A Human Folliculoid Microsphere Assay for Exploring Epithelial– Mesenchymal Interactions in the Human Hair Follicle

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The search for more effective drugs for the management of common hair growth disorders remains a top priority, both for clinical dermatology and industry. In this pilot study, we report a pragmatic organotypic assay for basic and applied hair research. The patented technique produces microdroplets, which generate human folliculoid microspheres (HFMs), consisting of human dermal papilla fibroblasts and outer root sheath keratinocytes within an extracellular matrix that simulates elements of the hair follicle mesenchyme. Studying a number of different markers (for example, proliferation, apoptosis, cytokeratin-6, versican), we show that these HFMs, cultured under well-defined conditions, retain several essential epithelial–mesenchymal interactions characteristic for human scalp hair follicle. Selected, recognized hair growth-modulatory agents modulate these parameters in a manner that suggests that HFMs allow the standardized preclinical assessment of test agents on relevant human hair growth markers under substantially simplified *in vitro* conditions that approximate the *in vivo* situation. Furthermore, we show by immunohistochemistry, reverse transcriptase–PCR, and DNA microarray techniques that HFMs also offer a useful discovery tool for the identification of target genes and their products for candidate hair drugs. HFM thus represent an instructive modern experimental and screening tool for basic and applied hair research in the human system.

INTRODUCTION

The loss of scalp hair (effluvium, alopecia) can be accompanied by severe psychological problems in a vastly underestimated number of afflicted patients (Hadshiew *et al.*, 2004). Therefore the development of ever new and hopefully more effective hair drugs for the management of common hair growth disorders remains a top priority both for clinical

dermatology and industry (Paus, 2006). The search for such agents is, however, severely handicapped by the lack of satisfactory three dimensional (3D) *in vitro* screening systems that sufficiently mimic important epithelial–mesenchymal interactions as they occur in human hair follicles (HFs). Therefore, pragmatic 3D screening systems are badly needed, whose preservation of native epithelial–mesenchymal interactions is superior to simple coculture assays for isolated HF cell populations in which these interactions are disrupted (Limat *et al.*, 1993; Roh *et al.*, 2004). Hence data produced with such cell culture studies reflect highly artificial conditions, carry very uncertain predictive value for the clinical situation, and are therefore inappropriate for the screening purposes in question here.

The complexity of epithelial–mesenchymal interactions that underlie HF growth and cycling (Paus and Cotsarelis, 1999; Stenn and Paus, 2001; Paus and Foitzik, 2004, Rendl *et al.*, 2005) makes it unlikely that these will ever be fully reproduced *in vitro*. Nevertheless, it should be possible to develop 3D systems that: (1) imitate at least some essential interactions characteristic for human HF biology; (2) show expected responses to recognized hair growth-modulatory agents; and (3) have a reasonable predictive value for how human HFs will respond to the same test agent *in vivo*. For preclinical research and development purposes, such simplified 3D folliculoid systems would offer a first-line screening tool for large-scale *in vitro* testing, to be followed by the organ culture of

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Abbreviations: CsA, cyclosporin A; DPC, dermal papilla fibroblast; HDF, human dermal fibroblast; HF, hair follicle; HFM, human folliculoid microsphere; HGF, hepatocyte growth factor; LDH, lactate-dehydrogenase; ORSK, outer root sheath keratinocyte; RT–PCR, reverse transcriptase–PCR; SCF, stem cell factor; TGFβ2, transforming growth factor-β2

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microdissected human anagen hair bulbs (Philpott *et al.*, 1990) and histocultures of hair-bearing skin, that is, microdissected normal skin (Li *et al.*, 1991, 1992; Lu *et al.*, 2007) for the most promising agents that have been identified in this manner as a second-line assay, and eventual clinical testing as the ultimate and only fully reliable test system.

Previously, we have defined basic criteria that all hair biology-related organotypic systems should meet to support the claim that they mimic the *in vivo* situation as closely as possible (Havlickova *et al.*, 2004). Briefly, these criteria are: outer root sheath keratinocytes (ORSK) and follicular dermal papilla fibroblasts (DPC) should be physically interacting; the extracellular matrix through which this occurs should contain basement membrane components; the epithelial HF cells should form cell aggregates; epithelial HF cells should show substantial proliferation as well as HF-type keratinization and a low level of apoptosis; mesenchymal cells should show minimal proliferation, minimal apoptosis and should display specialized HF-type secretory activities; and these organotypic systems should be cultured under continuously submerged culture conditions in well-defined media (that is, under serum-free conditions and with a defined concentration of calcium ions).

Using these criteria, we have developed two folliculoid organotypic systems (Havlickova *et al.*, 2004). In one of these systems, a 3D pseudodermis (collagen I mixed with human dermal fibroblasts; HDF) is first generated, then Matrigel with DPC is mixed and layered on top of the pseudodermis, followed by an ORSK cell suspension ("layered sandwich"). Alternatively, a mixture of Matrigel, DPC, and ORSK is placed on top of a pseudodermis ("mixed sandwich"). Comparison of these systems indicated that the "mixed sandwich" system cultivated under continuously submerged condition in serum-free, low-calcium medium meets all basic criteria and offers several advantages over previously available assays (Limat *et al*. 1994a, b; Havlickova *et al.*, 2004).

However, these assays are still very laborious and time consuming, and only a very limited number of such "sandwiches" can be generated at any time, thus hampering the usefulness of this system as a higher throughput screening tool. Therefore, in this pilot study, our goal was to develop a more easy-to-handle *in vitro* assay which:

(1) requires lower cell numbers (especially of DPC) per test unit;
(2) is easier and faster to prepare than previously published 3D folliculoid systems;
(3) is well reproducible with a large number of test units;
(4) is less expensive;
(5) responds to recognized hair growth-modulatory drugs in a manner that at least approximates the appreciated clinical response to these agents; and
(6) functions as a discovery tool for identifying new target genes and/or their protein products for candidate hair growth-modulatory agents.

In this pilot study, we report a pragmatic human folliculoid microsphere (HFM) assay that meets all the basic and modified prerequisites for *in vitro* higher throughput screening systems for preclinical candidate hair drug screening.

RESULTS

Characteristics of interacting human HF-derived epithelial and mesenchymal cells in HFM

We first investigated the characteristics of the cells within the HFM system (Figure 1a). We found that ORSK and DPC, forming spheroid cell aggregates, were indeed in close physical contact in HFM (Figure 1b and c) and that the employed extracellular matrix indeed contained basement membrane components such as fibronectin (Figure 1d and e). In addition, epithelial ORSK showed HF-type keratinization (that is, CK6 expression; Figure 1g, h, k, and l), substantial proliferation (that is, Ki67 expression in the CK6-positive cells; Figures 1k and 2a), and a low level of apoptosis (number of TUNEL-positive ORSK; Figures 1l and 2c). Instead, the HF mesenchymal DPC exhibited minimal proliferation and apoptosis (Figures 1m and n, 2b and d) but maintained their characteristic, specific secretory activity, that is, they displayed strong expression of the large proteoglycan versican (Soma *et al.*, 2005; Figure 1i, j, m, and n).

In most of our experiments, in accordance with our findings in the previously developed "sandwich" 3D systems (Havlickova *et al.*, 2004), we used serum-free culturing media that contained low concentrations of calcium (Ca) (0.15 mM). However, we also intended to determine the biological features of the cells in the HFM cultured in high-calcium concentration solutions (1.8 mM). During the culture period of 10 days, we found no difference in proliferation and apoptosis of ORSK in the two different media (Figure 2a and c). In contrast, both the proliferation and the apoptosis of DPC significantly increased in high-Ca medium compared to low-Ca solution (Figure 2b and d).

Furthermore, we investigated the processes of possible necrosis by measuring the lactate-dehydrogenase (LDH) release during the culture period of 10 days in low-Ca media. The maximum amount of LDH ($20\,U\,l^{-1}$) was seen on day 3 when the system was (most probably) still under the "stress" occurred during the preparation of HFM. On days 7 and 10, the LDH levels were normalized which suggested the full cell recovery in HFMs (Figure 2e).

We conclude, therefore, that HFMs meet all the basic criteria for organotypic folliculoid systems, that they can be successfully cultivated under continuously submerged culture conditions (most efficiently in low-Ca media) and that they remain vital for at least 10 days.

Hair folliculoid HFM show several advantages over folliculoid "sandwich" systems

Comparison of the current data with those obtained by previous "sandwich" systems also revealed that the preparation of microspheres:

(1) requires lower number, yet higher density of cells (especially DPC) per test unit. Although the density of ORSK was essentially the same (1×10^6 cells per ml) both in the "sandwich" and HFM systems, we could achieve double DPC density (2×10^6 cells per ml) in the HFM. In addition, the remarkably higher number of test units that can be obtained using 1 ml of cell suspension

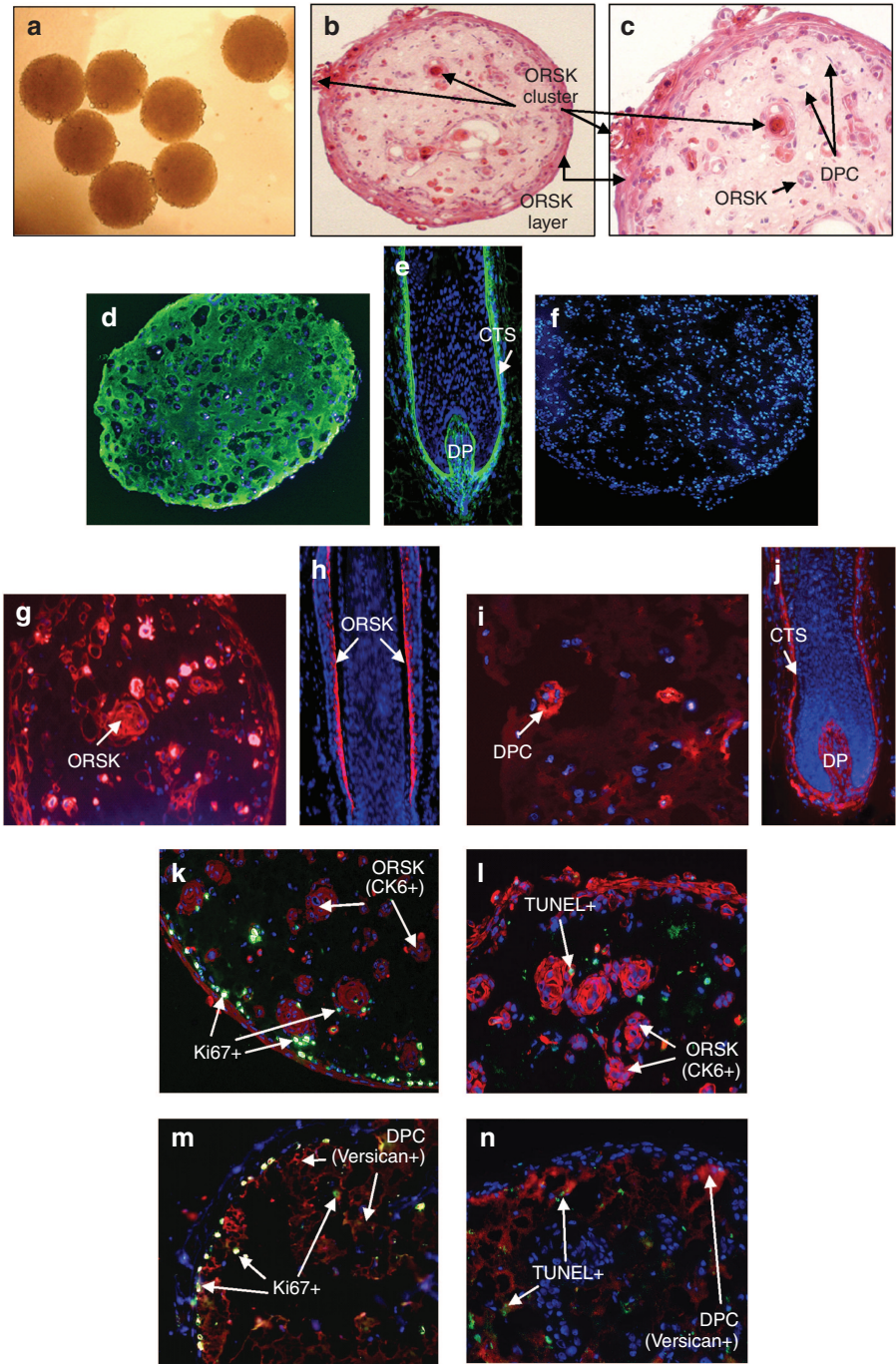**Figure 1. Microscopic photography and histology of the microspheres and the expression of certain markers in cells and matrix of HFM.** (**a**) Photomicrographs of HFMs in culture at day 10. (**b**, **c**) Histology of HFM structure (hematoxylin-eosin staining). ORSK, outer root sheath keratinocytes; DPC, dermal papilla cells. Note the physical contact of ORKS and DPC in HFMs. (**d**, **e**) Expression of fibronectin (as revealed by FITC immunostaining, green fluorescence) in HFMs (**d**) and in microdissected HF used as a positive control (**e**) DP, dermal papilla; CTS, connective tissue sheath. (**f**) Negative control, the primary antibody was omitted. (**g**, **h**) Expression of the ORSK marker CK6 (as revealed by rhodamine immunostaining, red fluorescence) in HFMs (**g**) and in microdissected HF (**h**). (**i**, **j**) Expression of the large proteoglycan versican (DPC marker; as revealed by rhodamine immunostaining, red fluorescence) in HFMs (**i**) and in microdissected HF (**j**). (**k**, **l**) Double immunolabeling of the ORSK marker CK6 (as revealed by rhodamine immunostaining, red fluorescence) with the proliferation marker Ki67 (**k**) or with the apoptosis marker TUNEL (**l**) (in both cases, FITC immunostaining, green fluorescence). (**m**, **n**) Double immunolabeling of the DPC marker versican (as revealed by rhodamine immunostaining, red fluorescence) with the proliferation marker Ki67 (**m**) or with the apoptosis marker TUNEL (**n**) (in both cases, FITC immunostaining, green fluorescence). Original magnification, ×40 for (**a**); ×100 for (**b**, **d–f**, **h**, **j**); ×250 for (**c**, **g**, **i**, **k-n**). For (**d–n**), nuclei were counterstained with DAPI (blue fluorescence).

in the HFM system (that is, 30–50 HFMs compared to the 10 ''sandwiches'') also suggests that the same amount of test units can be prepared using much fewer cells;

(2) is easier and faster. The average time to prepare the HFMs is only few hours compared to the approximately 5-day-long preparation time minimally needed for any
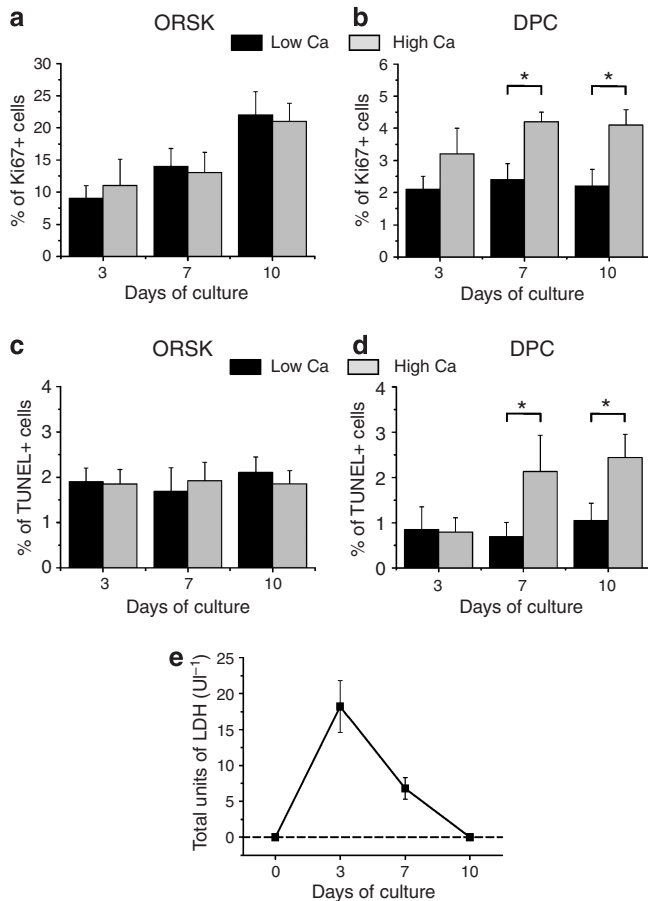
Figure 2. **Quantitative analysis of proliferation and apoptosis of ORSK and DPC in the HFM cultured in low or high calcium concentration media for 10 days.** Series of double immunolabeling were performed to define the number of Ki67-positive (proliferating, **a** and **b**) and TUNEL-positive (apoptotic, **c** and **d**) cells in CK6 expressing outer root sheath keratinocytes (ORSK, **a** and **c**) or versican expressing dermal papilla fibroblasts (DPC, **b** and **d**), as described in Materials and methods section. The numbers of double positive cells (Ki67+/CK6+, TUNEL+/CK6+, Ki67+/versican+, TUNEL+/versican+) in each group were determined, and expressed as a percentage of total number of cells expressing the respective marker for ORSK (CK6+) or for DPC (versican+). All data are shown as mean ± SD. Asterisks mark significant (*$P < 0.05$) differences. (**e**) Determination of level of the released lactate dehydrogenase (LDH) during culturing. All data are shown as mean value ± SD.

of the two ''sandwich'' systems presented before (Havlickova et al., 2004);

(3) is easily repeatable with a higher number of test units, which allows a higher degree of standardization and greatly facilitates automatization. In addition, the HFM system allows for the preparation of test units with variable sizes, depending on specific experimental needs;

(4) and due to the dramatic decrease in culturing time and consumables, HFM preparation is less expensive.

Negative regulators of HF growth differentially inhibit proliferation and induce apoptosis in cells growing in HFMs
In the next phase of our experiments, we assessed whether the HFM system is also suitable to analyze the actions of

recognized hair growth-modulatory drugs (the effects of all agents examined are summarized in Tables 1 and 2). We first investigated the effects of the potent HF growth inhibitory agents, that is, 10^{-6} M tretinoin (Foitzik et al., 2005), 25 ng ml^{-1} transforming growth factor-β2 (TGFβ2; Soma et al., 2002; Hibino and Nishiyama, 2004), and 10^{-7} M corticotropin-releasing hormone (CRH) (Slominski et al., 2000; Ito et al., 2005; Table 1). As expected, all agents significantly decreased the number of Ki67-positive (hence proliferating) cells in the CK6-positive ORSK (Figures 3a and 4a; Table 1a). In addition, tretinoin (Figure 3c) and TGFβ2 (Figure 4c), but not CRH (Table 1b), increased the number of TUNEL-positive ORSK, suggesting stimulation of apoptosis. Furthermore, TGFβ2 (Figure 4b) suppressed the number of Ki67-positive DPC, whereas the other two agents did not affect the proliferation of these cells (Figure 3b; Table 1a). Finally, tretinoin (Figure 3d) and TGFβ2 (Figure 4d) stimulated apoptosis in DPC as well, whereas CRH, similar to its lack of action as seen on ORSK, did not affect the process (Table 1b).

Positive regulators of HF growth stimulate proliferation of ORSK and DPC in HFMs without affecting apoptosis
We then investigated the effect of recognized HF growth-promoting agents, that is, insulin-like growth factor I (IGF-I) (Philpott et al., 1994), 1α,25 dihydroxyvitamin D3 (1,25(OH)2D3, calcitriol; Harmon and Nevins, 1994; Schilli et al., 1998; Vegesna et al., 2002), cyclosporin A (CsA; Kurata et al., 1996), and hepatocyte growth factor (HGF; Jindo et al., 1995; Table 2). Applications of 100 ng ml^{-1} of IGF-I, 10^{-8} M calcitriol, 10 ng ml^{-1} CsA, and 10 ng ml^{-1} HGF resulted in very similar modifications in the functions of the cells in the microspheres (Table 2). Namely, all agents increased the proliferation (elevated the number of Ki67-positive cells (Table 2a)) both in the ORSK (Figures 5a and c, 6a and c) and DPC (Figures 5b and d, 6b and d) populations without exerting any measurable effect on the apoptosis of these cells (Table 2b).

The effects of CsA and HGF are modified by the alteration in the calcium concentration of the culturing media
The above data, as mentioned before, were obtained in such experiments where low-Ca media were used to culture HFMs. Previous studies, however, suggested that the effects of CsA (Takahashi and Kamimura, 2001) and HGF (Sato et al., 1995) on the proliferation of keratinocytes were strongly affected by the calcium content of the culturing media. Therefore, these molecules were also tested on HFM cultured under high-Ca conditions. In marked contrast to data obtained in the low-Ca medium, 10 ng ml^{-1} CsA in high-Ca solution did not significantly stimulate the proliferation of ORSK and DPC (Table 2a) but, intriguingly, induced apoptosis in the ORSK (Table 2b). The effects of HGF (10 ng ml^{-1}) to stimulate proliferation of ORSK (Table 2a) and not to modify apoptosis of ORSK and DPC (Table 2b) were essentially the same in the two media. However, contrary to the low-Ca data, HGF was unable to promote proliferation of DPC in HFM cultured in high-Ca solution (Table 2a). These

Table 1. The effect of hair growth inhibitors on proliferation (a) and apoptosis (b) of ORSK and DPC in the HFM

(a) Effects of hair growth inhibitors on proliferation

Days of culture	Ki67-positive cells in ORS (% of control)		Ki67-positive cells in DPC (% of control)	
	Day 7	Day 10	Day 7	Day 10
Tretinoin	40.9 ± 23 ↓**	34.4 ± 26 ↓**	100.5 ± 23	98.2 ± 27
TGFβ$_2$	48.4 ± 19 ↓*	37.7 ± 23 ↓**	22.7 ± 14 ↓**	26.4 ± 27 ↓**
CRH	61.3 ± 19 ↓*	62.8 ± 15 ↓*	92.6 ± 37	93.1 ± 38

(b) Effects of hair growth inhibitors on apoptosis

	TUNEL-positive cells in ORS (% of control)		TUNEL-positive cells in DPC (% of control)	
Tretinoin	176.2 ± 22 ↑*	195.8 ± 24 ↑*	105.6 ± 29	226.8 ± 37 ↑**
TGFβ$_2$	185.4 ± 24 ↑*	245.5 ± 23 ↑**	188.1 ± 27 ↑**	225.23 ± 36 ↑**
CRH	115.8 ± 23	91.6 ± 32	96.9 ± 29	91.4 ± 36

CRH, corticotropin-releasing hormone; TGFβ$_2$, transforming growth factor-β2.
Series of double immunolabeling were performed to define the number of Ki67-positive (proliferating, **a**) and TUNEL-positive (apoptotic, **b**) cells in CK6 expressing outer root sheath keratinocytes (ORSK) or versican expressing dermal papilla fibroblasts (DPC). The numbers of double positive cells (Ki67+/CK6+, TUNEL+/CK6+, Ki67+/versican+, TUNEL+/versican+) in each group were determined, and expressed as mean ± SD values as a percentage of the control (non-treated) samples regarded as 100%. All data are shown as mean value ± SD. Asterisks mark significant (*$P<0.05$; **$P<0.01$) differences.

Table 2. The effect of hair growth stimulators on proliferation (a) and apoptosis (b) of ORSK and DPC in the HFM

(a) Effects of hair growth stimulators on proliferation

Days of culture	Ki67-positive cells in ORS (% of control)		Ki67-positive cells in DPC (% of control)	
	Day 7	Day 10	Day 7	Day 10
IGF-I	192.8 ± 37 ↑*	215.5 ± 28 ↑**	266.9 ± 38 ↑**	360.6 ± 42 ↑**
Calcitriol	193.7 ± 26 ↑**	198.1 ± 30 ↑**	220.9 ± 21 ↑**	117.5 ± 27
CsA, low Ca	170.8 ± 26 ↑*	195.4 ± 29 ↑**	212.8 ± 21 ↑**	252.9 ± 32 ↑**
CsA, high Ca	124.7 ± 38	116.3 ± 21	115.6 ± 19	123.8 ± 29
HGF, low Ca	197.5 ± 33 ↑*	169.5 ± 28 ↑*	106.7 ± 34	176.3 ± 30 ↑*
HGF, high Ca	167.1 ± 19 ↑*	196.9 ± 21 ↑**	116.8 ± 22	94.1 ± 26

(b) Effects of hair growth stimulators on apoptosis

	TUNEL-positive cells in ORS (% of control)		TUNEL-positive cells in DPC (% of control)	
IGF-I	87.3 ± 30	129.2 ± 55	97.6 ± 31	94.6 ± 27
Calcitriol	127.5 ± 39	112.7 ± 23	121.5 ± 27	125.9 ± 28
CsA, low Ca	117.9 ± 27	123.9 ± 33	93.5 ± 20	114.9 ± 28
CsA, high Ca	116.01 ± 31	215.7 ± 37 ↑*	119.39 ± 64	118.42 ± 62
HGF, low Ca	86.8 ± 31	92.3 ± 22	111.2 ± 26	84.7 ± 29
HGF, high Ca	90.9 ± 19	90.4 ± 18	91.8 ± 24	93.3 ± 16

CsA, cyclosporin A; HGF, hepatocyte growth factor; IGF-1, insulin-like growth factor 1.
Series of double immunolabeling were performed to define the number of Ki67-positive (proliferating, **a**) and TUNEL-positive (apoptotic, **b**) cells in CK6 expressing outer root sheath keratinocytes (ORSK) or versican expressing dermal papilla fibroblasts (DPC). The numbers of double positive cells (Ki67+/CK6+, TUNEL+/CK6+, Ki67+/versican+, TUNEL+/versican+) in each group were determined, and expressed as mean value ± SD values as a percentage of the control (non-treated) samples regarded as 100%. All data are shown as mean value ± SD. Asterisks mark significant (*$P<0.05$; **$P<0.01$) differences.
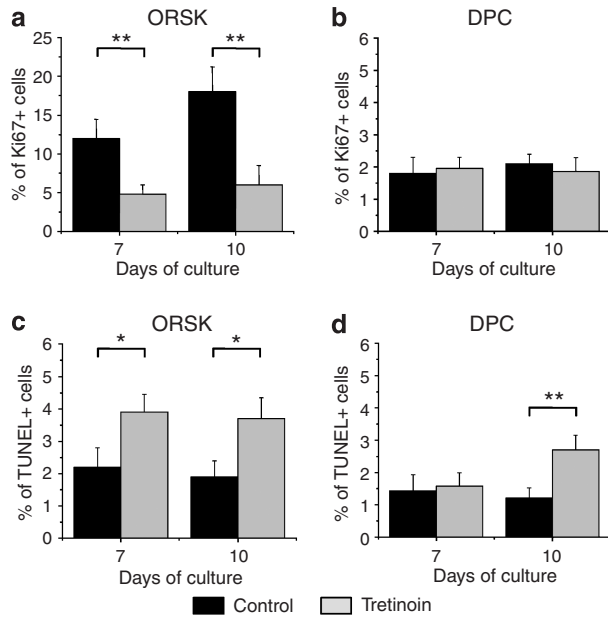
Figure 3. Effects of tretinoin on proliferation and apoptosis of ORSK and DPC in HFM. HFMs were treated with vehicle (Control) or with 10^{-6} M tretinoin for up to 10 days, then series of double immunolabeling were performed to define the number of Ki67-positive (proliferating, **a** and **b**) and TUNEL-positive (apoptotic, **c** and **d**) cells in CK6 expressing outer root sheath keratinocytes (ORSK, **a** and **c**) or versican expressing dermal papilla fibroblasts (DPC, **b** and **d**). The numbers of double positive cells (Ki67+/CK6+, TUNEL+/CK6+, Ki67+/versican+, TUNEL+/versican+) in each group were determined, and expressed as a percentage of total number of cells expressing the respective marker for ORSK (CK6+) or for DPC (versican+). All data are shown as mean value ± SD. Asterisks mark significant (*$P<0.05$; **$P<0.01$) differences.

Figure 4. Effects of TGFβ2 on proliferation and apoptosis of ORSK and DPC in HFM. HFMs were treated with vehicle (Control) or with 25 ng ml^{-1} TGFβ2 for up to 10 days, then series of double immunolabeling were performed to define the number of Ki67-positive (proliferating, **a** and **b**) and TUNEL-positive (apoptotic, **c** and **d**) cells in CK6 expressing outer root sheath keratinocytes (ORSK, **a** and **c**) or versican expressing dermal papilla fibroblasts (DPC, **b** and **d**). The numbers of double positive cells (Ki67+/CK6+, TUNEL+/CK6+, Ki67+/versican+, TUNEL+/versican+) in each group were determined, and expressed as a percentage of total number of cells expressing the respective marker for ORSK (CK6+) or for DPC (versican+). All data are shown as mean value ± SD. Asterisks mark significant (*$P<0.05$; **$P<0.01$) differences.

findings strongly suggest to carefully control and select the calcium concentration in the culture media for such studies.

HFMs are suitable to investigate the protein and gene expression of cytokines and growth factors involved in HF biology

We furthermore dissected whether the HFM system operates as a discovery tool for identifying new target genes (and their protein products) for candidate hair growth modulatory agents. Therefore, we measured the expression at protein and mRNA level of such biological markers in HFMs, which were previously described as important regulators in HF biology *in vivo*. Using immunofluorescence, we were able to detect the expression of TGFβ2 (negative regulator of hair growth) and stem cell factor (SCF, hair growth stimulator; Stenn and Paus, 2001; Peters *et al.*, 2003) in the cells of the microspheres (Figure 7a–d). In addition, employing reverse transcriptase (RT)–PCR on total RNA isolated from HFM, we could also identify the mRNA transcripts for these molecules (Figure 7e and f).

Positive and negative regulators of hair growth not only directly alter biological functions of individual HF cell populations, but also significantly modulate the complex cytokine/growth factor network of this mini organ. Therefore, using RT–PCR, we investigated the effects of some of those hair growth-modulatory agents on the expression of
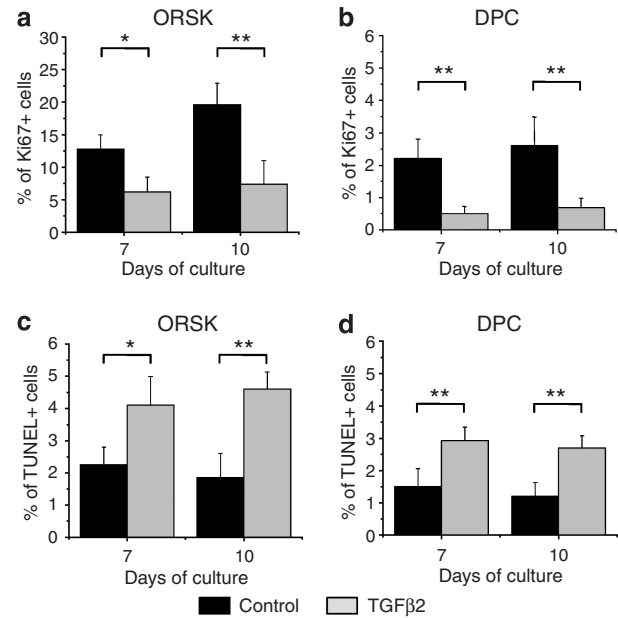
endogenous TGFβ2 and SCF which, when applied "exogenously", significantly affected proliferation and apoptosis of cells in HFMs (see above).

As shown in Figure 7g, 25 ng ml^{-1} "exogenous" growth inhibitor TGFβ2 (Figure 4; Table 1) remarkably upregulated the gene expression of "endogenous" TGFβ2 but did not affect the expression of SCF. Partly similar to these findings, 10^{-6} M tretinoin (another negative regulator; Figure 3; Table 1) also significantly increased the level of TGFβ2 mRNA transcripts; however, in contrast to the effect of "exogenous" TGFβ2, it also significantly decreased the expression of the growth-promoter SCF. Moreover, we were able to show that CsA (a positive regulator of HF growth; Figure 6a and b), significantly suppressed the expression of the growth-inhibitory TGFβ2 without affecting the level of SCF.

HFMs are suitable to investigate global gene expression profiles as well

Finally, we wished to analyze whether our model system allows the determination of changes of global gene expression profiles upon experimental manipulations. Therefore, in a pilot study, HFMs were treated with certain hair growth stimulatory agents (such as HGF, calcitriol, IGF-I, and 17β Estradiol; Philpott *et al.*, 1995; Thornton 2005; Conrad *et al* 2005; Ohnemus *et al.*, 2006) for 7 days, then a Microarray
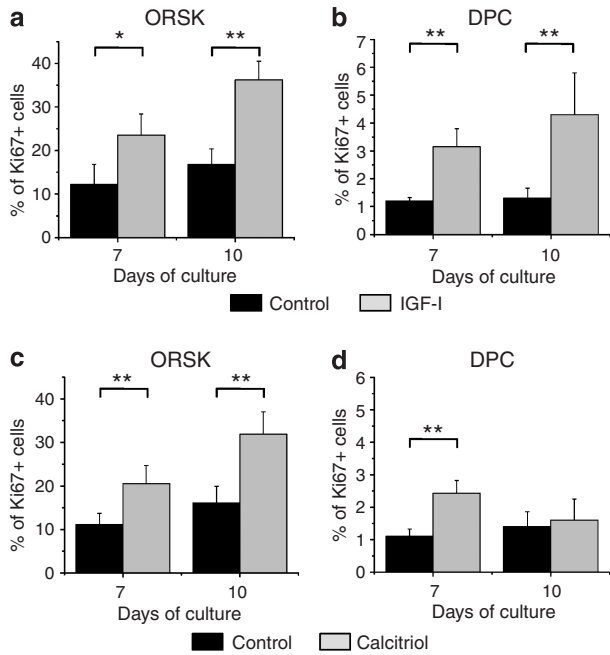
Figure 5. Effects of IGF-I and calcitriol on proliferation of ORSK and DPC in HFM. HFMs were treated with vehicle (Control), with 100 ng ml^{-1} of IGF-I (**a** and **b**), or with 10^{-8} M calcitriol (**c** and **d**) for up to 10 days, then series of double immunolabeling were performed to define the number of Ki67-positive (proliferating) cells in CK6 expressing outer root sheath keratinocytes (ORSK, **a** and **c**) or versican expressing dermal papilla fibroblasts (DPC, **b** and **d**). The numbers of double positive cells (Ki67 +/CK6 +, Ki67 +/versican +) in each group were determined, and expressed as a percentage of total number of cells expressing the respective marker for ORSK (CK6 +) or for DPC (versican +). All data are shown as mean value ± SD. Asterisks mark significant (*$P < 0.05$; **$P < 0.01$) differences.

Figure 6. Effects of Cyclosporin A and HGF on proliferation of ORSK and DPC in HFM. HFMs were treated with vehicle (Control), with 10 ng ml^{-1} Cyclosporin A (**a** and **b**), or with 10 ng ml^{-1} HGF (**c** and **d**) for up to 10 days, then series of double immunolabeling were performed to define the number of Ki67-positive (proliferating) cells in CK6-positive expressing outer root sheath keratinocytes (ORSK, **a** and **c**) or versican expressing dermal papilla fibroblasts (DPC, **b** and **d**). The numbers of double positive cells (Ki67 +/CK6 +, Ki67 +/versican +) in each group were determined, and expressed as a percentage of total number of cells expressing the respective marker for ORSK (CK6 +) or for DPC (versican +). All data are shown as mean value ± SD. Asterisks mark significant (*$P < 0.05$; **$P < 0.01$) differences.

analysis that represented the whole human genome (Agilent, 44K, G4112A) was performed to follow gene expression alterations. As seen in Supplementary Table S1 (showing changes of only selected genes), treatment of HFM with recognized growth stimulators induced remarkable changes in the expressions of certain genes with potential roles in regulation of HF growth, development, and cycling. Hence, although these preliminary findings remain to be repeated and confirmed by quantitative "real-time" PCR, our data (in accordance with the above findings, see Figure 7) further indicate that the HFM system is indeed a suitable hair research tool, since prototypic hair growth-modulatory agents that are recognized to alter gene and protein expression patterns in human HFs also do so in HFMs.

DISCUSSION

Studies of the epithelial–mesenchymal interactions in HF have been limited due to a lack of suitable *in vitro* screening systems that sufficiently mimic conditions as they occur in human HFs. So far, there are no available systems, which allow a higher throughput screening of candidate hair drugs than can be obtained by skin organ or histoculture techniques (Li *et al.*, 1992; Lu *et al.*, 2007) or by the classical organ culture of microdissected, amputated human scalp HF in the anagen VI phase of the hair cycle (Philpott *et al.*, 1990,
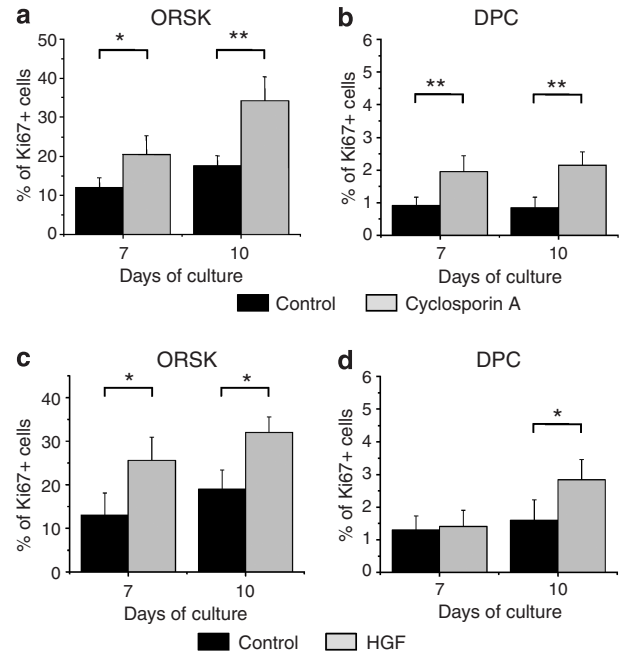
Magerl *et al.*, 2002). Even if this method, which is still the gold standard for *in vitro* analyses of human hair growth, is complemented by the addition of read-out parameters beyond hair shaft elongation (for example, Soma *et al.*, 2002; Foitzik *et al.*, 2005; Peters *et al.*, 2005), the very limited number of human HFs available severely restricts the number of agents that can be screened. Also, although our understanding of epithelial–mesenchymal interactions in murine HFs has recently progressed very substantially (for example, Rendl *et al.*, 2005), our corresponding understanding of these interactions in human HFs is still very rudimentary.

Some HF-like *in vitro* models derived from human follicular cells have been described (Limat *et al.*, 1994a, b; Stark *et al.*, 1999; Havlickova *et al.*, 2004; Krugluger *et al.*, 2005): however, so far none of them was prepared with relative ease in high amount to allow high-throughput screening and the proper investigation of epithelial–mesenchymal interactions. Our aim was, therefore, to develop a 3D *in vitro* system that allows a more detailed study of the basic molecular processes involved in HF growth and development with a robust reproducibility.

In this pilot study, we reported a very pragmatic organotypic assay, which imitates human HF-like epithelial–mesenchymal interactions and is prepared with comparative ease. Studying the expression of a number of

different markers (for example, proliferation, apoptosis, CK6, and versican expression), we show that, under culture conditions with a serum-free, low-Ca medium, these HFMs retain several essential characteristics for human scalp HFs.

The technique of the microsphere preparation by jellification process of the matrix and cell mixture sustains high cell viability. The inclusion of Matrigel (containing laminin and collagen IV) imitates the matrix environment of the follicular dermal papilla, namely that it more closely resembles that of basement membranes, rather than of interfollicular dermis and enables to very closely mimic an extracellular matrix of the HF mesenchyme. The low number of TUNEL-positive cells in later phases of the culture with normalized LDH level suggests the optimal nutrient supply to HFMs also when the cell number has increased. Culturing for more than 10 days is feasible when the number of cells, volume of extracellular matrix, and size of the HFM is increased during the preparation process. The system is highly reproducible, because we generated thousands of microspheres in 10 previous experiments.

The technique of the HFM preparation also facilitates cell migration within the microsphere compared to other spheroid systems. Nevertheless, although a mixture of isolated ORS and DPC with HF inductive potential (Reynolds and Jahoda, 1992) was used in our experiments and cell migration and matrix reorganization within the microsphere were indeed observed, new HF units did not spontaneously from under these assay conditions. This suggests that the interaction and signaling of the follicular cells with surrounding dermal microenvironment are crucial for morphogenesis and development of HF. However, the main aim of this study was to develop a simplified HF-like 3D *in vitro* system rather then to induce terminal HF formation.

All hair growth-modulatory agents investigated altered apoptosis, proliferation, protein, and gene expression in different cell populations within HFM system in a manner that suggests that HFMs allow the standardized preclinical assessment of test agents on relevant human hair growth markers under substantially simplified *in vitro* conditions that approximate the *in vivo* situation. We furthermore show by DNA microarray that HFM also offers a useful discovery tool for the identification of target genes for candidate hair drugs. Evidently, the HFM method still has limitations, especially in the rapid analysis of various read-out parameters. These shortcomings will hopefully be overcome in the near future with the ''automatization'' of the evaluation process by employing, for example, staining ''robots'', automated image analyzer software packages, and high-content screening devices. Nevertheless, our assay system is currently the only one that can claim to come at least close to overcoming the formidable remaining methodological challenges that have to be met before automatization.

In HFM, we were also able to identify several other markers important for the HF development and differentiation (for example, CK14, β-catenin, IGF-I, IGF-I receptor, alkaline phosphatase; data not shown). Due to the fact that there is no exclusively ORS-specific marker available (Langbein et al.,

2001, Langbein and Schweizer, 2005), the use of CK6 immunostaining as a ''HF-type keratinization marker for ORS'' is a reasonable approach, even though it must be kept in mind that CK6 is also expressed by activated (for example, wounded, inflamed or UV-irradiated) interfollicular epidermal keratinocytes and in the companion layer of HF (Langbein and Schweizer, 2005). The latter is unproblematic, because CK6 immunoreactivity originating from a companion layer-type epithelium within the microspheres would still reflect and confirm a HF-type keratinization pattern (the companion layer only exists within the HF epithelium). In addition, during our ORS isolation method and culture, no epidermal components were present, and pure ORSK cultures were generated (as confirmed by negative inner root sheath markers; data not shown).

In conclusion, our data suggest that HFMs represent a valuable system to study epithelial–mesenchymal interactions and their changes in response to treatment with various candidate hair drugs. HFMs, thus, offer not only a pragmatic basic screening tool, but also an instructive new experimental system for basic and applied preclinical hair research in the human system.

MATERIALS AND METHODS
Cell isolation and culture
ORS keratinocytes were isolated from plucked anagen HF by trypsinization (Limat and Noser, 1986). Primary cultures of ORSK were then cultured on feeder layer of X-ray irradiated HDF, obtained from human skin from deepidermized dermis using enzymatic digestion (Limat et al., 1989), in defined keratinocyte serum-free medium (Invitrogen, Paisley, UK) supplemented with 0.1 nM cholera toxin, $5\,\mu g\,ml^{-1}$ insulin, $0.4\,\mu g\,ml^{-1}$ hydrocortisone, $2.43\,\mu g\,ml^{-1}$ adenine, 2 nM triiodthyronine, $10\,ng\,ml^{-1}$ epidermal growth factor, 1 mM ascorbyl-2-phosphate, and antibiotics penicillin G, gentamicin (all reagent purchased from Sigma-Aldrich, Taufkirchen, Germany; Havlickova et al., 2004). Cells at early passage (2–4) were used.

After microdissection of anagen VI HF from scalp-skin biopsies using a method modified from Philpott et al (1990), isolation of DPC from HF was established according to Magerl et al (2002) and cultured in Chang's medium (Trinova, Santa Ana, CA) with 10% fetal bovine serum (Biochrom KG, Berlin, Germany) (Messenger et al., 1986). The passages of 1–2 were used.

Preparation of human folliculoid microspheres
As described in patent EP1231949, a beaker containing two phases of immiscible autoclaved liquids (lower phase: 250 ml of perfluorether (Fluorinert FC-40; 3M Corp., Germany); upper phase: 500 ml of defined triglyceride mixture (Miglyol, Hüls, Germany)) was prepared and warmed up to 37 °C with continuous magnetic stirring to prevent sedimentation and attachment of prepared HFM (see below).

Then a cell–matrix mixture of HFM was prepared, following the optimized protocol for the previously described (Havlickova et al., 2004) preparation of ''mixed sandwich'' 3D system. Mixture of collagen I and Matrigel Basement Membrane Matrix (ratio 4:1) was used as a matrix for cells. Ice-cold collagen type I extracted from rat tail tendons (BD Biosciences, Bedford, MA) at a final concentration of $4\,mg\,ml^{-1}$ was mixed with $10\times$ Hank's buffered saline
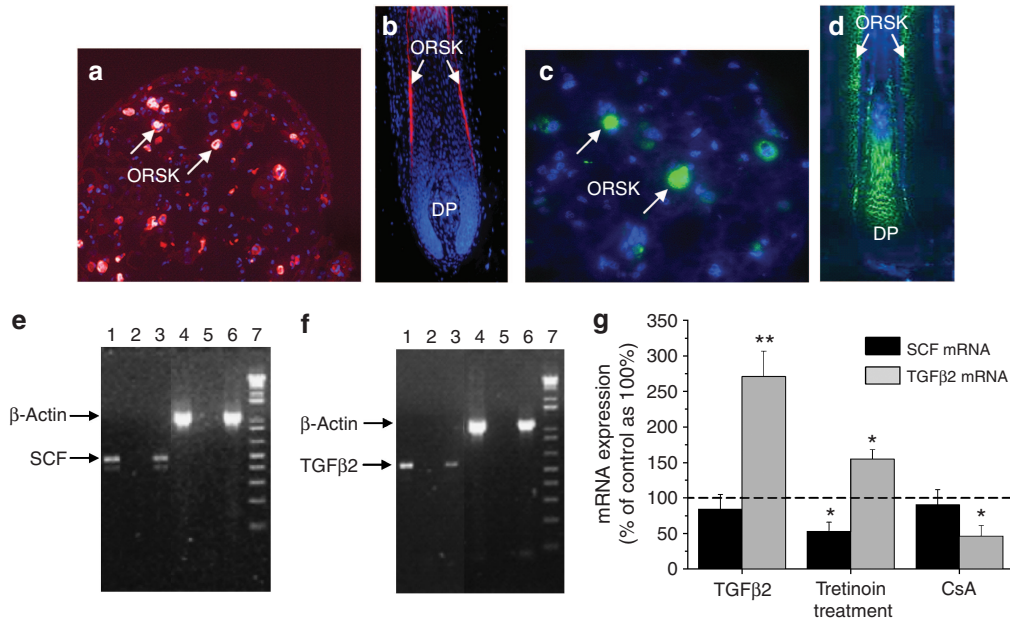
Figure 7. Expression of TGFβ₂ and SCF in HFM, RT–PCR analysis of SCF and TGFβ₂. (**a–b**) Expression of TGFβ₂ (as revealed by rhodamine immunostaining, red fluorescence) in HFMs (**a**) and in microdissected HFs used as a positive control (**b**). (**c, d**) Expression of SCF (as revealed by FITC immunostaining, green fluorescence) in HFMs (**c**) and in microdissected HFs (**d**). Original magnification, ×100 for (**a, b, d**); ×250 for (**c**). Nuclei were counterstained by DAPI (blue fluorescence). (**e–f**) Reverse transcriptase–PCR analysis of SCF (**e**) and TGFβ2 (**f**) (and β-actin, used as an internal control) mRNA expression in HFMs and in microdissected HFs (used as positive controls). Lanes, *1 and 4*, HF (positive control); *2 and 5*, reaction without template (negative control); *3 and 6*, HFM. (**g**) HFMs were treated with 25 ng ml⁻¹ TGFβ₂, 10⁻⁶ M tretinoin or 10 ng ml⁻¹ cyclosporin A (CsA) for 7 days and the expressions of TGFβ2- and SCF-specific mRNA were determined by RT–PCR. The amount of mRNA transcripts was then quantified by densitometry and the values obtained for TGFβ2 and SCF were normalized on the base of those for β-actin. Data of the treated groups, obtained in three independent experiments, are expressed as mean ± SD values as a percentage of the matched control samples regarded as 100% (line). Asterisks mark significant (*$P<0.05$; **$P<0.01$) differences.

(Invitrogen) followed by neutralization with 1 M NaOH (Invitrogen), then appropriate volume of Matrigel (BD Biosciences) was added and mixed. One volume of fetal bovine serum with resuspended cells (ORSK and DPC in the ratio 1:2) was then added to the matrix and mixed thoroughly (the cell density of ORSK was 1×10^6 cells per ml and of DPC was 2×10^6 cells per ml). A 1 ml syringe was then filled with the above cell–matrix suspension and, by gently pressing the syringe, small droplets entered into the Miglyol-FC-40 mixture and formed HFM by a gelling process at 37 °C (Bettermann and Hübner, 2000). HFMs were left in this liquid for 5 minutes (with continuous stirring), then removed by a net, washed immediately in the culture medium and placed in culture Petri dishes. HFMs were cultivated submerged in the aforementioned (see Cell isolation and cell culture) supplemented low-Ca (0.15 mM) serum-free medium for 10 days (Figure 1a). Under these conditions, the average size of the HFM was 1.73 ± 0.33 mm on day 0 and 1.03 ± 0.20 mm on day 7. In addition, to determine the effects of high-Ca content in growth media, in some experiments a medium with a calcium concentration of 1.8 mM was used. This (also serum-free) solution was composed of three parts of DMEM, one part of Ham's F 12 (both from Sigma), and the above additives.

The agents investigated were resuspended in the appropriate vehicle and were added to the medium on day 0. The medium was changed on days 3 and 7, and collected for LDH analysis (see Cytotoxicity assay). HFMs (20–30 per group) were cultured submerged in culture medium, and one third of the samples were collected on days 3, 7, and 10 each.

Histology

For morphological analysis of the HFM, samples were embedded in Thermo Shandon Cryochrome solution (Thermo Shandon Inc., Pittsburgh, PA) and frozen in liquid nitrogen vapor. Sections (10 μm) were then cut, and, after fixation in ice-cold acetone, the samples were stained with hematoxylin-eosin (Sigma).

Immunofluorescence

Sections were fixed for 10 minutes in acetone at −20 °C, rehydrated in phosphate-buffered saline, and then they were preincubated with 10% serum originating from species of the secondary antibody for 20 minutes. For immunofluorescence labeling, the following primary antibodies were employed: mouse anti-fibronectin (1:10; Acris, Hiddenhausen, Germany), mouse anti-cytokeratin-6 (CK6, clone KA12, 1:10; Progen, Heidelberg, Germany), mouse anti-c-kit/ scattered factor (SCF, 1:100; Santa Cruz Biotech, Santa Cruz, CA), and rabbit anti- TGFβ2 (1:50; Santa Cruz Biotech). Sections were then labeled with appropriate FITC- or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson Immuno-Research, West Grove, PA). For positive controls, cryosections of human HFs and tissues with specific expression of markers were stained similarly as described above.

To assess proliferation of different cell types in HFM, a double immunolabeling was performed. For detection of proliferation in the ORSK and DPC, sections were incubated overnight with the first primary antibody against the recognized proliferation marker Ki67 (1:10 of rabbit anti-human Ki67; Zymed, San Francisco, CA) at 4 °C,

then with the first secondary antibody (goat anti-rabbit, FITC-conjugated) for 45 minutes at room temperature. Then samples were incubated with either an antibody against CK6, which is not expressed by normal epidermal keratinocytes, yet is characteristic for normal ORSK (1:10 of mouse anti-human CK6; Progen; Langbein *et al.*, 2001; Langbein and Schweizer, 2005) or with an antibody against the DPC-specific extracellular matrix antigen, large proteoglycan versican (1:500 of mouse anti-human versican; Seikagaku Corporation, Tokyo, Japan; Kishimoto *et al.*, 1999, Soma *et al.*, 2005, Kim *et al.*, 2006) for 1 hour at 37 °C. Finally, the second secondary antibody (goat anti-mouse, rhodamine-conjugated) was applied for 45 minutes at room temperature. Sections were then washed in phosphate-buffered saline, counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (Sigma) and mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL).

For measurement of apoptosis in the ORSK and DPC, a similar double staining protocol was used. In this case, however, instead of Ki67 labeling, components of an ApopTag TUNEL (terminal dUTP nick-end labeling) apoptosis assay kit (Intergen, Purchase, NY) were employed following the instructions suggested by the manufacturer.

Cytotoxicity assay

To assess the presence of necrotic cells within the HFM, a LDH-based cytotoxicity assay was applied (Bio Vision, Mountain View, CA). Briefly, the culture media of HFM of all treated groups were collected on days 3, 7, and 10 and the amount of LDH, as a marker of necrotic cell death, was colorimetrically determined according to the protocol suggested by the manufacturer.

Semi-quantitative RT-PCR technique

The expression of mRNA for TGFβ2 and SCF in HFM (and in microdissected human scalp HFs, used as positive controls) was determined by semi-quantitative RT-PCR. The total RNA was extracted using the RNA easy kit (Qiagen, Hilden, Germany) and then was reverse transcribed with random primers and reverse transcriptase provided in first strand cDNA synthesis kit for RT–PCR (Boehringer, Mannheim, Germany). Subsequent PCR amplification (94 °C for 5 minutes; 30 cycles of 94 °C for 30 seconds, 57 °C for 60 seconds, 72 °C for 60 seconds; 72 °C for 10 minutes) was performed on the UNO-Thermoblock (Biometra, Göttingen, Germany) with the following primers (all from Sigma): TGFβ2, 5′-ATCC CGCCCACTTTCTACAGAC-3′ and 5′-CATCCAAAGCACGCTTCTTC C-3′ (GenBank accession number: Y00083); SCF, 5′-ATTCAA GAGCCCAGAACCCA and CTGTTAACCAGCCAATGTACG (GenBank accession number: M59964); β-actin, 5′-CGACAACGGCTCC GGCATGTGC-3′ and 5′-CGTCACCGGAGTCCATCACGATGC-3′ (GenBank accession number: NM001101). The PCR products were visualized on a 2% agarose gel with ethidium bromide, and the photographed bands were quantified by an Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Springs, MD).

Microarray gene expression analysis

The microarray experiment was based on a two-color ratio hybridization and a low RNA input fluorescent linear amplification kit (Agilent Technologies, Böblingen, Germany) for RNA labeling. In short, 500 ng of total RNA (isolated from control and treated HFM as described above) was reverse transcribed with an oligo(dT)-T7 promoter primer and Moloney murine leukemia virus-reverse transcriptase (Applera, Darmstadt, Germany) to synthesize first and second-strand of cDNA. Fluorescent antisense cRNA was synthesized with T7 RNA polymerase, which simultaneously incorporated either cyanine 3-cytidine 5′-triphosphate (3-CTP) or cyanine 5-CTP (both from Cy Scribe, Amersham, Freiburg, Germany). The purified products were quantified by absorbance at a 552 nm for cyanine 3-CTP and a 650 nm for cyanine 5-CTP, and labeling efficiency was verified with a nanodrop photometer (Kisker, Steinfurt, Germany). Before hybridization, 2 μg of each labeled cRNA product were fragmented and mixed with control targets and hybridization buffer according to the supplier's protocol (Agilent Technologies). Hybridizations were done overnight for 19 hours at 60 °C. The slides were then washed according to the manufacturer's manual, and the scanning of microarrays was performed with 5-μm resolution using a DNA microarray laser scanner (Agilent Technologies). Features were extracted with an image analysis tool version A 6.1.1 (Agilent Technologies) using default settings. Data analysis was conducted on the Rosetta Inpharmatics Platform Resolver Built 4.0. Expression patterns were identified by stringent data analysis using a two-fold expression cut-off and an exclusion of data points with a low *P*-value ($P < 0.01$). By using this strategy, data selection was independent of error models implemented in the Rosetta Resolver system.

Statistical analysis

To compare the proliferation and apoptosis of cells in HFM, the percentage of Ki67- or TUNEL-positive cells was determined (both in ORSK and in DPC using a double immunolabeling) in five samples per group for each experiment. After TUNEL staining the apoptotic cells were distinguished from necrotic cells according to the morphological criteria, and only apoptotic cells were assessed. The values were then averaged and were expressed as mean ± SD. Statistical analysis was performed using Mann–Whitney's nonparametric test.

SUPPLEMENTARY MATERIAL
Table S1. Alterations in the expression of selected genes expressed in HFM after 7 days of incubation with various recognized hair growth stimulators.

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