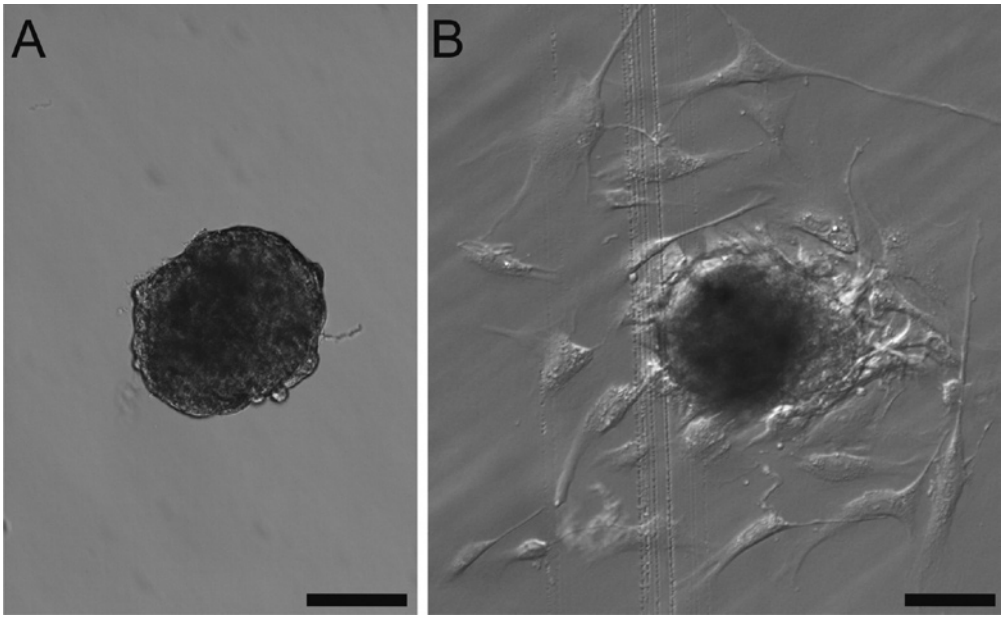

Fig. 5. hEPI-NCSCs were able to form 3-dimensional spheres when grown under non-adherent conditions. (A) A sphere after 5-week cultivation. (B) When transferred onto a collagen-coated culture dish, spindle-shaped cells emigrated from the sphere within 12 h. Scale bars = 100 μm

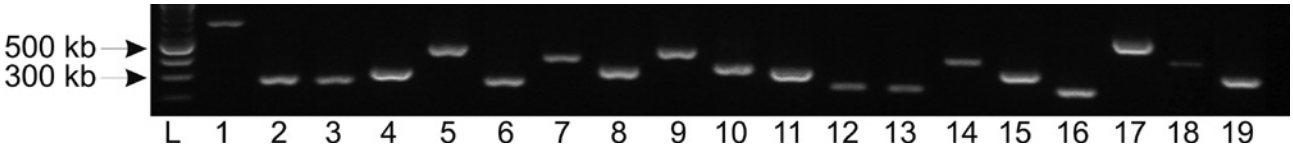

Fig. 6. RT-PCR verification of expression of all EPI-NCSC molecular signature genes. 1 – *MSX2*, 2 – *H1FX*, 3 – *PCBP4*, 4 – *THOP1*, 5 – *VARS2*, 6 – *MYO10*, 7 – *SELH*, 8 – *ETS1*, 9 – *PYGO2*, 10 – *ADAM12*, 11 – *TSEN15*, 12 – *VDAC*, 13 – *CALR*, 14 – *CRYAB*, 15 – *AGPAT6*, 16 – *CRMP1*, 17 – *UBE4B*, 18 – *PEG10*. 19 – housekeeping gene *GAPDH*. L = 100 kb ladder.

mately 94 %, exhibited intensity levels higher than the background (Supplementary Table S2).

Of 19 genes constituting the molecular signature of EPI-NCSCs (supplementary Table S2, bold), 15 had probes present on the chip and 14 of them had intensity levels significantly higher than negative controls. *MSX2* was not detected.

RT-PCR analysis was performed to confirm and extend our data about the expression of molecular signature genes. The GenBank and Unigene Databases were screened to find the corresponding human genes. The search was successful with 18 out of 19 genes. Fig. 6 shows that all of the tested genes were amplified by RT-PCR, including *MSX2*. Thus, 100 % of the tested murine EPI-NCSC molecular signature genes were expressed in hEPI-NCSCs. There was no amplification when no-reverse-transcriptase samples were used as a template (not shown).

hEPI-NCSCs express regulators of NC development

We screened our microarray results for the expression of genes that are up-regulated during NC induction (Gammill and Bronner-Fraser, 2002; Adams et al., 2008). Of 163 genes with assigned function, 147 had probes present on the BeadChip, 130 probes, i.e. approx. 88 %, had intensity levels significantly higher than the background (supplementary Table S3).

hEPI-NCSCs express iPS cell genes

Takahashi and Yamanaka (2006) reprogrammed adult murine fibroblasts into embryonic-like pluripotent cells via viral introduction of four transcription factor genes – *Pou5f1* (*Oct3/4*), *Sox2*, c-*Myc* and *Klf4*. Subsequently, reprogrammed cells were selected according to *Fbxo15* or *Nanog* (Okita et al., 2007) promoter activity and were designated induced pluripotent stem (iPS) cells. Analysis of our microarray data showed that all of the genes mentioned above were expressed in hEPI-NCSCs. Expression of *POU5F1, SOX2, MYC* and *NANOG* was further confirmed by RT-PCR (Fig.7).

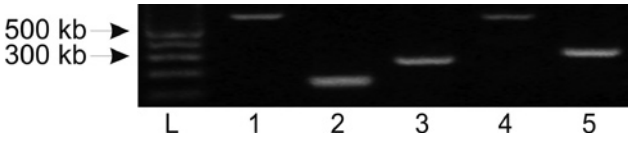

Fig. 7. RT-PCR verification of expression of genes that were used for induction of pluripotency in adult somatic cells. 1 – *POU5F1*, 2 – *SOX2*, 3 – *MYC*, 4 – *NANOG*. 5 – housekeeping gene *GAPDH*. L = 100 kb ladder.

Discussion and Conclusion

Here we report the isolation and characterization of human EPI-NCSCs using the method based on the migratory ability of these cells. We used a similar method for bulge region isolation and the same culture conditions as for murine EPI-NCSCs described by Sieber-Blum et al. (2004). They have shown that explantation of the bulge region of whisker follicle changes the environment of the stem cell niche in a way that activates proliferation and emigration of NCSCs, so that more than 80 % of emigrated cells are multipotent. We obtained a population of cells that expressed both NC marker SOX10 and immature cell marker nestin. Other NC markers, including *BMP4*, *SLUG, SNAIL*, *TWIST* and *SOX9*, were detected in cultured cells at the mRNA level. These findings support the presumption of NCSC identity of isolated human cells. Most of the emigrated cells also expressed nanog, a marker of stem cells (Chambers et al., 2003). They were also able to grow under non-adherent conditions as 3-dimensional spheres, which proved their clonogenic potential. All of this is consistent with their stem character.

Fetal calf serum and embryonic extract-containing culture medium promotes both survival/proliferation and differentiation into some types of NC progeny (Ito and Sieber-Blum, 1993). In agreement with this data, spontaneous differentiation of hEPI-NCSCs occurred after prolonged cultivation. Besides neurons and glial or melanocyte precursors, smooth muscle cells also appeared among the cultured cells. Our results further showed that the medium composition promoted differentiation into only a subset of NC progeny. We obtained only precursors of melanocytes, as shown by the expression of *MITF*, but not its downstream target gene *TYRP1*, and we did not obtain Merkel cells as shown by the absence of *CK20* expression.

Supplementation of culture medium with neuregulin 1 promoted differentiation into Schwann cells in increased cell numbers compared to cultivation in neuregulin 1-free medium. Nevertheless, unlike murine EPI-NCSCs, hEPI-NCSCs did not differentiate into chondrocytes following supplementation of culture medium with BMP2. This fact could have several explanations:

1. It was shown previously that NCCs of cranial and trunk origin respond differently to the same signals (Abzhanov et al., 2003). We tested targeted differentiation with cells originating in trunk NC, while murine EPI-NCSCs originated in cranial NC. The difference in chondrogenic potential could thus be explained by different behaviour of NCCs of trunk (hEPI-NCSCs) and cranial (murine EPI-NCSCs) origin. Although targeted differentiation into chondrocytes was already achieved in NCCs of non-cranial origin, these experiments were performed under different culture conditions than those used in our work.

2. hEPI-NCSCs could lose their responsiveness to BMP2 during subculture.

Future studies should test other culture conditions for the cells originating in the trunk NC or test hEPI-NCSCs originating in the cranial NC under conditions used in this work.

To further extend our knowledge about emigrated cells we analysed their transcriptome. Whole-genome microarray revealed that emigrated cells expressed 94 % of genes that have been detected as the key EPI-NCSC genes in mice (Hu et al., 2006). Of these key genes, 19 have been selected as being EPI-NCSC unique in the hair follicle milieu and they have represented the EPI-NCSCs molecular signature genes. All but one (which could not be tested) of these genes were detected in human cells, showing their similar nature with murine EPI-NCSCs.

We also compared microarray data with the list of genes that are up-regulated in newly induced chick premigratory and migratory embryonic NCSCs (Gammill and Bronner-Fraser, 2002; Adams et al., 2008). Of these, 88 % were detected in hEPI-NCSCs, showing their close resemblance with embryonic NCSCs.

We further tested the emigrated cells for the expression of genes that have been used for reprogramming adult somatic cells into pluripotent state (Takahashi and Yamanaka, 2006). Microarray data and following confirmation of results by RT-PCR showed that all the genes used for reprogramming and subsequent selection were expressed in hEPI-NCSCs. Sieber-Blum and Hu (2008) have tested expression of these genes in murine (adult) EPI-NCSCs and embryonic stem cells and compared the levels of their expression between these two cell types. They have found that although all of the genes are detected in both cell types, their levels differ with c-*Myc* and *Klf4* elevated in murine EPI-NCSCs and *Sox2, Pou5f1* and *Nanog* elevated in embryonic stem cells. Significantly lower expression of *Nanog* and *Pou5f1* is considered to be the reason why EPI-NCSCs, unlike embryonic stem cells, do not form tumours after transplantation (Sieber-Blum et al., 2006). According to our microarray data, expression of *NANOG* and *POU5f1* in hEPI-NCSCs was also very low, while expression of *MYC* was much higher. We thus could anticipate that in the case of transplantation, hEPI-NCSCs like mEPI-NCSCs would not form tumours.

All NCSCs isolated previously from murine and human epidermis and/or dermis have been obtained by dissociation of donor tissue and have been cultivated as 3-dimensional spheres and in different types of medium (Fernandes et al., 2004; Amoh et al., 2005; Wong et al., 2006; Toma et al., 2005; Yu et al., 2006). Recently, Yu et al. (2010) have cultivated whole human hair follicles in embryonic stem cell culture medium and observed formation of 3-dimensional spheroid structures within the bulge region. They have used human hair scalp follicles after dispase-mediated separation of dermal and epidermal structures. They have found that their follicle-derived cells do not grow under culture conditions used in the work of Sieber-Blum et al. (2004) and in our work. This has led them to the suggestion that there is a difference in biological behaviour between murine and human follicle stem cells and that it is not possible to isolate human EPI-NCSCs by the method introduced by Sieber-Blum et al. (2004). Nevertheless, according to our results there was no significant difference in the ability to emigrate from explanted follicle and grow as a monolayer between murine and human EPI-NCSCs. This was an important finding showing that murine and human EPI-NCSCs share very similar properties and that the results obtained with murine cells could be translated into human biology. The inability of emigration from explanted follicle observed by Yu et al. (2006) could be caused by dispase treatment. It was shown that dispase inhibits the ability of melanoma cells (one type of the NC progeny) to attach to the substrate (Whalen and Ingber, 1989). As the method described here was based on the emigration of cells, their attachment ability was crucial. When Yu et al. (2006) used non-adherent conditions, dispase treatment did not affect their cells growing as spheres. Finally, we proved that our cells were also able to grow under non-adherent conditions.

All the results presented in this work strongly support the NC identity and multipotent potential of isolated human EPI-NCSCs. It was shown that murine EPI-NCSCs can be successfully grafted into spinal cord lesions (Sieber-Blum et al., 2006). The advantage of these cells is that upon transplantation they do not form tumours. The other advantage is that the cells do not need targeted differentiation prior to grafting, because they differentiate spontaneously according to microenvironmental cues. As hEPI-NCSCs exhibited highly similar properties to murine EPI-NCSCs, it is possible to anticipate their similar behaviour after grafting. In conclusion, we propose that hEPI-NCSCs could be good candidates for stem cell therapy in regenerative medicine, especially thanks to the easy accessibility of donor tissue.

Supplementary material

Supplementary material is linked to the online version of the paper at:

Supplementary Table S1: http://www.folia-biologica.org/supplements/fb-2010-4-s1.pdf

Supplementary Table S2: http://www.folia-biologica.org/supplements/fb-2010-4-s2.pdf

Supplementary Table S3: http://www.folia-biologica.org/supplements/fb-2010-4-s3.pdf

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