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Towards expansion of human hair follicle stem cells *in vitro*

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Abstract

Objectives: Multipotential human hair follicle stem cells can differentiate into various cell lineages and thus are investigated here as potential autologous sources for regenerative medicine. Towards this end, we have attempted to expand these cells, directly isolated from minimal amounts of hair follicle explants, to numbers more suitable for stem-cell therapy.

Materials and methods: Two types of human follicle stem cells, commercially available and directly isolated, were cultured using an in-house developed medium. The latter was obtained from bulge areas of hair follicles by mechanical and enzymatic dissociation, and was magnetically enriched for its $CD200^+$ fraction. Isolated cells were cultured for up to 4 weeks, on different supports: blank polystyrene, laminin- and Matrigel^{TM}-coated surfaces.

Results: Two-fold expansion was found, highlighting the slow-cycling nature of these cells. Flow cytometry characterization revealed: magnetic enrichment increased the proportion of $CD200^+$ cells from initially 43.3% (CD200+, CD34: 25.8%; CD200+, CD34+: 17.5%) to 78.2% (CD200+, CD34: 41.5%; CD200+, CD34+: 36.7%). Enriched cells seemed to have retained and passed on their morphological and molecular phenotypes to their progeny, as isolated $CD200^+$ presenting cells expanded in our medium to a population with 80% of cells being $CD200^+$: 51.5% ($CD200^+$, $CD34^-$) and 29.6% ($CD200^+$, $CD34^+$).

Conclusions: This study demonstrates the possibility of culturing human hair follicle stem cells without causing any significant changes to phenotypes of the cells.

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Introduction

Clinical conditions causing hair loss (such as androgenetic alopecia, alopecia areata and scarring alopecia), or skin burns can be psychologically devastating to individuals. These frequently occurring conditions are currently being addressed by restorative hair follicle transplantation. The hair follicle (Fig. 1) is a mini-organ that is totally distinct from other tissues in the human body, as it is characterized by cycles, in which all of the hair growth machinery is lost, then fully reconstituted, from its pool of intrinsic stem cells (1). The importance of the hair follicle in skin biology, however, does not rest solely on its ability to produce hair. Hair follicles are also believed to be involved in regeneration of the epidermis and play a crucial role in wound healing (2–5). Despite this regenerative capacity, often the degree of degeneration is so severe that the permanent potion of the follicle is lost, the adult human body being unable to regenerate the hair follicle and restore homoeostasis. In these cases, hair transplantation may no longer be a viable option for treatment, as there might not be sufficient donor hair follicles (available from the scalp or other areas) that can be transplanted without causing collateral damage elsewhere. Moreover, type and quality of follicle from other body parts than the scalp do not give rise to the same type of hair, with long shafts as usually grow on the scalp. Thus, stem-cell therapy to restore bald scalp areas has become an emerging focus in addressing this medical condition.

In this context, the use of human hair follicle stem cells (HFSCs) may have a number of advantages over other sources of stem cells, such as embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. ES cells have the potential to provoke teratoma formation at the site of application (6) and are also ethically challenged. Furthermore, success of this type of treatment cannot be guaranteed for all individuals, due to the inherent

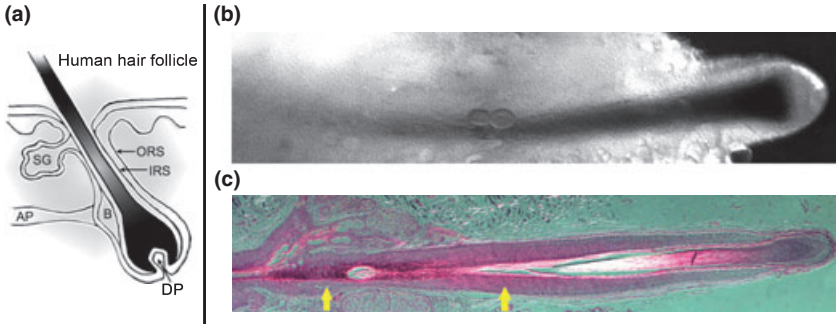

Figure 1. Images of intact hair follicle. (a) Scheme of a human hair follicle illustrating areas of the mini-organ: arrector pili muscle (AP), bulge (B), dermal papilla (DP), inner root sheath (IRS), outer root sheath (ORS) and sebaceous gland (SG). (b) Image of a human hair follicle, taken using a light microscope, immediately after plucking from skin tissue during the dissection process. (c) H&E-stained section of intact human hair follicle visualized using light microscopy. Yellow arrows indicate areas that are the bulge region of the hair follicle.

possibility of graft-versus-host disease (7). On the other hand, iPS cells, which are created through genetic modification of adult cells by viral transfection, or as recently reported, by direct delivery of reprogramming proteins (8), circumvent issues surrounding ES cells. However, iPS cells currently face their own challenges concerning therapeutic application and may not be available for clinical use in the near future (9). In contrast, HFSCs (i) do not bear any known risk of oncogenesis, (ii) are immune-privileged as autologous cells, and (iii) are located in well-known and easily accessible locations of the human body. Furthermore, beyond their innate capabilities for complete self-renewal, these cells have been characterized by their multipotency to differentiate into various lineages (10,11). This important attribute makes HFSCs valuable candidates for regenerative medicine beyond hair and skin regeneration, and topical wound healing.

The location of intrinsic stem cells within the hair follicle was first suggested by Cotsarelis *et al.* (12), as the outer root sheath layer of the bulge area of mouse vibrissae (whisker) hair follicles. Since then, other researchers have reported similar findings regarding location of HFSCs within both human and murine hair follicles (13–18). Research in murine hair follicle stem cells is quite advanced, and different groups have shown the multipotency of HFSCs by differentiating them into various lineages (19–22). The potential of using HFSCs in regenerative medicine, other than those immediately involved with hair or skin, has been highlighted by Amoh *et al.* (23), this group having used cells derived from murine HFSCs for structural and functional repair of spinal cord lesions. Unlike mouse hair follicles, where the bulge area is easy to detect due to its distinct projection, localization of the bulge area of human hair follicles has proved to be challenging, due to lack of obvious anatomical features that can be visualized using light microscopy (24). For this reason, along with relatively small size of the hair follicle, manual dissection of HFSCs from hair follicles has been difficult, and research into human HFSCs has been limited, although one group has reported validation of the cells' multipotency (25). As a solution, Ohyama *et al.* (26) suggested utilizing flow cytometry for sorting of cells from the bulge area, and isolating human HFSCs using CD200 mostly known for its role in autoimmunity (27) as the putative cell surface marker. Enrichment of cells using this marker has technical advantages over utility of cytokeratin-15 (CK15), a well-established but cytosolic marker of human and murine HFSCs, as the marker is not suitable for sorting of naïve live cells without their genetic manipulation (13,28). Comparing expression of CK15 and other known markers for HFSCs, Ohyama *et al.* characterized human HFSCs as being $CD200^{+}$, $CD24^{-}$, $CD34^{-}$, $CD71^{-}$ and $CD146^{-}$. Interestingly, CD34 has previously been reported as a putative marker of murine HFSCs (29). Exclusion was made for the human system, as $CD34^{+}$ cells have been mostly localized in the outer root sheath of the hair follicle below the insertion point of arrector pili muscle and therefore do not represent the bulge area (30). Subsequently, investigators have confirmed that human HFSCs can best be described as $CD200^{+}$ (31–33). For our purposes, we have decided to follow this approach in obtaining HFSCs, that is by isolating $CD200^{+}$ cells in the bulge area of human hair follicles.

Stem-cell therapy provides a powerful concept for regenerative medicine. However, in reality, it is often limited by (lack of) availability of multipotential stem cells that could be introduced, either systemically or directly to a site of injury; this also applies to HFSCs – the number of hair follicles available is, in most cases, restricted. Thus, there is considerable interest in expansion of HFSCs *in vitro*, prior to their subsequent autologous reintroduction back into the donor. It appears that, at present, this

matter is under-investigated, judged by the limited number of publications in this area; from this we assume that large-scale culture of hair follicle stem cells has not yet been attempted. Most previous publications using human HFSCs have been focused on the cells' colony-forming capabilities or their multipotential ability to differentiate into other lineages (25,26,33). Others have tried to increase numbers of bulge area cells *in toto*, without specific separation out of HFSCs (34). Here, we comprehensively report our efforts to expand these cells in culture, starting from minimal numbers of explanted human hair follicles to cause least possible collateral damage, as intended for a real-life situation, and with special attention to retention of the cells' multipotency over possible differentiation, as they proliferate.

Materials and methods

Culture of pre-derived stem cells

Commercially available human hair follicle stem cells (Celprogen, San Pedro, CA, USA), herein abbreviated as C-HFSCs, were cultured with in-house developed medium (see section following, for further details) to assess feasibility of growing hair follicle stem cells. As control, C-HFSCs were also cultured with Human Hair Follicle Maintenance Medium (Celprogen). For both types of media, all cultures were performed in two types of culture flasks, in parallel: (i) specially coated flasks designed for culture of C-HFSCs (Celprogen) and (ii) uncoated flasks (BD Biosciences, San Jose, CA, USA). In all conditions, culture media were replaced every 3–4 days, and cells were passaged every 10 days to support maximal growth. Cells were regularly monitored for both phenotype and cell count; phenotype was evaluated by light microscopy and cell counting was performed at time of each passage, with a haemocytometer, and using trypan blue staining to exclude dead cells from propagation statistics. For immunophenotyping, fractions of hair follicle stem cells were subjected to fluorescence-activated cell sorting (FACS).

Preparation of culture media

An in-house developed medium was used consisting of 3:1 mixture of Dulbecco's modification of Eagle's medium (DMEM) and Ham's F-12 medium (Mediatech, Manassas, VA, USA) supplemented with 5% foetal bovine serum (Mediatech), 1% Gluta-MAX supplement (Invitrogen, Carlsbad, CA, USA), 1% β-mercaptoethanol (Sigma, St Louis, MO, USA), 1% non-essential amino acids (Invitrogen) and 0.1% penicillin-streptomycin (Mediatech). 20 ng⁄ml basic fibroblast growth factor (Invitrogen) and 10 ng⁄ml epidermal growth factor (Invitrogen) were added immediately prior to feeding or passaging, to prevent adsorption of growth factors to medium storage containers.

Isolation of hair follicle stem cells

Hair follicles were originally obtained in the form of transected or intact hair follicles during microscopic hair transplant procedures, following obtaining informed consent. Hair follicles were derived from scalp skin of the occipital region of patients undergoing strip method hair transplant surgery. Protocols for processing human specimens were approved by the Institutional Review Board of the Cedars-Sinai Medical Center (IRB # Pro00012075). Isolation techniques were designed from previously established protocols for isolating bulge area cells from tissues and modified for specific aims of this research (25,26).

Human scalp tissues were first rinsed in lactated Ringer's solution and excess adipose tissue was removed, prior to treatment with 10 mg⁄ml dispase (BD Biosciences) overnight. Individual hair follicles were then extruded from tissues, and only bulge areas – located between isthmus and suprabulbar regions of the hair follicles – were isolated by use of spring scissors (Fig. 1a). Collected bulge areas were treated with 0.05% trypsin-EDTA (Mediatech) for up to 45 min at 37 °C. Following digestion, the bulge areas were physically triturated using a Pasteur pipette to generate a suspension of bulge area cells. Suspensions were subsequently filtered through 100 and 40 μm cell strainers (BD Biosciences) to remove tissue remnants. Fractions of such suspensions were characterized by FACS for cell population assessment.

Bulge area cells were further processed by magnetic selection with antibodies against CD200, the putative cell surface marker for HFSCs, using CELLection Biotin Binder Kit (Invitrogen), as previously reported (35). Briefly, bulge area cells were treated first with biotin-conjugated mouse monoclonal anti-CD200 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then $CD200^+$ cells were enriched using streptavidin-coated magnetic beads. $CD200^+$ cells were then detached from the magnetic beads using DNase I for enzymatic cleavage of a DNA linker between streptavidin and the beads. Hereafter, we will refer to this directly isolated population of human hair follicle stem cells as I-HFSCs, to distinguish them from C-HFSCs.

Histochemical tissue staining

Explanted hair follicles with attached surrounding tissue were fixed in 10% buffered formalin, and embedded in paraffin wax, then 5–10 μm sections were stained with

haematoxylin and eosin (H&E) by standard procedures for visual identification and assessment of structural integrity of hair follicles.

Culture of isolated stem cells

I-HFSCs were cultured on three types of substrate: bare polystyrene surface, laminin, and Matrigel basement membrane matrix (BD Biosciences). For laminin-coated surfaces, tissue culture plates were incubated at 37 °C with a solution of laminin in DMEM for 2 h to provide coating concentration of 5 μg/cm^2, followed by washing the culturing surface in phosphate-buffered saline (PBS) to remove any non-adsorbed protein. For Matrigel-coated surfaces, tissue culture plates were incubated at 37 °C in 1:1 mixture of Matrigel in culturing medium until it gelled. Before use, plates were washed in PBS, to remove non-solidified material.

Cultures were maintained for up to 4 weeks after initial seeding, media being changed every 3–4 days, and cells were passaged once every 2 weeks. Cells were counted at time of each passage. As before, all cell counts were performed using the trypan blue exclusion assay. For bare surfaces, entire medium was retrieved and centrifuged during media changes and passaging to prevent significant loss of cells, due to their weak attachment to bare plastic surfaces. For laminin and Matrigel-coated surfaces, half the culture medium was replaced with fresh medium during each medium change. For passaging, cells were dissociated from laminin-coated surfaces by treatment with 0.05% trypsin-EDTA for 5 min, while for Matrigel-coated surfaces, Matrigel was disintegrated by treatment with 1 mg/ml dispase in DMEM at 37 °C for 30 min, followed by aggressive mechanical trituration using Pasteur pipettes. To characterize cells, FACS was performed prior to seeding and after 4 weeks in culture to test for changes in phenotype.

Immunophenotyping

Immunophenotyping of cells was performed by FACS. Cells were fixed in 4% paraformaldehyde, blocked with 3% bovine serum albumin in PBS, and simultaneously stained with biotin-conjugated mouse monoclonal anti-CD200 and goat polyclonal anti-CD34 (GeneTex Inc., San Antonio, TX, USA). Cells were subsequently incubated in streptavidin-PE/Cy5 (BioLegend, San Diego, CA, USA) and donkey anti-goat IgG conjugated to PE/Cy7 (Santa Cruz Biotechnology), respectively. Stained cell populations were analysed by flow cytometry (Dako CyAn, Carpinteria, CA, USA) and retrieved data were analysed using the corresponding software (Dako Summit, Carpinteria, CA, USA).

Results

The objective of our study was to isolate human hair follicle stem cells from their original whole tissues and to culture these cells to ultimately characterize their population growth *in vitro*. For this purpose and to control and manipulate cell proliferation and differentiation, it was necessary to use medium whose components were known to us. To ensure that our new medium formulation would be successful in promoting cell proliferation, we set up and performed parallel cultures with a commercially available cell system.

Propagation of pre-derived stem cells

C-HFSCs were cultured for 8 weeks in two types of medium, maintenance medium provided by the supplier of the C-HFSCs, and our in-house developed medium. Over culture periods, we did not observe significant differences in cell proliferation between cultures using different media (data not shown). Expansion rates and related doubling times of C-HFSCs cultured with our in-house developed medium are shown in Table 1. As mentioned above, cultures grew on two different surfaces. In all four combinations (two media, two surfaces), we found one commonality between the various culturing conditions – C-HFSCs did not attach strongly to culture substrates. Instead, these cells settled on culture-flask surfaces, forming only loose attachment, and did not develop typical adhesion cell surface features; this characteristic was observed over the entire duration of the cultures. Thus, to prevent any significant loss of cells during medium exchange, all media were retrieved and centrifuged. Trypan blue exclusion assays of cells showed that the majority of counted cells were intact, verifying that C-HFSCs were still viable, despite lack of solid attachment to substrates.

FACS of C-HFSCs were performed at two different time points, 3 and 50 days in culture after initial seeding, for cells grown in our in-house-developed medium. Analysis of results showed no significant differences in signal/cell distribution (Fig. 2), suggesting that phenotype of the cells did not change over duration of cultures. This observation was consistent with evidence obtained by visual monitoring of the cells, where we saw no significant changes in shape and/or size of cells, nor in their attachment behaviour.

In summary, our results indicated that our in-house-developed culture medium was able to allow propagation of C-HFSCs, albeit at a low rate, but without differentiation. We therefore concluded that our newly derived medium was suitable as base medium for the following studies with tissue extracted cells (I-HFSC).

Table 1. Expansion of C-HFSC. Expansion of C-HFSCs calculated as ratio of growth rate from day 0, and respective doubling time of the cells

Days	0	4	11	15	21	25	32	38
Growth rate (D0 = 1)	1	1.27 ± 0.18	1.39 ± 0.13	1.45 ± 0.14	1.90 ± 0.32	2.35 ± 0.40	2.62 ± 0.43	2.88 ± 0.63
Doubling time (days)	–	11.7	23.1	27.8	22.7	19.5	22.3	24.9

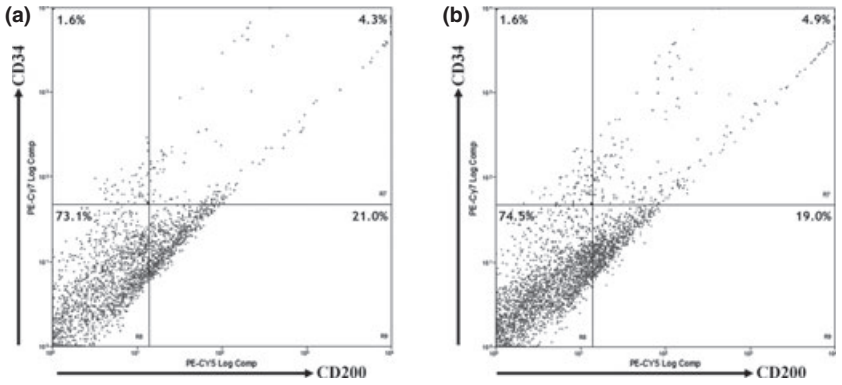

Figure 2. FACS analysis of C-HFSC. For dual-fluorophore plots, *X*-axis represents CD200 conjugated with PE-Cy5 and *Y*-axis represents CD34 conjugated with PE-Cy7. Cultures were grown in in-house developed medium on bare tissue culture flask for (a) 3 days, and (b) 50 days after initial seeding. During this time, percentage of $CD200^+$ cells did not change significantly (25.3% at day 3 and 23.9% at day 50).

Population growth behaviour of extracted hair follicle stem cells

I-HFSCs were extracted from bulge areas of human hair follicles – the area known to contain purported hair follicle stem cells – as highlighted in H&E-stained images of intact hair follicles, as shown in Fig. 1c. On average, 60–100 intact hair follicles were obtained and dissected for each experiment. Around 1000 cells were obtained per intact hair follicle following completion of the entire isolation process.

Separation of $CD200^+$ cells was accomplished using appropriately coated magnetic beads. As illustrated in Fig. 3a–d, there were significant differences in population homogeneity between bulge area cell suspensions that contained all extracted cells from the bulge area and a subpopulation of cells enriched for CD200 presentation. Effects of separation can be seen most distinctly in respective distributions in forward scatter of flow sorting (Fig. 3b,d): wide distribution in forward scatter is evident for extracted mixed population, prior to magnetic separation, while $CD200^+$-enriched population is represented by narrower forward scatter region. This result suggests that cells that are smaller in size (a description that better matches stem cells in comparison to other tissue cells present in the excised bulge area, such as epithelial cells and keratinocytes), are the ones that are positively selected. Concordantly, our approach enriched CD200-positive cells from initially 43.3% of total isolated cells to 78.2% in the purified population.

As mentioned earlier, pre-derived C-HFSCs did not attach strongly to culture surfaces; this phenomenon was also observed with I-HFSCs. To avoid problems of having to retain waste materials throughout the culture, we decided to expand cells on a substrate that would promote strong attachment of cells to the support surface and would be inert enough to not influence growth or induce differentiation. We have solved this problem by introducing either laminin or Matrigel as a supporting substrate. These surfaces were able to provide good attachment for cells compared to uncoated culture flask surfaces. Rate of expansion and related doubling times of I-HFSCs proliferating on different substrates are shown in Table 2 and Fig. 4. Cells were grown as a monolayer on both blank and laminin-coated substrates, while they were embedded in Matrigel-coated substrates. Images of I-HFSCs in culture on different substrates are shown in Fig. 5. As seen in these images, there were no significant changes in apparent morphology of the cells over culture duration.

Overall, Matrigel-coated surfaces provided best cell proliferation and retention, as demonstrated by the highest growth rates of the three substrates, with average doubling rate of around 38 days. Matrigel-coated surface allowed cell population expansion without potential of cumulative losses of significant numbers of cells during subsequent medium changes or cell passages, which was important considering the general dearth in numbers of starting material. Even with laminin-coated substrates, we encountered some cell loss during processing, as evidenced by lower growth statistics compared to I-HFSCs grown on Matrigel-coated surfaces.

Similar to the results we obtained for pre-derived stem cells, there were no significant changes in morphological and molecular phenotypes of cells during culture, as

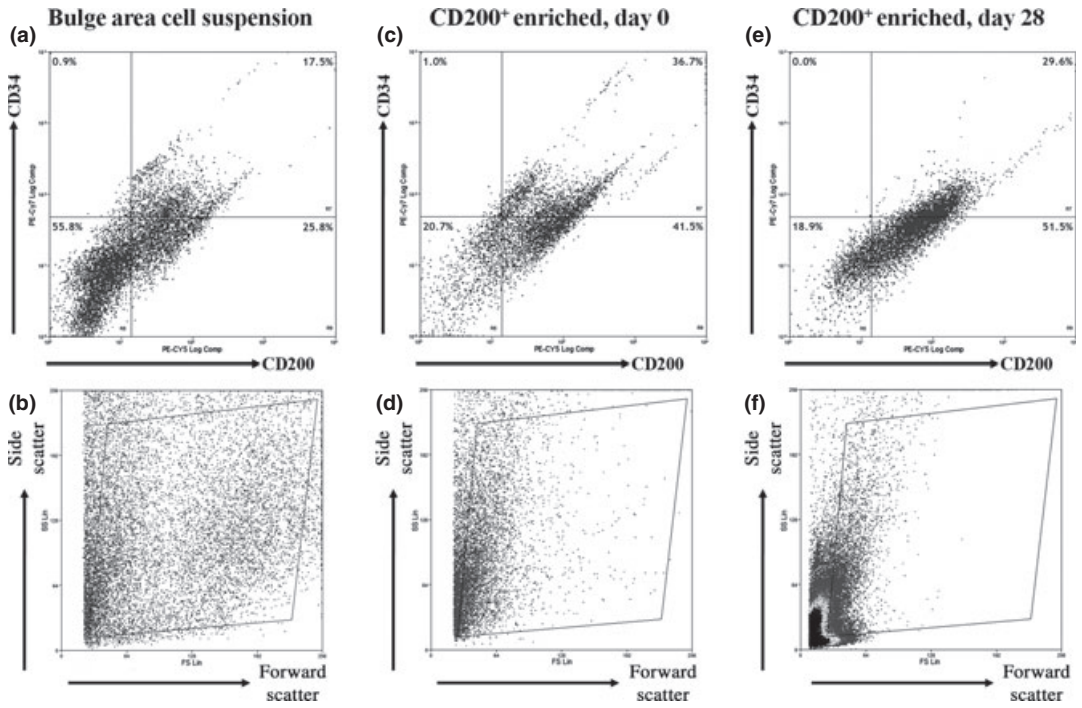

Figure 3. FACS of I-HFSC. For dual-fluorophore plots, *X*-axis represents CD200 conjugated with PE-Cy5 and *Y*-axis represents CD34 conjugated with PE-Cy7. For scatter plots, *X*-axis represents forward scatter and *Y*-axis represents side scatter. (a, b) Bulge area cell suspensions were subjected to flow cytometry after dissection to generate plots shown. (c, d) Similarly, $CD200^+$-enriched cell populations were analysed immediately after magnetic separation. (e, f) I-HFSCs grown for 28 days on Matrigel-coated substrates were recovered and subjected to flow cytometry. Proportions of $CD200^+$ were greater in (c) I-HFSCs immediately after magnetic separation and (e) I-HFSCs cultured for 28 days, compared to (a) bulge area cell suspension. There are no significant differences in distribution of proportions for (c) I-HFSCs immediately after magnetic separation and (e) after 28 days in culture. Also, comparing scatter information, forward scatter of (b) the bulge area cell suspension differs from both, (d) I-HFSCs immediately after magnetic separation and (f) I-HFSCs cultures for 28 days.

Table 2. Expansion of I-HFSC. Expansion of I-HFSCs is calculated as ratio of growth rate from day 0 and respective cell doubling time

Days	0	14	28
Blank			
Growth rate (D0 = 1)	1	1.20	1.44
Doubling time (days)	–	52.3	52.7
Laminin			
Growth rate (D0 = 1)	1	1.34 ± 0.18	1.53 ± 0.07
Doubling time (days)	–	38.3	46.3
Matrigel			
Growth rate (D0 = 1)	1	1.35 ± 0.15	1.73 ± 0.22
Doubling time (days)	–	38.5	38.0

revealed by light microscopy (Fig. 5) and verified by flow cytometry (Fig. 3). Comparison of FACS results in Fig. 3c,e illustrates that there were no significant changes in proportion of stem cells between the cell population analysed immediately after isolation and separation, and the same population after 28 days culture on Matrigel-coated substrate. Furthermore, distributions of forward scatter, that is, size of cells in the population, were similar (Fig. 3d,f). Compared to forward scatter distribution for bulge area cell suspension, shown in Fig. 3b, distributions for both isolated $CD200^+$ samples (before and after 4 weeks in culture) were quite different. These results underline the fact that with our culture media formulation, I-HFSCs remained viable without differentiation, over the 4 weeks of cell culture.

Discussion

The original focus of our work was to expand human hair follicle stem cells *in vitro* to become numbers suitable for purposes of stem-cell therapy, usually introduced through direct injection into the site of application. Number of cells injected, as reported for such treatment have been in the magnitude of millions of cells (36,37). As an individual intact hair follicle contains putative stem cells in the order of hundreds of cells (15,38), we were specially interested in cell number expansion without differentiation. An important consideration in our objective was to obtain numbers of multipotential cells needed for regenerative medicine, while causing the least amount of collateral dam-

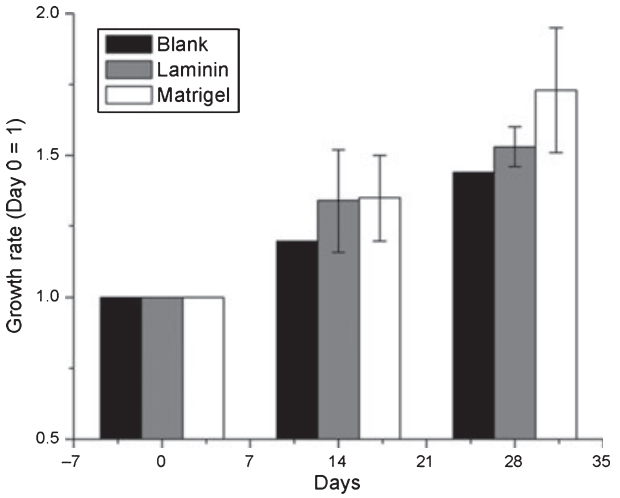

Figure 4. Expansion of I-HFSC. Values are expressed as ratio of total cells from initial seeding level at day 0 of cultures and related error bars. I-HFSCs cultured on blank polystyrene culture surfaces (black bars) had lowest level of population growth, while cells cultured on laminin-coated surfaces (gray bars) Matrigel-coated surfaces (white bars) showed the highest level of population growth. Differences in cell population growth rates between those on uncoated and coated surfaces are highly attributable to weak attachment of I-HFSCs to uncoated surfaces.

age; in other words, while it would also be possible to extract cells from eventually several thousands of intact hair follicles, an attractive therapy should not lead to significantly more follicle-depleted areas on the scalp beyond the occipital region. For this reason, we focused only on a limited quantity of starting material, to stay within the scope of medical demand. Another important factor in our attempts to expand numbers of hair follicle stem cells was to prevent differentiation. Hair follicle stem cells have previously been differentiated into various lineages other than cells involved in the hair growth mechanism. These include neuronal, glial and smooth muscle cells, as well as keratinocytes and melanocytes (17,21,25). As many reports indicate, this multipotency may become significantly reduced if cells begin to differentiate into precursor cells of a particular lineage. Thus, in the search for an appropriate medium for non-differentiating propagation of hair follicle-derived stem cells, we developed a culture medium closely related to maintenance media for human embryonic stem cells. As these kinds of media usually lack factors that support differentiation and subsequent exponential proliferation, a trade-off had to be made in favour of retaining multipotency of these cells, and this may have attenuated their proliferative capacity. This new medium formulation was first assessed using commercially available pre-derived stem cells (C-HFSCs), as materials from human hair follicles are extremely valuable and not abundant enough for testing purposes. These cells mostly remained loosely attached, even in specially coated culture flasks purchased from the supplier of the cells and using supplier's expansion medium. Nevertheless, through observation with trypan blue exclusion assay, we verified that the majority of cells still remained viable cultures of C-HFSCs, maintained for up to 50 days, during which the cell population's growth rate remained relatively consistent, and FACS results showed no significant change in proportions of $CD200^{+}$ and $CD34^{-}$ cells. This fraction was believed to contain hair follicle stem cells, as previously

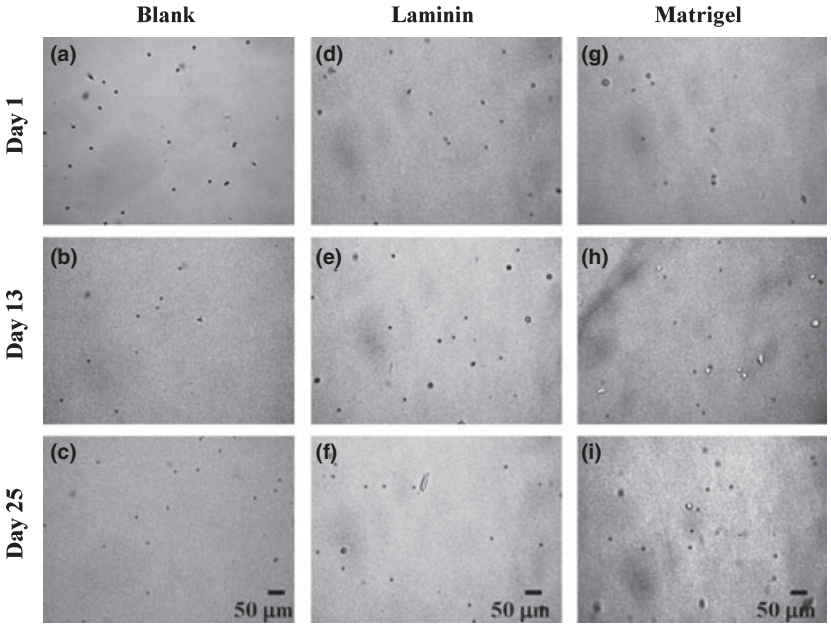

Figure 5. Cultured I-HFSC. I-HFSCs were cultured on blank polystyrene culture surfaces (a–c), laminin-coated surface (d–f), and Matrigel-coated surface (g–i). Images of cultured cells were taken during active culture within their respective flasks. Images were magnified using a 20× objective lens and taken (using a camera attached to a light microscope) at 1 day, 13 days, and 25 days after initial seeding. No significant differences in attachment behaviour and morphology are visible in the respective culture conditions.

reported in several publications (26,31–33,39). We conclude that the culture medium we have adapted to grow hair follicle stem cells showed potential for expanding cell numbers, without detectably altering their morphology, and disparate representation of the two cell markers in the growing population, over a long time period.

The main component of our investigations, hair follicle stem cells, were isolated from intact human hair follicles obtained from superfluous material after hair transplant surgery, and as such, amounts of starting material were limited (60–100 hair follicles per session). This limitation closely mimics real-life situations in which follicles utilized as an autologous source for multipotential adult stem cells, need to be expanded for cell-based therapy. HFSCs are well-known to be highly concentrated in the bulge region of the hair follicle, and therefore, we only targeted this area by manual dissection, followed by cell extraction and enrichment for $CD200^+$ cells. As seen in FACS results (shown in Fig. 3a,c), we succeeded in enriching these cells from 43.3% in the bulge area cell suspension to 78.2% in $CD200^+$-enriched population of cells, by magnetic separation. The fraction of $CD200^+$, $CD34^-$ cells, which would be considered the purest population of hair follicle stem cells, as previously mentioned, also increased from 25.8% in the bulge area cell suspension to 41.5% in the $CD200^+$-enriched population of cells. Furthermore, enriched cells had limited range of forward scatter in FACS, suggesting relatively uniform size distribution among $CD200^+$ cells compared to the global fraction of bulge area cells. This further proves that positive selection results in a much more uniform cell population.

Previous publications document attempts to isolate hair follicle stem cells using a two-step magnetic process, involving positive-isolation of $CD200^+$ cells followed by negative exclusion of cells with surface markers CD24, CD34, CD71, or CD146 (26). In our study, we decided to perform only $CD200^+$ isolation with twofold justification. First, it would have been challenging to yield effective negative selection with such low numbers of total cells as we had obtained from the first positive isolation step (given the limited amount of starting material). Second and more importantly, currently there is no conclusive evidence that $CD200^+$ cells with co-presentation of the aforementioned markers would exclude them from being multipotential stem cells. Promiscuity in cell surface marker presentation is a general matter in stem-cell characterization and application that is being seriously investigated in the field of stem-cell biology in humans and model organisms (40). According to previously published works on mouse vibrissa follicle stem cells, there are two distinct populations of stem cells – basal and suprabasal bulge cells with different surface marker characteristics (10). Another recent hypothesis has suggested existence of both multipotential and monopotential populations of cells from the bulge area of the mouse (41). Furthermore, CD34, which has previously been suggested as putative marker for hair follicle stem cells in mouse, is not expressed in human hair follicle stem cells, while CD200 is expressed in both human and mouse hair follicle stem cells (42). In our opinion, more studies are necessary to conclude the nature and applicability of $CD200^+$ cells (from the bulge area), which are positive for other cell surface markers, including CD34. Therefore in our study, we considered $CD200^+$ and $CD34^+$ as part of I-HFSCs. This led to a larger fraction of isolated and proliferating double-positive cells indeed, as seen in Fig. 3c,e. Our results show that proportions of these cells did not change *in vitro* over the period of culture, even though sum of the two types of cells slightly increased: (a) bulge area cell suspension, 25.8% ($CD200^+$, $CD34^-$) and 17.5% ($CD200^+$, $CD34^+$) and (b) magnetically enriched cells, 41.5% ($CD200^+$, $CD34^-$) and 36.7% ($CD200^+$, $CD34^+$). We therefore assume that both types of $CD200^+$ cells grew at the same rate and gave rise each to its own progeny.

Cultures of I-HFSCs (maintained for several weeks) led to similar results, as experienced with pre-derived C-HFSCs: the media we developed did not cause any change in parameters we assessed over the entire duration of the cultures (Fig. 3e). More precisely, cell population diversity remained similar, and specifically, the proportion of $CD200^+$ cells remained constant at around 80% (Fig. 5d,f). These results suggest that there was no biased proliferation of cells of different sizes and that morphology of $CD200^+$-enriched hair follicle stem cells did not detectably change under the conditions provided *in vitro*. Overall, growth rate was lower in directly isolated hair follicle stem cells compared to the purchased counterparts. Some of these differences may be attributed to the proportion of $CD200^+$ cells in each group $\sim$80% of I-HFSCs, compared to only $\sim$25% for C-HFSCs. As the proportion of $CD200^+$ cells did not change significantly over time for either cell source, differences did not suggest higher population growth rate of $CD200^+$ over $CD200^-$ cells. As the biological sources of the supplied C-HFSCs were unknown to us, there is no need to proceed into further speculation about them.

To the best of our knowledge, there is no published evidence regarding large-scale expansion of human hair follicle stem cells *in vitro*. Previously published studies mostly reported ability of the cells to form colonies and did not describe any large-scale cell proliferation (26,33,34,38,43). There have been studies with epithelial stem cells originating from human foreskin that focused on their population expansion in culture and subsequent differentiation, but these types of cells are not identical in their behaviour to the hair follicle stem-cell models used

here (44–46). There have also been studies in which hair follicle stem cells were immortalized and grown as a cell line (47,48). In this sense, our research has highlighted differences from previous publications in both scope and focus. Our initial motivation to grow human hair follicle stem cells *in vitro* – without affecting their morphology and $CD200^+$ phenotype, as potential indicators of multipotency – towards adequate numbers for cell-based therapy, was challenged by the fact that growth rate of the HFSCs was much lower than we had anticipated. The initial expectation was to isolate up to 2×10^4 multipotent stem cells from around 100 donor hair follicles and expand them up to 100-fold in number so that we could obtain a therapeutically reasonable dose for injection. What we have observed in our investigation is along the lines of a two-fold expansion over around 30 days, which necessitates donation in excess of 1500 hair follicles, a conclusion that may interfere with the attraction of this type of approach for regenerative medicine. Nevertheless, we have discovered two properties of the $CD200^+$ cells: (i) they proliferated at an extremely low pace and (ii) the possibility of maintaining HFSCs *in vitro* over significant periods without altering their above mentioned parameters; therefore, it is tempting to speculate that they may also retain their multipotency during extended culture times. However, more criteria need to be considered in future cell monitoring, including more comprehensive immunophenotyping before and after induced lineage-specific differentiation and karyotype analysis to assess consistency of the cells during *in vitro* propagation. If proven to be true, the so far revealed properties of our culture regimen can be beneficial to investigative areas in biomedical research other than for direct application in regenerative medicine. Especially, when considering current difficulties in culturing stem cells, while avoiding spontaneous differentiation during *in vitro* proliferation, our results suggest that it is possible to keep this type of stem-like cells in a slowly proliferating state with the medium formulation we have developed. HFSCs cultured in our media formulation could also provide a valuable robust model system for study of biological mechanisms by live-cell and time-lapse imaging. As stem cells are becoming important tools in drug discovery and disease treatment (49), this combination of cells and medium can be utilized in drug discovery, when compounds are tested in cell-based assays that span several days and, eventually, weeks, in which spontaneous differentiation that might skew the biological data, needs to be avoided.

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