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Skin equivalents in basic and applied research:

Development of human hair equivalents

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Development of human hair equivalents

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1 List of abbreviations

ACTH	Adrenocorticotropic hormone
AGA	Androgenetic alopecia
APM	Arrector pili muscle
ATRA	All-trans retinoid acid
BDNF	Brain-derived nerve growth factor
BMP	Bone morphogenic protein
CL	Club hair
CK6	Cytokeratin 6
CRH	Corticotrophin-releasing hormone
CsA	Cyclosporin A
CTS	Connective tissue sheath
CTSL	Cathepsin L
DAPI	4′,6-Diamidin-2-phenylindol fluorescent stain
DMEM	Dulbecco's modified Eagle's medium
DP	Dermal papilla
DPC	Dermal papilla cells / fibroblasts
E2	17-beta estradiol
ER	Estrogen receptor
FBS	Fetal bovine serum
FC	Fetal clone
FDA	Food and Drug Administration
FGF-7	Fibroblast growth factor 7
GDNF	Glial cell line-derived neurotrophic factor
HaCaT	Human keratinocyte cell line
HDF	Human dermal fibroblasts
HE	Hematoxylin-eosin
HF	Hair follicle
HFM	Human folliculoid microspheres
HGF	Hepatocyte growth factor
HR	Hairless gene
IFNy	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IRS	Inner root sheath

LEF-1	Lymphoid enhancer-binding protein 1
LDH	Lactate-Dehydrogenase
MSH	Melanocyte-stimulating hormone
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
NHEK	Normal human epidermal keratinocytes
ORS	Outer root sheath
ORSK	Outer root sheath keratinocytes
P75NTR	Low affinity neurotrophin receptor
PBS	Phosphate-buffered saline
PRL	Prolactin
PRLR	Prolactin receptor
RAR	Retinoid acid receptor
RC	Rough coat
RT- PCR	Real-Time-quantitative Polymerase Chain Reaction
RXR	Retinoid X receptor
SCF	Stem cell factor
SG	Sebaceous gland
SHH	Sonic hedgehog
STAT-3	Signal transducer and activator of transcription 3
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
TSP-1	Thrombospondin 1
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEGF	Vascular endothelial growth factor
VR-1	Vanilloid receptor 1
WNT	Wingless gene
3D	Three-dimensional

2 Introduction

The loss of scalp hair (effluvium, alopecia) can be accompanied by severe psychological problems in a vastly underestimated number of afflicted patients[57].Therefore the development of ever-new and hopefully more effective drugs for the management of common hair growth disorders remains a top priority both for clinical dermatology and the industry[143].

Most hair growth disturbances seen in clinical practice primarily result from changes in hair follicle cycling. Thus, a more profound understanding of the molecular controls of hair follicle cycling and its underlying disturbances promises to lead to the development of more effective “hair drugs,” one of the prime challenges of modern hair research.

The search for such hair drugs 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 (HF). Therefore, pragmatic 3D screening systems are urgently needed, as preservation of native epithelial-mesenchymal interactions is superior to simple co-culture assays for isolated HF cell populations in which these interactions are disrupted[103,178], and hence data produced with such cell culture studies reflect highly artificial conditions, provide very uncertain predictive value for the clinical situation, and are therefore inappropriate for the screening purposes discussed here.

For pre-clinical R&D (research and development) purposes, a simplified 3D folliculoid systems should provide a first-line screening tool for large-scale *in vitro* testing, to be followed by organ culture of micro-dissected human anagen hair bulbs[159] and histocultures of hair-bearing skin, i.e. micro-dissected normal skin[97,98,110] 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.

The main aim of this study was therefore to develop a human tissue-engineered hair equivalents for investigative dermatology that provides well-defined basic parameters for detailed exploration of epithelial-mesenchymal interactions in the human hair follicle.

3 Skin Equivalents

3.1 Tissue Engineering

Decades ago, the possibility of growing replacement tissues to repair human skin was considered science fiction. When substitution of the skin was required, the only options were split or full-thickness skin grafts, tissue flaps and free-tissue transfers[166]. Over the last 30 years, tissue-engineered skin equivalents has developed and progressed at a very rapid rate. Today, there are some tissue engineered skin equivalents approved for use by the US Food and Drug Administration (FDA) and many others undergoing testing and development. Tissue-engineered skin substitutes offer tissue replacement without requiring a donor site and may produce better healing[39]. Tissue engineering was defined in 1987 by the National Science Foundation bioengineering panel meeting in Washington, DC, USA, as 'the application of the principles and methods of engineering and the life sciences toward the development of biological substitutes to restore, maintain, or improve function[46].

Key materials for tissue engineering are cells, matrix material (scaffold) and growth factors. The cell synthesizes matrices of new tissue, while the scaffold provides the appropriate environment for cells to be able to effectively accomplish their missions. The function of growth factors is to facilitate and promote cells to regenerate new tissue[73]. Future directions include genetic modification of transplanted cells to improve wound healing transiently or to deliver gene products systemically[17].

A short review of some engineered skin equivalents are listed here.

3.2 Human Tissue- Engineered Skin Equivalents commercially available

3.2.1 Cultured Epidermal Autografts (Epicel™)

In 1975, Rheinwald and Green developed a technique that permitted epidermal keratinocytes to be cultured *in vitro* using a feeder layer of irradiated murine fibroblasts[177]. From a skin biopsy, dermis and subcutaneous tissue were removed, epidermis was minced and trypsinized, and a cell suspension was seeded on lethally irradiated 3T3 mouse fibroblasts (which can not multiply and inhibit growth of human fibroblasts from the biopsy). The standard culture medium was enriched by cholera toxin, epidermal growth factor, adenosine, insulin and hydrocortisone[56,177]. Small colonies formed within days after a cell suspension was plated. When cultures reached confluence, the keratinocytes were released with dispase and attached to a non-adherent gauze dressing[56]. Two to three weeks were required before the cultured graft was available. Using this technique, cultured epidermal autografts (Epicel™), were first produced in 1988. The advantages of cultured epidermal autografts are the potential provision of permanent wound coverage[66,132], a decreased requirement for donor sites, pain relief, and a better functional and cosmetic outcome. The disadvantages are the requirement for a skin biopsy (or biopsies), a 3-week delay for graft cultivation, the lack of a dermal component, and high costs. Cultured epidermal autografts have been used to treat burns[54,132], chronic leg ulcers[66], epidermolysis bullosa[23], cutaneous wounds resulting from excision of giant congenital nevi[55], vitiligo[164], chronic mastoiditis[169], congenital hypospadias[179], pressure ulcers[157], and corneal replacement[150]. However, cultured epidermal autografts are not a complete bi-layered skin, and the scar contraction and unstable attachment to the wound bed is attributed to the absence of a dermal substrate[58].

3.2.2 Cultured Epidermal Allografts

Cultured epidermal allografts are derived from unrelated allogeneic donors, such as newborn foreskin, can be grown in advance and may be stockpiled. They promote granulation formation and stimulate epithelialization from wound edges and from adnexal structures in the dermal bed of superficial wounds, probably through growth factor release[154]. The advantage of using cultured epidermal allografts is immediate graft availability. The disadvantages are that they do not survive permanently on the wound bed, and there is a possibility of disease transmission, which can be minimized with an extensive screening. Cultured epidermal allografts have been used to treat burns[34,72,67,114], chronic leg ulcers[37,96,155,156], donor sites[158], and epidermolysis bullosa[118]. Cultured epidermal allografts can be cryopreserved and stored at –70°C to –120°C and thawed at room temperature before using[12].

3.2.3 Non-living Allogeneic Acellular Dermal Matrix (Alloderm®)

Alloderm® is an acellular matrix material, processed directly from fresh cadaver skin that is treated with high salt to remove the epidermis and extracted with a solution to remove the cellular material. It is then freeze dried, leaving an immunologically inert acellular dermal matrix with intact basement membrane complex. It has been used to treat burns since 1992. It can be glycerol preserved or lyophilized. The advantages of this dermal matrix include: it is acellular and immunologically inert; it provides a template with natural dermal porosity for regeneration with the presence of an intact basement membrane; and it allows the use of thinner autografts. The disadvantages include risk of transmitting infectious diseases[203].

3.2.4 Non-living Extracellular Matrix of Collagen and Chondroitin-6-Sulfate (Integra®)

An *in vitro* dermal replacement, Integra® is an artificial skin developed by Burke and Yannas[22]. It consists of an artificial dermis (matrix of bovine collagen and chondroitin-6-sulfate, a shark-derived glycosaminoglycan) and a disposable silicone sheet (artificial epidermis). The matrix becomes vascularized, then the disposable epidermis is removed and can be replaced with a split thickness skin graft. In 1996, the FDA approved its use in burns. The advantage of using this artificial dermis is that it allows a neo-dermis to develop. The disadvantages are the collection of fluid under Integra® with the possibility of unnoticed infection, and the lack of a real epidermal component[20].

3.2.5 Cultured Skin Substitute

A collagen and glycosaminoglycan dermal skin substitute was studied as substrate for cultured human epidermal keratinocytes[18]. Hansbrough[61] further developed this skin substitute, which is prepared *in vitro* and transferred as a unit to the wound bed. This skin substitute is composed of autologous cultured keratinocytes as the epidermal component, and collagen and glycosaminoglycan substrate inoculated with autologous fibroblasts as the dermal component. Formation of rete ridge interdigitation, and basement membranes proteins (laminin and type IV collagen), were identified immunohistochemically.The disadvantage of this skin substitute is the need to wait 3 to 4 weeks to produce the cultured grafts. Later Boyce[21] added human melanocytes to the skin culture composed of human epidermal keratinocytes, dermal fibroblasts and collagen glycosaminoglycan substrates, and demonstrated restoration of pigmentation and inhibition of wound contraction after transplantation to athymic mice. More studies are needed to determine the effectiveness of this composite skin substitute.

3.2.6 Living Allogeneic Dermal Fibroblasts (Dermagraft®)

A living dermal replacement (Dermagraft®) can be obtained by culturing human neonatal fibroblasts on a polyglyactin mesh. This method was developed by Cooper[26]. Dermal fibroblasts from neonatal foreskin are seeded on a bioabsorbable mesh, in a sterile bag with circulating nutrients. These cells attach, multiply and begin secreting collagens and growth factors. The cells and the collagen proteins form a solid tissue by covering the mesh inside and outside. The mesh is made of biodegradable material and disappears after 3 to 4 weeks. This fibroblast collagen matrix can be used alone or as a base for meshed autografts or possible epidermal cultures[154]. The advantages of this skin substitute include good resistance to tearing, ease of handling, and lack of rejection[60,165].

3.2.7 Extracellular Matrix of Allogeneic Human Dermal Fibroblasts (Transcyte™)

Transcyte™ is a laboratory-grown temporary skin replacement. Neonatal (allogeneic) fibroblasts are cultured and proliferate on nylon fibers that are embedded into a silastic layer for 4 to 6 weeks, forming a dense cellular 'tissue' which contains high levels of secreted human matrix proteins as well as multiple growth factors[60]. The fibroblasts are rendered non-viable by freezing after synthesizing collagen extracellular matrix and growth factors. TransCyte™ is approved for the treatment of burn wounds, and advantages include immediate availability and easy storage[171].

3.2.8 Living Allogeneic Bilayered Skin Construct (Apligraf®)

A living human skin equivalent was first developed by Bell et al[7]. Apligraf® (also known as 'Graftskin') is a bilayered skin construct or living human skin equivalent, composed of living keratinocytes and dermal fibroblasts, derived from neonatal foreskin and propagated in culture. Initially, dermal fibroblasts are combined with type I bovine collagen. Subsequently, keratinocytes overlying the epi-

dermis are exposed to an air-liquid interface to promote formation of a stratum corneum[208]. Apligraf® resembles human skin histologically, produces matrix proteins and growth factors, and can heal itself, following an injury[45]. Apligraf® gained FDA approval for the treatment of venous ulcers based on its efficacy in a controlled, randomized, multicenter study performed in 293 patients with venous ulcers. It has also been used to treat patients with epidermolysis bullosa[43,44] and in acute, partial or full-thickness excisional wounds made mostly by excision of skin cancer[38]. The advantages of using Apligraf® include ease of application, ability to apply the graft as an outpatient procedure, and avoidance of a surgical procedure that leaves a donor site wound. The disadvantages are its short shelf life (5 days) and cost.

3.2.9 Composite Cultured Skin (OrCel™)

Another living skin equivalent is the composite cultured skin, which consists of allogeneic fibroblasts and keratinocytes grown *in vitro* and seeded on opposite sides of a bilayered matrix of bovine collagen[129]. The collagen matrix consists of a cross-linked bovine collagen sponge coated with an overlay of pepsinized insoluble collagen. Keratinocytes are seeded over a non-porous collagen gel (insoluble collagen) and the fibroblasts are seeded on the underside of the porous sponge. They are cultured between 10 to 15 days. Composite cultured skin has been used as a partial substitution for autografts on digits and over donor sites in the course of 16 operations performed on 7 children with recessive dystrophic epidermolysis bullosa, syndactyly and flexor contracture of the fingers[41]. The advantage of this skin substitute is its immediate availability; the disadvantage is the little clinical data to support its use.

3.3 Tissue-Engineered Hair Equivalents

3.3.1 Hair equivalents for wound healing

An autologous epidermal equivalent generated *in vitro* from the patient's hair (the outer root sheath cells of the hair follicle [ORSK]) has been grafted successfully in a pilot study performed on 11 chronic leg ulcers[105]. The advantages of this graft include the easy isolation of precursor cells for epidermal keratinocytes from plucked scalp hair follicles and that these precursor cells retain a high proliferative capacity irrespective of the age of the hair follicle donor. This epidermal equivalent is commercially available in Europe, as EpiDex™.

3.3.2 Neogenesis of the hair follicle

Many approaches for neogenesis of the hair follicle (creation hair follicle *de novo*) have been tested recently, e.g. autologous dermal cells from dissociated hair follicle expanded in the culture and then in combination with competent epithelial cells re-implanted to the scalp or forming hair follicle as mini organs *in vitro* and then transplanting the newly generated follicles back to the alopetic scalp[194]. However, due to tissue engineering challenges the creation of new hair follicles for the treatment of alopecia has not been achieved yet.

3.3.3 Hair equivalents for investigative dermatology

Many different 3D organotypic assay systems based on human skin and hair cell populations have been developed for investigative dermatology[5,101,104,193,211]. These systems allow so far limited study of the function of diffusible factors affecting epithelial–mesenchymal interactions, the dynamics of basement membrane formation and the testing of the effects of various modulators, growth hormones, nutrients, etc.[112,113,188-190].The human skin equivalents have become an indispensable alternative to animal models in pharmacological and toxicological *in vitro* testing[167].

Historically, normal human epidermal keratinocytes (NHEK) were first used in organotypic 3D systems[112,189,190,193] and later ORSK were also employed [101,102,104]). In these systems cells were layered above collagen I, or collagen I mixed with human dermal fibroblasts (HDF), generating 'pseudodermis' [193]. Later, these collagen I gels or a pseudodermis were covered with various cell and matrix mixtures, which contained basement membrane and extracellular matrix components (Matrigel™ basement membrane matrix;), NHEK or ORSK, plus mesenchymal cells (HDF or DPC) in a number of different designs[101,193]. Ultimately, in previously reported 3D systems, comprehensive data on the ratio of proliferation/apoptosis of ORSK and DPC and/or on HF-like differentiation markers have not been assessed in the classical studies that have pioneered the field[101,113,188,189,193].

A human tissue-engineered skin equivalent in for the investigation of transfollicular penetration has been developed by Michel[125]. It is produced from human fibroblasts and keratinocytes obtained from healthy adult skin specimens removed during reductive breast surgery, and does not contain any synthetic material. Previously extracted pilosebaceous units are then inserted in the dermal equivalent in which small holes have been previously made. The product has histological, immunological, immunohistological, and ultrastructural properties similar to healthy human skin. This new *in vitro* hair model could be used to further broaden the understanding of the transfollicular route in the transcutaneous delivery of active substances.

In order to overcome numerous challenges, further progress needs to be made in understanding the molecular pathways activated during hair follicle embryogenesis and hair cycling. Eventually, this understanding should lead to the generation of new pharmaceutical agents that specifically target these pathways[194].

4 The Biology of the hair follicle

4.1 Human hair follicles

The hair follicle is one of the most complex mini-organs of the human body. This exquisitely productive protein fiber factory, which doubles as a sensory organ and serves as an instrument of psychosocial communication, excretion, and protection, undergoes cyclic transformations between phases of rapid growth (anagen), apoptosis-driven regression (catagen), and relative quiescence (telogen)[35]. With this "hair cycle," the follicle demonstrates the unique ability to cyclically regenerate itself during our lifetime, based on epithelial–mesenchymal interactions that drive waves of daughter cell populations, derived from resident epithelial, neural, and mesenchymal stem cells, into defined strata of differentiation[88,146,195]. The human scalp, eyebrows, and lashes consist of long, thick, medullated and pigmented terminal hair shafts, whereas the body is covered with short, thin and often unpigmented vellus hairs. Each of us displays an estimated total number of 5 million hair follicles, of which 80,000 to 150,000 are located on the scalp. The hair length is defined by the duration of anagen, which lasts for 2 to 6 years. Approximately 85% to 90% of all scalp hairs are within anagen follicles. Catagen lasts only for a few weeks, followed by the telogen phase, which lasts 2 to 4 months. The usual growth of scalp hair follicles (i.e. the rate of hair shaft elongation) lies between 0.3 and 0.5 mm per day and is dependent on proliferation and subsequent follicular-type differentiation of the matrix keratinocytes in the hair bulb. The thickness of the hair shaft is related to the size of the hair bulb[33], which in turn is dictated by the volume of the hair follicle's mesenchymal component[199].

4.2 Hair Follicle Anatomy

The mature anagen hair follicle is composed of a multicylindric stem that contains the hair shaft in its center and originates as an oval hair bulb proximally (**Fig 1**)[207]. Embraced by the hair bulb lies an onion-like structure, called the dermal papilla (DP) (sometimes referred to as the "follicular papilla" to avoid confusion with the most superficial region of the dermis). The DP functions as the "command

center" of the hair follicle and determines thickness, length, and likely the hair cycle itself[146].

Each hair follicle consists of epithelial and mesenchymal parts. The epithelium is divided into an upper permanent region, distal to the arrector pili muscle (APM) and an inferior region (including the hair bulb), which dramatically reforms itself over the cycle (**Fig.1,2**). Apart from serving as the hair shaft factory, the anagen hair bulb also provides the hair shaft's trichocytes with melanin granules. Within the hair bulb is a population of cells with the highest proliferation rate in the human body: the keratinocytes of the hair matrix. These can differentiate into trichocytes, or cells of the inner root sheath (IRS). The outer root sheath (ORS), hair matrix, and hair shaft derive from the epithelial stem cells in the bulge area, functioning as a pluripotent epithelial stem cell population for the skin (**Fig. 2**) [9,28,130].

The size of the anagen hair bulb, the duration of anagen, and the hair shaft diameter are determined by the volume, the number of cells, and the secretory activity of the DP[82,148]. Stringent coordination between epithelial and mesenchymal portions is needed to maintain the cyclic hair follicle growth[195]. Mesenchymal stem cells within the tissue sheath serve as a recruitment pool for new DP cells. Apart from mesenchymal stem cells, the hair follicle also contains mast cell precursors[81,90,134] and neuronal stem cells, the latter of which can develop into neurons and blood vessels[1]. The large numbers of stem cells make the hair follicle a fascinating organ in the field of stem cell biology.

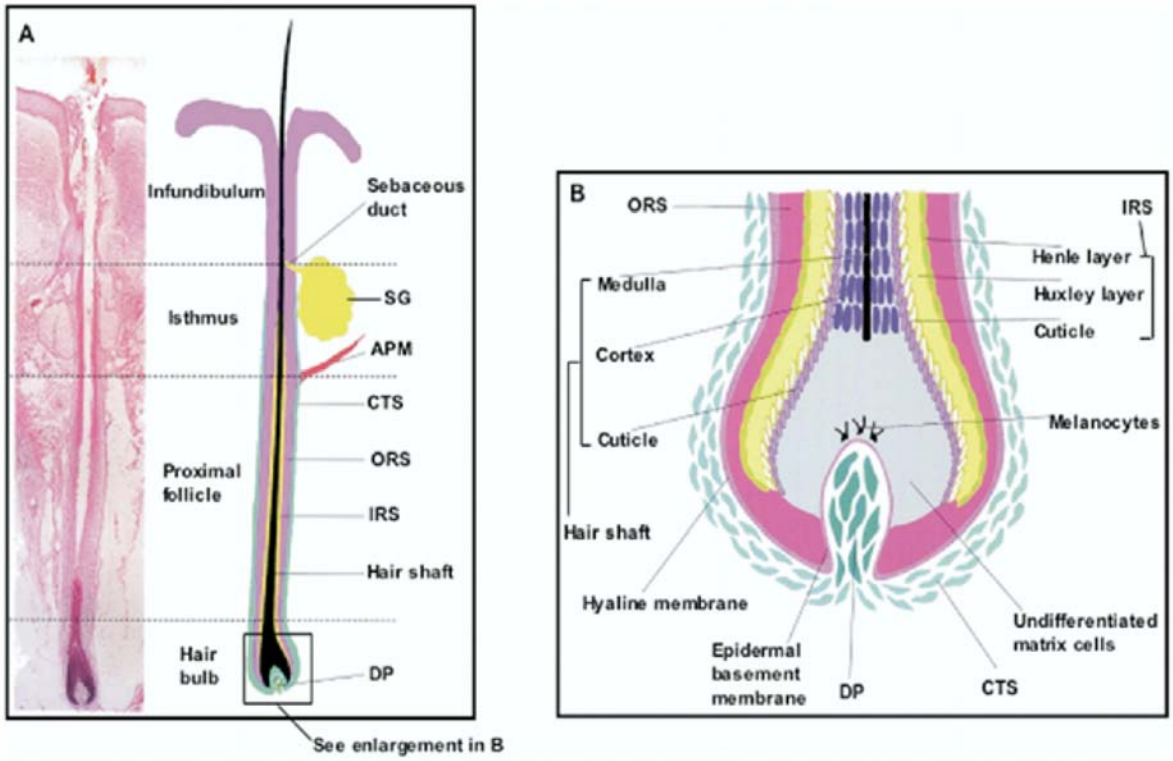


Figure 1. **Hair follicle anatomy**

A) Anagen VI hair follicle (IV is morphologic phase of anagen cycle). Histologic longitudinal section on the left hand side. Schematic drawing of an anagen VI follicle with anatomical details on the right hand side. B) Anagen VI hair bulb in detail (enlargement of schematic drawing in A). APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; IRS, inner root sheath; ORS, outer root sheath; SG, sebaceous gland[88] .

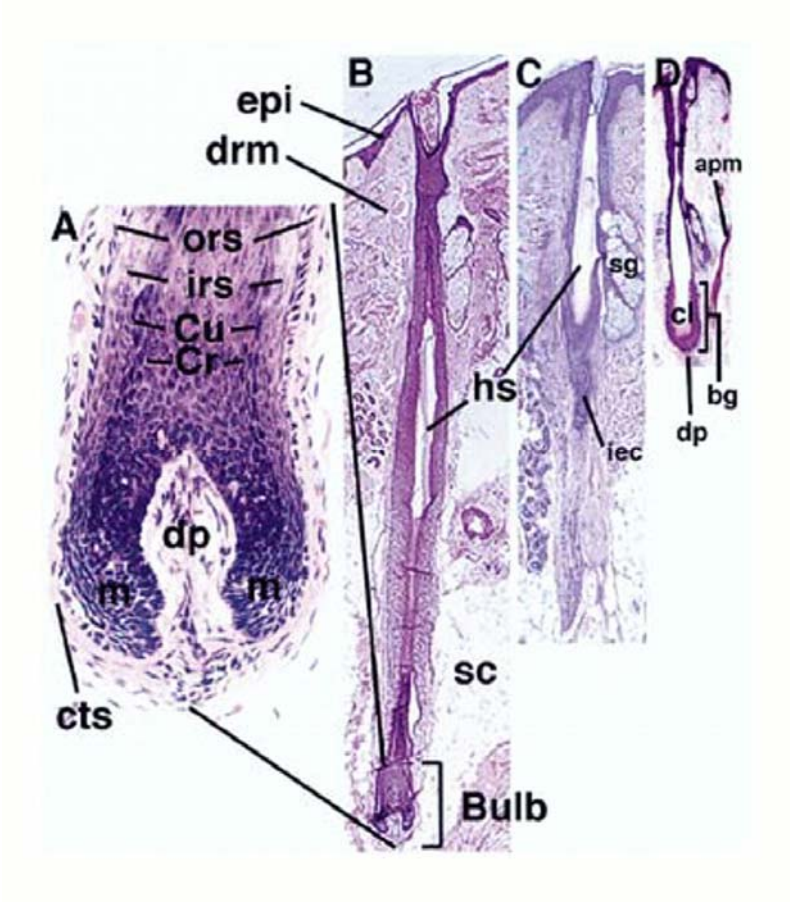


Figure 2. Structure of hair follicles during anagen, catagen and telogen stages of cycling (Hematoxylin and Eosin).

A) a high magnification of hair bulb during the anagen (growth) stage (x100). B) a scalp-hair follicle during anagen stage (x25). C) a scalp-hair follicle during catagen (involutional) stage (x40). D) a scalp-hair follicle during telogen (resting) stage (x25). In these panels, apm denotes arrector pili muscle, bg bulge, cl club hair, Cr cortex, Cu cuticle, cts connective-tissue sheath, dp dermal papilla, drm dermis, epi epidermis, hs hair shaft, iec involuting epithelial column, irs inner-rooth sheat, m matrix cells, ors outer root sheath, sc subcutaneous fat and sg sebaceous gland[145]

4.3 The Hair Cycle

Hair cycling is the rhythmic change of the hair follicle through phases of growth (**anagen**), regression (**catagen**), and rest (**telogen**). *Synchronized* hair follicle cycling (in mammals) prepares the hair coat for seasonal changes in habitat conditions as well as procreational activities[195]. The purpose of hair cycling in mammals with individual (*asynchronous*) follicle waves (e.g. humans) is not as obvious, but may include cleaning the skin surface of debris and parasites, and excretion of deleterious chemicals by encapsulation within trichocytes[195]. In addition, follicle cycling might serve as a regulator of paracrine or even endocrine secretion of hormones and growth modulators produced within the follicle and secreted into the skin or circulation[146]. Finally, hair follicle cycling may act as a safe-guard against malignant degeneration by protecting rapidly dividing keratinocytes from oxidative damage by deletion during catagen[88,144,146].

4.3.1 Anagen

Anagen (the growth phase of the hair cycle) is divided into 6 different stages defined by specific morphologic criteria[148] (**Fig. 3**). During anagen, epithelial stem cells differentiate into at least 8 different cell lines, forming the ORS, companion layer, Henle's layer, Huxley's layer, cuticle of the IRS, cuticle of the hair shaft, shaft cortex, and shaft medulla. The ORS probably is established by the downward migration of the regenerating epithelium[174]. IRS and hair shaft are tied together by their interlocked cuticle structures. The IRS-packaged shaft uses the innermost layer of the ORS (companion layer) as a slippage plane for orientation to move straight toward the skin surface[76,136].

Epithelial stem cells are located in the bulge area of the follicle. From there, in anagen stem cells ascend into the interfollicular epidermis and descend to differentiate into ORS cells. One hypothesis suggests that derivatives of stem cells from the bulge area reach the hair germ, transform into matrix keratinocytes, and rebuild the hair shaft[139]. During catagen, this stem cell population is situated lateral to the DP, protected from apoptosis and able to proliferate again in early anagen to produce a new hair shaft.

Hair shaft pigmentation only takes place in anagen. The cyclic reconstruction of an intact hair follicle pigmentary unit works optimally in scalp follicles during the first 10 hair cycles, meaning until approximately 40 years of age. Afterward there appears to be a genetically regulated exhaustion of the pigmentary potential of each individual follicle leading to “hair greying”[201].

4.3.2 Catagen

The anagen period ends with a highly controlled involution of the hair follicle resulting in apoptosis and terminal differentiation. This process, called catagen, consists of 8 different stages. The hair follicle epithelium, neuroectodermal cell populations (melanocytes and Merkel cells), the mesenchyme, the perifollicular vascular system, and the follicular innervation all show cyclic changes in proliferation, differentiation, and apoptosis[107,119,151]. During catagen, the DP condenses, moves upward, and comes to rest beneath the bulge. The hairless gene (Hr) is responsible for the strong connection between the condensing DP and the diminishing hair follicle epithelium in catagen and telogen follicles. In its function as a safeguard of apoptosis control during catagen[140], Hr operates as a negative transcription repressor and insures that apoptosis only takes place in certain tissues in the correct order.

4.3.3 Telogen

After regression, the hair follicle enters telogen, a phase of relative quiescence regarding proliferation and biochemical activity. The follicle remains in this stage until it is reactivated by intrafollicular and extrafollicular signals. The unpigmented club hair often remains stuck in the hair canal and is shed from the follicle during combing or washing. Most people lose 50 to 150 scalp hairs per day. The telogen stage typically lasts for two to three months before the scalp follicles reenter the anagen stage and the cycle is repeated.

The percentage of follicles in the telogen stage varies substantially according to the region of the body (e.g. 5 to 15 percent of scalp follicles are in the telogen stage at any one time, as compared with 40 to 50 percent of follicles on the trunk)[145].

The chronology of hair follicle cycling is:

catagen→telogen→anagen→catagen.

Contrary to common misconception, morphologically, hair follicle cycling does not begin with anagen, but with catagen (**Fig. 3**) i.e., shortly after hair follicle morphogenesis has been completed. The initiation of hair follicle cycling via catagen induction is followed by a first, short phase of telogen, after which the first anagen development is seen[195].

The hair follicle has only one irreversible physiologic mechanism to break out of the hair cycle: programmed cell death, e.g. perifollicular inflammation that destroys the bulge region and therefore the follicle's capacity to cycle[40]. This targeted destruction probably serves to remove degenerated and nonfunctioning hair follicles.

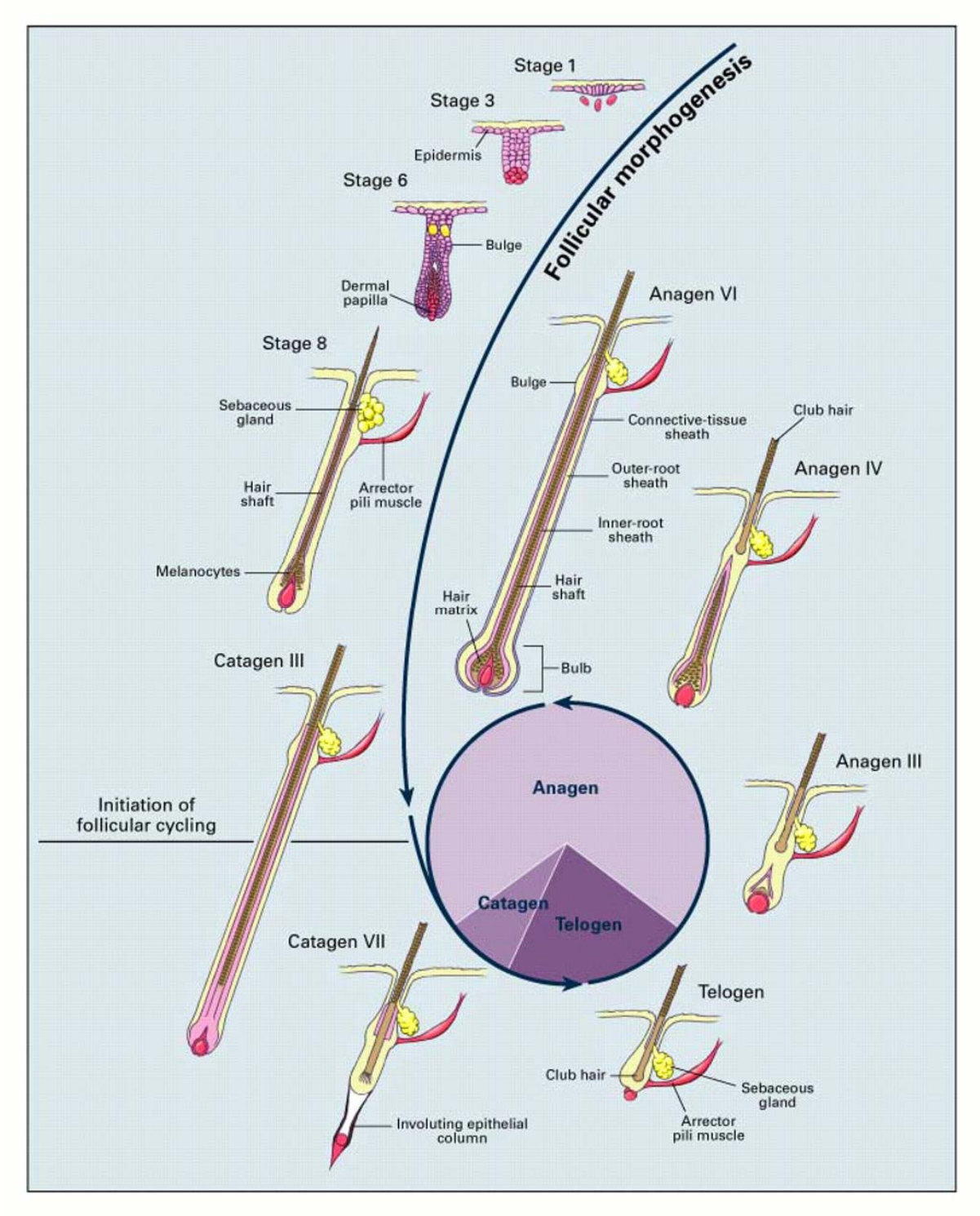


Figure 3. Development and cycling of hair follicles.

Selected stages of morphogenensis of hair follicles and three stages of follicular cycling (anagen, catagen and telogen) are shown. The roman numerals indicate morphologic sub-stages of anagen and catagen. The pie chart shows the proportion of time the hair follicle spends in each stage[145].

4.4 Locally Produced Growth Factors, Hormones, and Proteins

The hair follicle is not only a very productive source of pigmented hair shafts (keratins and melanin) but also of many growth-, pigment-, and immunomodulators. It can synthesize or metabolize an enormous number of hormones, neurotransmitters, neuropeptides and growth factors. For example, growth factors like TGF-β1/2, IGF1, HGF [48,75,108] and hormones like CRH, prolactin, cortisol, and melatonin[47,77,87] are all synthesized in the hair follicle. Androgens are metabolized to dihydrotestosterone or 17β-estradiol, and proopiomelanocortin to ACTH, alpha-MSH, or β-endorphin within the hair follicle[195]. The exact biological functions of the locally generated factors are not well understood. The hair cycle depends on this complex activity and on the expression of the specific matching receptors, suggesting that these actions function as autocrine and paracrine mechanisms.

As the hair follicle is regulated by diverse systemic extrafollicularly generated hormones and growth factors and by a variety of self-generated substances, it is no surprise that even small changes in this sensitive milieu can lead to a shortening of anagen, an induction of catagen, and to an increased number of telogen follicles, resulting in telogen effluvium.

4.5 Key Factors in Hair Follicle Cycling

It is now widely accepted that hair follicle transformation during cycling is caused by alterations in the local signaling milieu. There are key regulators that build up local gradients with competing stimulating and inhibitory signals (**Fig. 4**). Rhythmic changes of signal transducers in the key compartments of the follicle (bulge, secondary hair germ, dermal papilla) are thought to drive cyclic hair follicle transformation.

Key factors known to induce anagen include soluble proteins of the WNT family, activation of the corresponding β-Catenin pathway, noggin, and the transcription factor STAT3[14,128]. Sonic hedgehog, HGF, and FGF7 (KGF) support this process and stimulate the subsequent steps of anagen development[32,108,182].

DP-induced keratinocyte differentiation occurs via β-catenin/lef1 signaling[178]. Hair shaft differentiation seems to be mediated, at least in part, by desmoglein[124]. WNT signals (WNT3a and WNT7a) are capable of keeping the dermal papilla in anagen. IGF1, HGF, glial cell-derived neurotrophic factor, and vascular endothelial growth factor can prolong anagen (**Fig. 4**)[42,69,108,111,119,128,135,149,161]. During the anagen–catagen transformation of the hair follicle, the transcription factor Hr is a central, indispensable element of navigation and coordination of signal transduction. Loss of Hr function leads to rapid degeneration of the hair follicle[138]. Certain members of the homeobox gene family also seem to control some of the named factors. TGF-β1, TGF-β2, FGF-5, the neurotrophins NT3, NT4, BDNF, p75, also retinoids, prolactin, and several other candidates like thrombospondin 1 and vanilloid receptor 1 induce catagen[10,13,47,48,50,79,192,196,210]. An essential inhibition/disinhibition system in anagen development is the neutralization of BMP4 by noggin[15]. Anagen is terminated by the upregulation of hair growth inhibitors (TGF-β1, TGF-β2, FGF-5) and downregulated by anagen preserving factors (IGF-1, HGF, FGF-5S) at the same time.

It seems confusing that some hair growth modulators have growth-stimulating effects during morphogenesis but inhibitory effects in the hair cycle. TGF-β2, follistatin, and NT3, for example, accelerate hair follicle morphogenesis, but are catagen-inducing in mature anagen follicles[14,49,131,146]. Some of the very potent signal transducers of anagen induction or termination could lead to the development of specific pharmacologic agents that could manipulate the human hair cycle and treat hair growth disturbances more efficiently. However, none of these factors seems to be a key element of the hair cycle clock itself, which directs the factors to execute the cyclic hair follicle transformations.

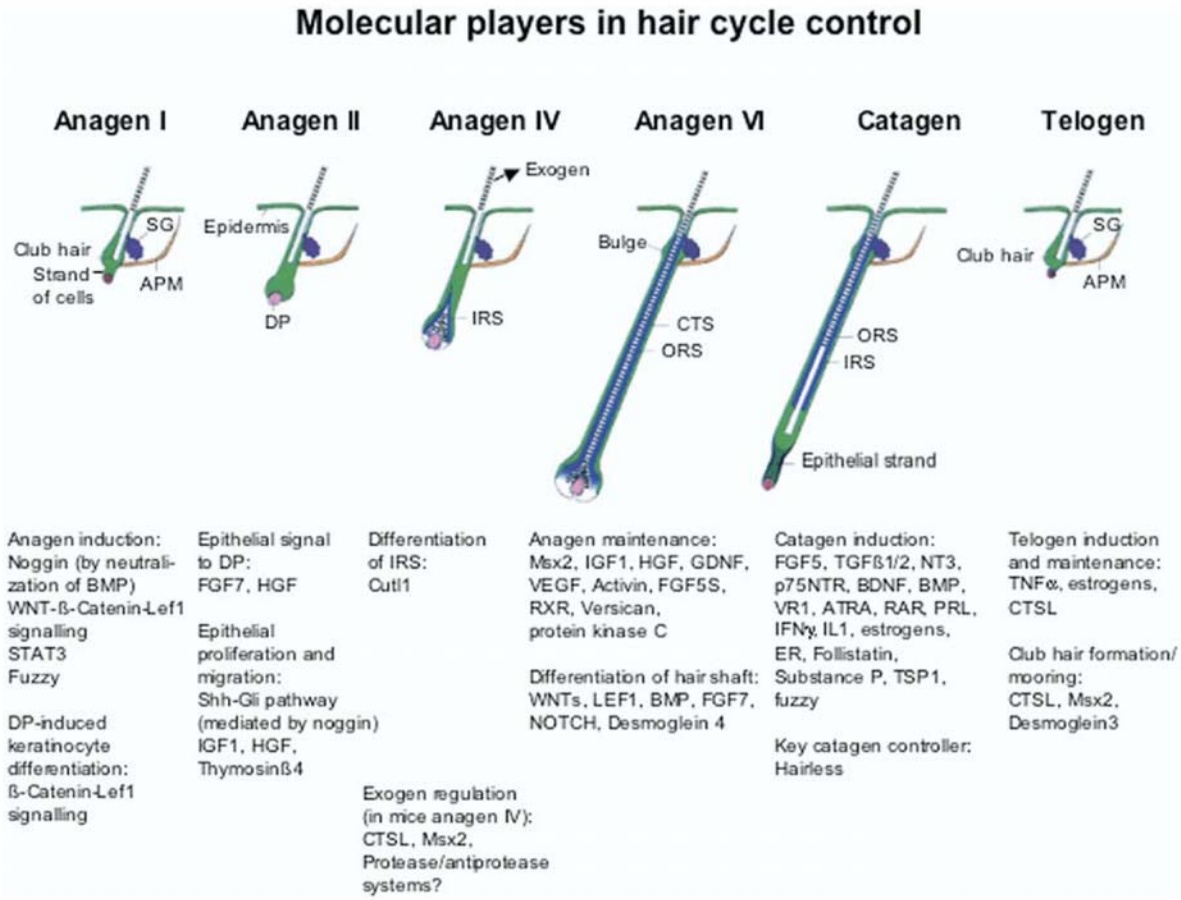


Figure 4. Molecular players in hair cycle control.

The figure shows key factors of hair follicle cycling being employed by the hair cycle control to drive the hair follicle from one stage to the next one or to keep it in a given stage. However, none of the named mediators are known to be key elements of the central pacemaker. (For references, see text). APM, arrector pili muscle; CTS, connective tissue sheath; DP, dermal papilla; IRS, inner root sheath, ORS, outer root sheath; SG, sebaceous gland; BMP, bone morphogenic protein; WNT, wingless; STAT3, signal transducer and activator of transcription 3; FGF7, fibroblast growth factor 7; HGF, hepatocyte growth factor; Shh, sonic hedgehog; IGF1, insulin like growth factor; CTSL, cathepsin L; cutl, transcriptional repressor; GDNF, glial cell line-derived neurotrophic factor; BDNF, brain-derived nerve growth factor; VEGF, vascular endothelial growth factor; ATRA, all-trans retinoid acid; RXR, retinoid x receptor; RAR, retinoid acid receptor; NGF, nerve growth factor; Lef1, lymphoid enhancer-binding protein; TGFβ, transforming growth factor β; p75NTR, low affinity neurotrophin receptor; PRL, prolactin; PRLR, prolactin receptor; IFNγ, interferon γ; ER, estrogen receptor; IL1, interleukin 1; VR1, vanilloid receptor 1; TNFα, tumor necrosis factor α; TSP1, thrombospondin 1; (modified after Paus and Peker[149]).

4.6 Sex Hormones as Potent Hair Growth Modulators

Androgens are very potent, yet non-essential, hair growth modulators. In hair growth regulation, different types of hair follicles in diverse body areas have different underlying cycle control mechanisms. A common example is the paradoxical effect of androgens on terminal follicles of the scalp compared with vellus follicles on other parts of the body. Androgens stimulate hair growth in non-scalp areas like the beard, breast or abdomen (at least in part by upregulation of gene expression and secretion of IGF1)[75]. In contrast, androgen sensitive hair follicles of the scalp become smaller under the influence of androgens (miniaturization) leading to the typical changes of androgenetic alopecia[33,70]. Inhibition of hair growth in the fronto-temporal region can be demonstrated by TGF-β stimulation[74]. Current theories suggest that scalp and body hair follicles react to androgen stimulation differently by triggering programmed gene regulation of defined hormones. These gene programs lead to potent hair growth stimulation in one follicle population and growth inhibition in others[117].

Localization and gender-specific regulation of hair follicle gene expression has also been demonstrated for estrogens. *In vitro* experiments show an inhibition of hair shaft elongation and anagen prolongation in human female occipital hair follicles, whereas in male frontotemporal scalp follicles, 17β-estradiol (E2) stimulates hair shaft elongation[25]. In vivo E2 also leads to anagen prolongation in human hair follicles[186].

4.7 Management of Hair Growth Disorders

Hair loss, as well as unwanted hair growth (hirsutism, hypertrichosis), is a widespread problem. According to one calculation, androgenetic alopecia on its own eventually affects approximately 50% of the world's adult population[145,206]. The hair shaft, the main product of the hair follicle, serves as an instrument of social communication, a protective device, and as a container for sequestering and excreting unwanted compounds[145,195].

Given the role of hair in psychosocial communication, (as a symbol of youth, health, fertility, and sexual potency) hair loss often has an underestimated psychosocial impact on an individual's self-esteem, interpersonal relationships, and positioning within society[57]. Telogen effluvium, androgenetic alopecia, and alopecia areata, the 3 most frequent hair loss disorders encountered in clinical practice, exemplify how a range of negative psychological and social experiences translate into significant stressors that possibly conspire to further aggravate hair loss[2-4,57]. The majority of the known hair growth disorders are a consequence of changes in the hair cycle. The most frequent growth disorder in men and women is **androgenetic alopecia** (AGA). AGA is characterized by a shortening of the anagen phase and a prolongation of telogen, combined with miniaturization of hair follicles[33]. These changes are androgen dependent and genetically determined. The underlying molecular mechanism depends on the conversion of testosterone to dihydrotestosterone by 5α-reductase. Dihydrotestosterone binds to androgen receptors of the hair follicle and leads to a shortening of anagen and a reduced cell hair matrix volume[31,120]. Men and women with AGA have a higher activity of 5α-reductase type II and androgen receptors in the frontal scalp area compared with the occipital area[170]. However, simply removing androgens does not usually result in the conversion of miniaturized follicles to terminal ones; thus, current treatments for advanced androgenetic alopecia, including minoxidil and finasteride, are usually ineffective.

The transient shedding of hair - **telogen effluvium** - that is associated with drugs, fever, endocrine abnormalities, parturition, anemia, and malnutrition occurs when an increased number of hair follicles prematurely enter the telogen stage and then shed their hair shafts. Transient shedding typically begins two to four months after the inciting event and lasts for several months[145]. Regrowth routinely follows, barring any metabolic or nutritional deficiency.

In contrast to androgenetic alopecia, **hirsutism and hypertrichosis** result from an extended anagen stage with an abnormal enlargement of hair follicles. Small vellus hairs are transformed into large, terminal hairs. Depilatory creams and waxes, the usual treatments, alleviate the problem only temporarily, because irritation or plucking rapidly induces the anagen stage and hair-follicle growth[145].

Electrolysis and selective photothermolysis with the use of lasers destroy the hair shaft, outer root sheath, bulge, and dermal papilla of the hair follicles[145]. The extent of the destruction determines whether the follicle regenerates.

Some types of **inflammatory alopecias** (such as those caused by lichen planopilaris and discoid lupus erythematosus) are scarring and permanent, whereas others (such as alopecia areata) are nonscarring and reversible[145]. In scarring alopecias, the inflammation usually involves the superficial portion of the follicle, including the bulge area, suggesting that the stem cells necessary for the regeneration of the follicle are irreversibly damaged. In contrast, the acute follicular inflammation in alopecia areata attacks the hair bulb in the subcutaneous fat[145]. This inflammation terminates the anagen stage, forcing the follicles into the catagen stage. However, because the bulge area is spared, a new hair bulb and hair shaft grow at the start of the anagen stage, once the inflammation has subsided or has been blunted with glucocorticoids.

Antineoplastic drugs disrupt the rapidly proliferating bulb matrix cells. As a result, hair production ceases, and the hair shaft becomes narrower, with subsequent breakage and loss of the hair. Because the hairs that are lost are those in the anagen stage, this phenomenon is called anagen effluvium. The stem cells of the hair follicles are spared, presumably because of their slow cycling, and they subsequently generate a new hair bulb. Radiation therapy can also result in reversible anagen effluvium. However, high doses of radiation (50 to 60 Gy) typically cause permanent alopecia, probably because of the destruction of the epithelial stem cells or the dermal papilla[145].

Figure 5 illustrates how common hair growth disorders can be managed by manipulating the hair cycle at different time points. AGA and telogen effluvium could be treated by inhibiting premature catagen transition and/or stimulating the telogen/anagen transformation. Catagen induction and arrest of follicles in a prolonged telogen stage may be a therapy for hypertrichosis and hirsutism.

A better understanding of the stem cell rich bulge region and the induction of secondary hair germ by DP signals could help narrow the search for tools that would limit the hair follicle miniaturization and loss seen in AGA. The discovery of

the enigmatic hair cycle clock is still the greatest challenge in hair research, and the one most likely to result in satisfactory treatment options in the field of hair growth disorders.

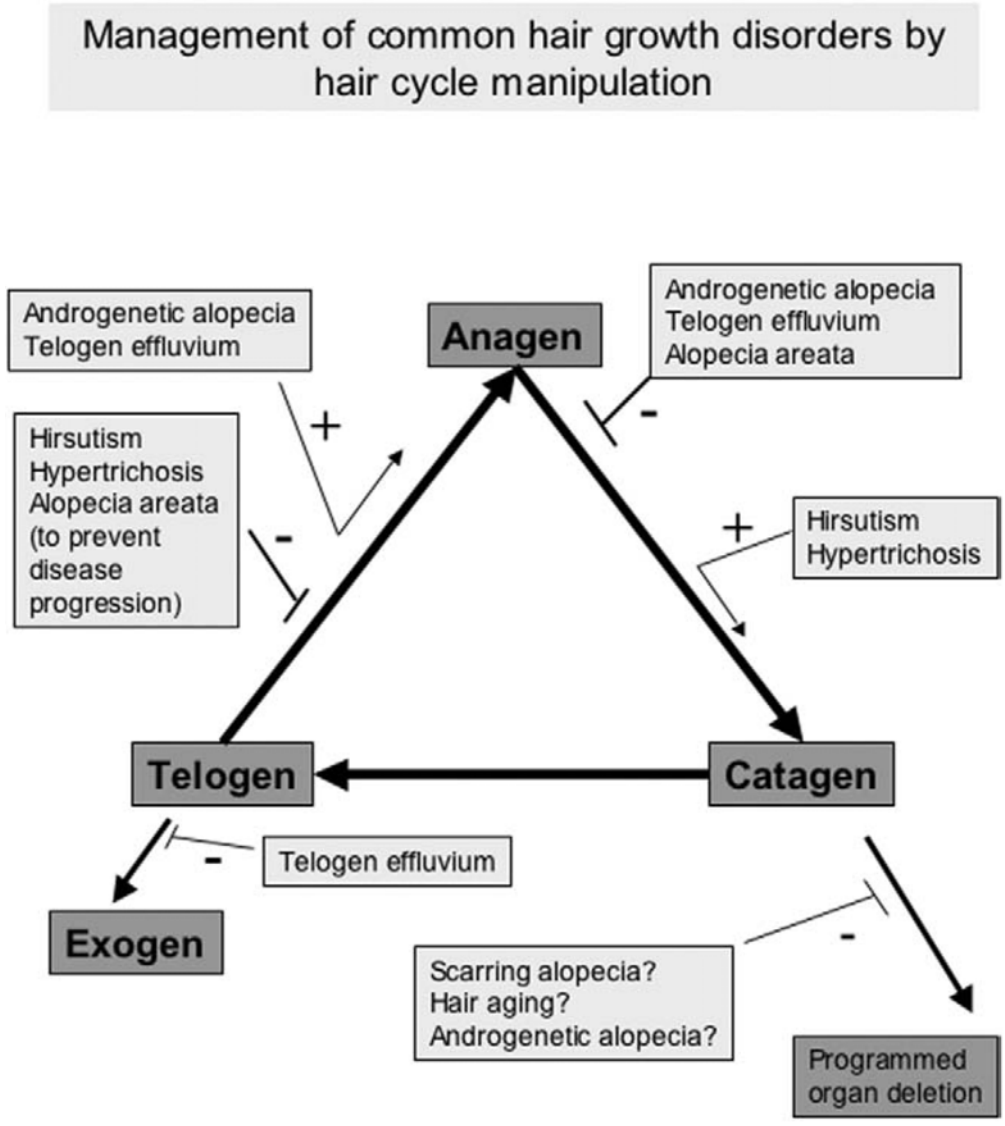


Figure 5. Management of hair growth disorders by hair cycle manipulation.

Common hair growth disorders frequently arise from changes of the hair cycle. They can be managed by manipulating the length of the different hair cycle stages. Anagen: growth phase with active production of a pigmented hair shaft, maximal length and volume of the follicle; catagen: apoptosis-driven phase of hair cycle regression with cessation of hair shaft production and pigmentation and club hair formation; telogen: phase of relative quiescence of hair follicle activity, exogen: active shedding of club hair; +, stimulate; −, inhibit (modified after Paus[145]).

5 Study Aims

"The Development of an Organotypic Folliculoid Assay Systems for the Exploration of Epithelial-mesenchymal Interactions in the Human Hair Follicle"

Worldwide annual investments made by consumers into manipulating their hair growth by pharmaceutical agents, cosmetics and/or nutraceuticals represent a major incentive for the pharmaceutical industry to develop new, and hopefully more effective hair growth-modulatory agents.

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 (HF).

The main aim of this study was therefore to develop an organotypic folliculoid (hair follicle-like) assay system (equivalent) that provides well-defined basic parameters for detailed exploration of epithelial-mesenchymal interactions in the human hair follicle. Furthermore the assay system should also serve as a screening tool for potential hair drugs modulators.

5.1 Criteria for the Development of Folliculoid Equivalent

The complexity of epithelial-mesenchymal interactions that underlie HF growth and cycling makes it unlikely that these will ever be fully reproduced *in vitro*. Therefore, when designing a human folliculoid *in vitro* system it is important to define the criteria that such an organotypic system should meet in order to mimic *in vivo* situation as closely as possible. Our 3D system should fulfill the following basic criteria:

1. Imitate at least some essential epithelial-mesenchymal interactions characteristic for human HF biology;
2. Show expected responses to recognized hair growth-modulatory agents; and
3. Provide a reasonable predictive value on how human HF will respond to the same test agent *in vivo*.

The work during this study was divided into 2 major parts including several objectives:

Part I:

To develop an organotypic system, which meets given criteria and provide well-defined basic parameters for quantitatively assessing the modulatory effects of a given test agent.

Part II:

To optimize existing system into high-throughput screening assay to allow to test standard hair growth- modulatory agents and to study in more detail the basic molecular processes involved in HF growth and development.

Objectives:

- To establish hair follicle cell cultures
- To design a structure of the folliculoid equivalents
- To produce well-reproducable hair equivalents with a large number of test units
- To optimize the culture condition of the equivalents
- To prove the suitability of the systems for investigation of the effects of hair growth-modulatory drugs
- To prove the suitability of the systems as a discovery tool for the identification of the target genes and the global gene expression profile

6 Materials and Methods

6.1 Cell isolation and cell culture

ORS keratinocytes were isolated from plucked anagen HF by trypsinization[106]. Primary cultures of ORSK were then cultured on feeder layer of X-ray irradiated human dermal fibroblasts (HDF), obtained from human skin from de-epidermized dermis using enzymatic digestion [102], in defined keratinocyte medium (SFM, Invitrogen, Paisley, UK) supplemented with 0.1 nmol L^{-1} cholera toxin, 5 μg mL^{-1} insulin, 0.4 μg mL^{-1} hydrocortisone, 2.43 μg mL^{-1} adenine, 2 nmol L^{-1} triiodthyronine, 10 ng mL^{-1} epidermal growth factor, 1 mmol L^{-1} ascorbyl-2-phosphate, and antibiotics penicillin G, gentamicin (all reagents purchased from Sigma-Aldrich, Taufkirchen, Germany). Cells at early passage (2-4) were used.

After microdissection of anagen VI HF from scalp-skin biopsies using a method modified according to Philpott [159], isolation of DPC from HF were established according to Magerl[115] and cultured in Chang medium (Trinova, Santa Ana, CA, USA) with 10% fetal bovine serum (FBS, Biochrom KG, Berlin, Germany) [123,204]. The cell passages of 1-2 were used.

6.2 Preparation of human organotypic folliculoid "sandwich" systems

Two different organotypic co-cultures were prepared based on previously described 3D systems by Limat[104] and Stark[193], with the basic modifications indicated in **Fig. 6B and C**.

In the first system ("layered sandwich", **Fig. 6B**), dermal equivalents were prepared from collagen type I extracted from rat tail tendons (BD Biosciences, Bedford, MA, USA) at a final concentration of 4 mg mL^{-1}. Eight volumes of ice-cold collagen solution were mixed with 1 volume of 10x Hank´s buffered saline (Invitrogen) followed by neutralization with 1 M NaOH. One volume of FBS was added with suspended HDF (passages 2-8) and mixed thoroughly resulting in final con-

centration of 3.2 mg mL^{-1} collagen and 2.5 x 10^5 cells mL^{-1}. Of this mixture, 1.5 mL each were poured into a 24-multiwell dish (Corning Costar, Corning, NY, USA) and allowed to gel at 37°C. After polymerization, Dulbecco's modified Eagle's medium (DMEM, Invitrogen) with 10% FBS was added up to the upper edge of the well [52,188]. The gels were cultivated under submerged conditions for 5 days, with changes of medium every other day. After this period the gels were maximally contracted and were used as pseudodermis. The pseudodermis was transferred by forceps into tissue culture transwells (pore size 0.4 μm, polycarbonate, Corning Costar) and overlaid with 0.1 mL of suspension of DPC in Matrigel™ Basement Membrane Matrix (BD Biosciences) at a concentration of 1x10^6 DPC mL^{-1}. The suspension geled within a few minutes at 37 °C, and then Chang medium[204] was added into the wells and cultured for 24 hours submerged. After this, the medium was removed and 1x10^5 ORSK (passage 2-4) in 0.1 mL of different media (see below) were seeded on top of the gels, 5 samples per each medium were prepared. Another 24 hours later, when the ORSK already had formed a sheet and had attached to the top of the pseudodermis with Matrigel™ + DPC layer, more medium was added until the entire sandwich system was submerged. Culture was performed for 14 days with medium changes every other day (note that previously published systems were cultured at the air-liquid interface[101,193] [see **Fig. 6A**], as opposed to the submerged conditions described herein).

The second, newly developed "folliculoid" 3D system ("mixed sandwich") (**Fig. 6C**) was prepared in an identical manner to the one described above, until the contracted pseudodermis had been transferred into tissue culture transwells. Then, a mixture of ORSK (1x10^6 cells mL^{-1}, passage 2-4), DPC (1x10^6 cells mL^{-1}, passage 1-2) in 1 volume of fetal clone II (FC, Perbio Science, Erembodegem-Aalst, Belgium), and the Matrigel™ matrix (9 volumes) was prepared and 0.1 mL of this suspension was poured on the top of the pseudodermis. The suspension geled within a few minutes at 37 °C and the different culture media were added into the wells (5 samples per each group) and the culture was submerged as described above. The experiments were repeated 3 times.

Throughout the experiments, three different culture media were used:

Culture medium 1. Composed of 3 parts of DMEM, mixed with 1 part of Ham´s F12 (Sigma-Aldrich) supplemented with 10% fetal clone (FC), 0.1 nmol L^{-1} cholera toxin, 5μg mL^{-1} insulin, 0.4 μg mL^{-1} hydrocortisone, 2.43 μg mL^{-1} adenine, 2 nmol L^{-1} triiodthyronine, 10 ng mL^{-1} human recombinant epidermal growth factor, 1 mmol L^{-1} ascorbyl-2-phosphate and antibiotics penicillin G, gentamicin. (modified from Smola[21]) The calcium concentration of this medium was 1.9 mmol L^{-1}.

***Culture medium 2*.** Similar component found in medium 1 but without FC. The calcium concentration of this medium was 1.8 mmol L^{-1}.

***Culture medium 3*.** Defined keratinocyte medium SFM (Invitrogen) supplemented with 0.1 nmol L^{-1} cholera toxin, 5 μg mL^{-1} insulin, 0.4 μg mL^{-1} hydrocortisone, 2.43 μg mL^{-1} adenine, 2 nmol L^{-1} triiodthyronine, 10 ng mL^{-1} epidermal growth factor, 1 mmol L^{-1} ascorbyl-2-phosphate, and antibiotics (penicillin G, gentamicin). The medium did not contain FC and the calcium concentration[45] was 0.15 mmol L^{-1}.

6.3 Preparation of Human Folliculoid Microspheres (HFM)

The following described method of HFM preparation has been patented under patent EP1231949. A beaker containing two phases of immiscible autoclaved liquids [lower phase: 250 ml of perfluorether (Fluorinert™ FC-40, 3M, 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[64] preparation of the “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, USA) at a final concentration of 4 mg/ml was mixed with 10x Hank´s buffered saline (Invitrogen) followed by neutralization with 1 M NaOH (Invitrogen), then an appropriate volume of Matrigel™ (BD Biosciences) was added and mixed. One volume of FBS 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 x 10^6 cells mL^{-1} and of DPC was 2 x 10^6 cells mL^{-1}). A 1 ml syringe was filled with the above cell-matrix suspension and, by gently pressing the syringe, small droplets were placed into the Miglyol-FC-40 mixture and formed HFM by way of a gelling process at 37 °C [8]. The HFM were left in this liquid for 5 min (with continuous stirring), then removed with a net, washed immediately in the culture medium and then placed in culture Petri dishes. The HFM were cultivated submerged in the aforementioned (see cell isolation and cell culture) supplemented low-calcium (0.15 mM) SFM for 10 days (**Fig. 13A**). Under these conditions, the average size of the HFM was 1.73 ± 0.33 mm on day 0 and 1.03 ± 0.20 on day 7. In order to determine the effects of high calcium content in growth media, a medium with a calcium concentration of 1.8 mM was used in some experiments. This (also serum-free) solution was composed of three parts of Dulbecco's Modified Eagle's Medium (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). HFM (20-30 per group) were cultured submerged in culture medium, and one third of the samples were collected on days 3, 7 and 10.

6.4 Histology and quantitative histomorphometry

For morphological analysis of folliculoid "sandwich" systems, the specimens were fixed in 4% formaldehyde (in phosphate-buffered saline, PBS, pH 7.4) for 24 hours. After dehydration, samples were embedded in paraffin and 5 µm sections were stained with hematoxylin-eosin (HE). On these sections, the quantitative evaluation of the spheroid diameter was also performed. Using a calibrated ocular eye-piece of a light-microscope (Olympus Optical Co., Japan), the largest diameter of the spheroids was measured in 3-5 samples per group (at least 75 spheroids

from 3-5 different folliculoid systems were measured per group). The statistical analysis was performed as described in section 6.9.

For morphological analysis of the HFM, samples were embedded into Thermo Shandon Cryochrome solution (Thermo Shandon Inc., Pittsburgh, PA, USA) and frozen in liquid nitrogen vapor. 8 µm thick sections were then cut, and, after fixation in ice-cold acetone, the samples were stained with hematoxylin-eosin (Sigma).

6.5 Immunofluorescence

Samples were embedded in Thermo Shandon Cryochrome solution (Thermo Shandon Inc., Pittsburgh, PA, USA) and frozen in liquid nitrogen vapor. Cryosections, made at 8µm settings, were mounted on silane coated slides, and air dried. Sections were then fixed for 10 minutes in acetone at –20 °C, rehydrated in phosphate-buffered saline (PBS), and then were pre-incubated with 10% serum species of the secondary antibody for 20 minutes. For immunofluorescence labeling, the following primary antibodies were used: mouse anti-cytokeratin 6 (CK6, clone KA12, 1:10, Progen, Heidelberg, Germany) to identify ORSK; mouse anti-large proteoglycan versican (Versican, 1:500, Seikagaku Corporation, Tokyo, Japan) to identify DPC; mouse anti-fibronectin (1:10, Acris, Hiddenhausen, Germany); mouse anti-c-kit/scattered factor (SCF, 1:100, Santa Cruz Biotech, Santa Cruz, CA, USA); rabbit anti-transformig growth factor-β2 (TGFβ2, 1:50, Santa Cruz Biotech). Sections were then labeled with appropriate fluorescein isothiocyanate-(FITC) or rhodamine-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). For positive controls for both CK6 and versican, cryosections of human HF[11,92,93] were used and stained similarly as described above.

To assess proliferation of different cell types in organotypic co-cultures, 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, USA) at 4 °C, then with the first secondary antibody (goat anti-rabbit, FITC-conjugated) for 45 minutes at room temperature. The samples were then incubated with either an antibody against CK6, which is not expressed by normal epidermal keratinocytes, yet characteristic for normal ORSK (1:10 of mouse anti-human CK6, Progen) [93,94] or with an antibody against the DPC-specific extracellular matrix antigen, Large proteoglycan Versican (Seikagaku Corporation, Tokyo, Japan) [84,85,191] for 1 h 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 PBS, counterstained with DAPI (4′,6-Diamidin-2-phenylindol fluorescent stain) (Sigma) and mounted with Fluoromount G (Southern Biotechnology Associates, Birmingham, AL, USA).

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, USA) were used following the instructions suggested by the manufacturer.

6.6 Cytotoxicity assay

To assess the presence of necrotic cells within the HFM, a Lactate-Dehydrogenase (LDH)-based Cytotoxicity Assay was applied (Bio Vision, Mountain View, California,USA). Briefly, the culture media of the 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.

6.7 Semi-quantitative RT-PCR technique

The expression of mRNA for TGFβ2 and SCF in HFM (and in microdissected human scalp hair follicles, used as positive controls) was determined by

semi-quantitive 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 1st Strand cDNA Synthesis Kit for RT-PCR (Boehringer Mannheim, Mannheim, Germany). Subsequent PCR amplification (94 °C for 5 min; 30 cycles of 94 °C for 30s, 57 °C for 60s, 72 °C for 60s; 72 °C for 10 min) was performed on the UNO-Thermoblock (Biometra, Göttingen, Germany) with the following primers (all from Sigma): TGFβ2, 5'-ATC CCG CCC ACT TTC TAC AGA C-3' and 5'-CAT CCA AAG CAC GCT TCT TCC-3' (GenBank accession number: Y00083); SCF, 5'-ATT CAA GAG CCC AGA ACC CA and CTG TTA ACC AGC CAA TGT ACG (GenBank accession number: M59964); β-actin, 5'-CGA CAA CGG CTC CGG CAT GTG C-3' and 5'-CGT CAC CGG AGT CCA TCA CGA TGC-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 Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Springs, MD, USA).

6.8 Microarray gene expression analysis

The microarray experiment was based on 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 (MMLVRT, Applera, Darmstadt, Germany) in order 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 A552 nm for cyanine 3-CTP and A650 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 was fragmented and mixed with control targets and hybridization buffer according to the supplier's protocol (Agilent

Technologies). Hybridization was 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 2-fold expression cut-off and 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.

6.9 Statistical analysis

In order to compare the proliferation and apoptosis of cells in the organotypic co-cultures, the percentage of Ki67-positive (proliferating) or TUNEL-positive (apoptotic) cells was determined (both in ORSK and in DPC) in at least 5 samples per group for each experiment. From 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 nonparametric test. Two types of statistical comparison were carried out for sandwich system. First, to reveal the differences between the different media within the given co-culture, the values obtained in the 3 different media were compared to one another either in the "layered" or in the "mixed" cultures. Then, to assess differences between the two co-cultures, values obtained in the given culturing medium in the "layered" sandwich were compared to those of the "mixed" one cultured in the same medium. P values < 0.05 were regarded as significant. A similar comparison was performed during the determination of the averaged cluster size and the average number of CK6+ ORSK and Versican+ DPC.

7 Experimental work

7.1 Experimental Part I - "Sandwich" system

Development of a Basic Organotypic Assay System that Imitates Human Hair Follicle-like Epithellial-Mesenchymal Interactions.

7.1.1 Introduction

The cyclic growth and regression activity of the HF is the result of bi-directional epithelial-mesenchymal interactions[27,82,120,122,139,145,176,195]. Even though the complexity of these interactions makes it unlikely that they can be fully reproduced *in vitro*, simplified in vitro-assay systems are required that attempt to imitate at least some key aspects of these interactions in a three-dimensional (3D) organotypic context[52,101,102]. Such assay systems hopefully will not only offer (urgently needed) hair research alternatives to the use of intact, organ-cultured human scalp HF [159], whose supply is very limited, but should also facilitate dissection of the as yet obscure molecular signaling principles that govern epithelial-mesenchymal interaction in *the human HF* (as opposed to the murine system, where due to the availability of numerous instructive mouse mutants with defined hair growth abnormalities[27,131] the molecular basis of epithelial-mesenchymal interactions in the HF during morphogenesis and cycling is already much better understood [27,145,195]).

Many different 3D organotypic assay systems on the basis of human skin cell populations have been developed for investigative dermatology[5,101,104,116,193,211]. These allow to study, for example, the role of diffusible factors affecting epithelial-mesenchymal interactions, the dynamics of basement membrane formation and to test the effects of various modulators, growth hormones, nutrients, etc.[19,112,113,188-190]. These human skin equivalents have become an indispensable alternative to animal models in pharmacological and toxicological *in vitro* testing[53,167]. As long as human HF epithelial stem cells and human hair matrix keratinocytes cannot be easily dissected, cultured and propagated *in vitro* without

loosing their *in vivo*-characteristics[95], it would be naive to expect that human HF-like structure can be reconstructed *in vitro* with such relative ease as is now the case for human epidermis and dermis. However, the characteristic mesenchymal component of the human HF, the highly specialized, inductive dermal papilla fibroblasts (DPC), can readily be dissected, cultured and propagated[115,123] as is the case for human outer root sheath keratinocytes (ORSK) [101,102,104,106]. Therefore, these cell populations offer themselves to be employed as the basis for any human folliculoid *in vitro* system.

When designing a human folliculoid *in vitro* system it is important to define the criteria that such an organotypic assay should meet in order to support the claim that it imitates the *in vivo* situation as closely, yet also as pragmatically and economically, as possible. Based on our current limited knowledge of human HF biology [80,122,139,145], we propose that the following minimal criteria should be met:

1. Given that direct interactions of the HF epithelium and the HF mesenchyme via native extracellular matrix are crucial for normal HF functions[104,109] , ORSK and DPC should be physically interacting under 3D conditions.
2. The extracellular matrix through which these interactions are occurring should also contain basement membrane components, since HF epithelium and mesenchyme are interacting via a basement membrane-like extracellular matrix [29,30,121,205].
3. Morphologically, the 3D system should allow the epithelial HF cells to form - ideally concentric - cell aggregates in order to mimic compact, functionally linked epithelial tissue compartments as they occur in the outer and inner root sheaths or the hair matrix of the HF *in vivo*.
4. While the epithelial HF cells should show substantial proliferation, HF-type keratinization (e.g. cytokeratin-6 [CK6] expression[92,93]) and a low level of apoptosis, the HF mesenchymal cells used in such a 3D system should show minimal proliferation and minimal apoptosis (as

is characteristic for the follicular dermal papilla *in vivo* [27,107,199] and should display secretory activities that distinguish HF fibroblasts from interfollicular dermal fibroblasts such as strong expression of versican. This proteoglycan, the expression of which correlates well with the hair inductive ability of DPC, was shown to act as a specific marker of matrix changes in the unique papilla extracellular matrix to promote HF development and cycling[36,86].

5. As *in vivo*, the interaction of HF epithelium and mesenchyme should be supported by a fibroblast-contracted collagen type I gel that resembles the interfollicular dermis.
6. Finally, in contrast to skin equivalents, "folliculoid" 3D systems should not be cultured at the air-liquid interface, but should be kept under continuously submerged culture conditions, since almost the entire HF epithelium lives under fully "submerged" conditions *in vivo*.

Historically, normal human epidermal keratinocytes (NHEK) were first used in organotypic 3D systems[112,189,190,193] and later ORSK were also employed[101,102,104]. In these systems were cells layered above collagen I or collagen I mixed with human dermal fibroblasts (HDF) forming pseudodermis[15,193]. Later, these collagen I gels or a pseudodermis were covered with various cell and matrix mixtures, which contained basement membrane and extracellular matrix components (Matrigel™), NHEK or ORSK, plus mesenchymal cells (HDF or DPC) in a number of different designs (see **Fig. 6A**) [101,193]. Ultimately, all these previously reported 3D systems with HF cell populations were cultured at the air-liquid interface. The comprehensive data on the ratio of proliferation/apoptosis of ORSK and DPC in these systems, and on HF-like differentiation markers (e.g. CK6 for ORSK, versican for DPC [36,86]) were not assessed in the classical studies that have pioneered the field [101,104,113,189,190,193] .

The aim of the present study, therefore, was to develop the first organotypic "folliculoid" 3D system that meets all of the above six criteria (as pragmatically and

economically as possible), and provides well-defined basic parameters for quantitatively assessing the modulatory effects of a given test agent.

In order to achieve this, we developed two continuously submerged 3D systems, whose basic design principle is outlined in **Fig. 6B and C**: In the *"layered sandwich"* (**Fig.6B**), the pseudodermis (collagen I mixed with HDF) was first layered by Matrigel™ mixed with DPC; over this, an ORSK cell suspension was layered 24 hours later. In the *"mixed sandwich"* (**Fig. 6C**) a mixture of Matrigel™, DPC, and ORSK was placed on top of a pseudodermis. Since proliferation and differentiation of most skin cells, especially of NHEK, are strongly affected by the presence of serum components and the calcium concentration in the culture medium[6,11,19,51,137,180,188,189,193], we investigated the effects of serum withdrawal and of switching from a high to a low calcium concentration in the culture media on the characteristics of both systems. Both systems were then compared by histology, quantitative histomorphometry and/or immunohistology with respect to overall morphology, arrangement and number of epithelial cell clusters, ORSK and DPC proliferation or apoptosis, CK6 or Versican expression.

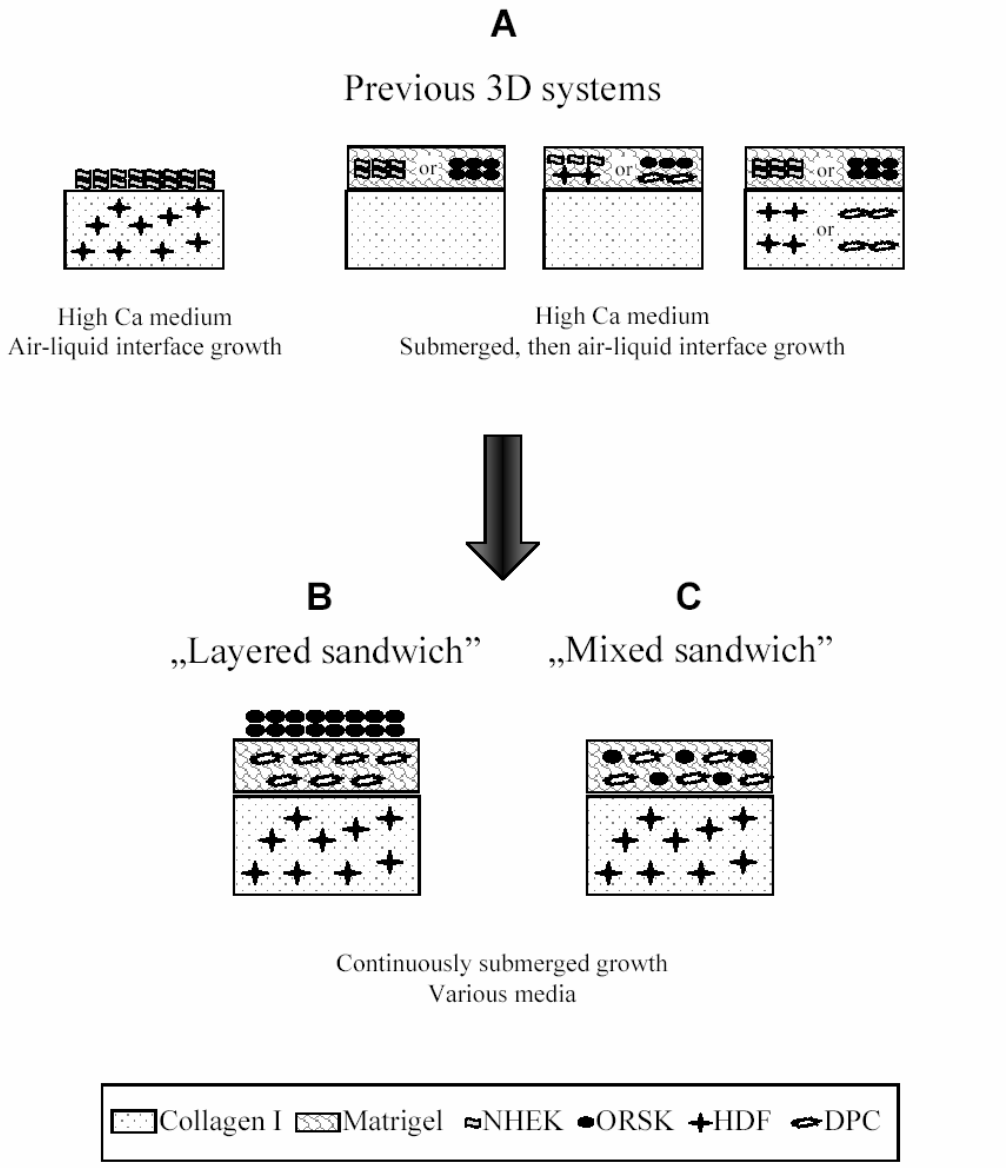


Figure 6. Schematic drawing of previously described organotypic co-cultures and the newly developed systems.

Normal human epidermal keratinocytes (NHEK) were used in previous organotypic 3D systems layered above collagen I mixed with human dermal fibroblasts (HDF). Also only collagen I gels or collagen I mixed with HDF or dermal papilla fibroblasts (DPC) were used and covered with various cell and matrix mixtures. Matrigel™ mixed with NHEK or outer root sheath keratinocytes (ORSK), and Matrigel™ mixed with epithelial and mesenchymal cell populations NHEK and HDF or ORSK and DPC was used (panel A) [101,193]. All these 3D systems were cultured at the air-liquid interface. Our new "layered sandwich" was prepared from collagen I mixed with HDF (pseudodermis) and layered firstly with Matrigel™ with DPC above and after with ORSK on the top

(panel B). In the “mixed sandwich”, we used pseudodermis (collagen I with HDF) layered above with the mixture of DPC and ORSK in Matrigel™ matrix (panel C).

7.1.2 Results

The „mixed” and the „layered” 3D systems meet all basic criteria for „folliculoid” organotypic systems that imitate human hair follicle epithelial-mesenchymal interactions.

Both the „mixed” and the „layered” folliculoid 3D systems are the first organotypic assays that meet all six minimal criteria proposed above that any *in vitro* organotypic assay system should fulfill, which aims at imitating basic principles of epithelial-mesenchymal interactions in the human HF:

1. ORSK and DPC are in physical contact under 3D conditions (see **Fig. 7B**) .
2. The use of Matrigel™, which contains laminin, collagen IV, heparan sulfate proteoglycans, entactin, nidogen (BD Bioscience product specification sheet), ensures that the extracellular matrix, through which ORSK-DPC interactions occurr, also contains basement membrane components.
3. Both 3D systems allow the epithelial HF cells (here: ORSK) to form spheroid cell aggregates, thus at least vaguely mimicking the compact epithelial tissue compartments as they occur in the outer and inner root sheaths or the hair matrix of the HF *in vivo* (**Fig. 7A, B**).
4. The epithelial HF cells (ORSK) show substantial proliferation (**Fig. 8A**)**,** HF-type keratinization (i.e. CK6 expression; **Fig. 9**) and a low level of apoptosis (**Fig. 8C**). Instead, as required, the HF mesenchymal cells (DPC) show minimal proliferation (**Fig. 8B**) and minimal apoptosis (**Fig. 8D**)**,** thus approximating the characteristically minimal basal rate of proliferation of the follicular dermal papilla and the virtual absence of fibroblast apoptosis, *in vivo*. In addition, the DPC maintain in both sandwich systems their characteristic secretory activities that distinguish HF fibroblasts from interfollicular dermal fibroblasts (i.e. they display strong expression of the large proteoglycan, versican [**Fig. 10A**]).

5. As *in vivo*, the interaction of ORSK and DPC is supported by a fibroblast-contracted collagen type I gel that resembles the interfollicular dermis (**Fig. 7A**).
6. The "folliculoid" 3D systems can be successfully cultured under continuously submerged culture conditions.

In both the "layered" and "mixed" folliculoid systems, the ORSK formed spheroid epithelial cell aggregates which retained their characteristic keratin expression pattern (**Fig. 9A**), while the DPC were cumulated in clusters and remained versican+ (**Fig. 10A**). In the following, differences between the two different "folliculoid" systems and between the use of three different media (high/low calcium, presence/absence of serum) are delineated.

The "mixed" sandwich model generates outer root sheath keratinocytes aggregates with a smaller diameter, while the outer root sheat keratinocytes and dermal papilla cell numbers are higher than in the "layered" sandwich model.

As revealed by HE staining of the histological sections of the 3D systems, significant differences among the morphological characteristics of the two cultures were found (**Fig. 7A,B**). Although, in both systems, the ORS cells formed multicellular spheroid clusters (similarly to those described by Limat [101]), the spheroids in the "layered" sandwich entered much less to the Matrigel™, whereas the ORS clusters were well distributed in the Matrigel™ matrix of the "mixed" sandwich.

In addition, the "layered" system, in media 1 (high Ca with FC) and 2 (high Ca without FC), showed much larger spheroids than in medium 3 (low Ca without FC) (**Fig.11**): the mean diameter of the multicellular aggregations was 0.522 ± 0.12 mm in medium 1; 0.44 ± 0.21 mm in medium 2; and 0.183 ± 0.08 mm in medium 3 (all expressed as mean ± SD). In the "mixed" sandwich, however, when compared to results with the "layered" sandwich (**Fig.11**), much smaller clusters

were formed in media 1 (0.313 ± 0.13 mm, mean ± SD) and 2 (0.239 ± 0.078 mm, mean ± SD) but not in medium 3 (0.206 ± 0.041 mm, mean ± SD), indicating that modifications of the culture media affected the morphological characteristics of the "layered" system much stronger than that of the "mixed" sandwich. Although this was not evaluated quantitatively, high calcium conditions appeared to favor cornification in the ORSK aggregates in the "layered" sandwich assay (see **Fig. 9A**, left panel)

As microscopic comparison of immunofluorescent pictures of different sandwiches revealed no noticeable differences among various culture media, the number of CK6 and Versican positive cells were measured in the multicellular clusters. As seen in **Fig. 12A**, the number of (CK6+) ORSK in media 2 and 3 was much greater in the "mixed" sandwich than in the "layered" one. Furthermore, in the "layered" co-culture, but not in the "mixed" one, the withdrawal of serum and/or the decrease in calcium content of the culture media remarkably suppressed the number of cells. In addition, the number of (versican+) DPC was also much greater in the „mixed" sandwich in all three media applied (**Fig. 12B**). However, we found significantly suppressed DPC numbers in medium 3 in both sandwiches.

Outer root sheat keratinocytes proliferate better in the „mixed" than in the „layered" sandwich system, regardless of the calcium or serum content of the media, whereas the proliferation and apoptosis of dermal papilla cells, in both sandwich systems, strongly depends on the amount of serum and calcium in culture media.

The proliferation and apoptotic properties of ORSK and DPC (**Fig. 8**) after 14 days in the culture were compared. In medium 1 (high- calcium medium, presence of FC), the growth properties of ORSK were significantly greater in the „mixed" sandwich compared to the "layered" one, as revealed by immunohistochemical detection of the proliferation marker Ki67 and by determining the percentage of Ki67 and CK6 positive cells (**Fig. 8A**). In contrast, such differences

were not observed in the case of DPC; namely, in medium 1, the percentage of Ki67 and versican positive cells were very similar both in the „layered" and in the „mixed" systems (**Fig. 8B**).

In medium 2 (high-calcium medium without FC), the expression of Ki67 in CK6 positive cells behaved very similarly to that seen in sandwiches cultured in medium 1 (**Fig. 8A**). Cellular proliferation of ORSK in the „mixed" sandwich significantly increased when compared to the „layered" one. However, although some non-significant tendencies for higher proliferation in serum-free medium could be observed, we found no marked differences among data obtained in the two media in either sandwich.

In contrast, the Ki67 expression in DPC was remarkably affected by the serum content of the medium (**Fig. 8B**). In both sandwiches, the withdrawal of serum significantly reduced the percentage of Ki67 in Versican positive cells suggesting that the presence of serum is a growth-promoting factor for the proliferation of DPC. Furthermore, we observed a significantly decreased Ki67 expression in DPC (cultured in medium 2) in the „mixed" sandwich compared to the „layered" one.

The behavior of cells in the sandwiches in low calcium concentration and serum-free medium (medium 3) were also examined. Essentially, very similar findings were observed for both the ORSK and DPC with those in the experiments performed in medium 2 (**Fig. 8A and B**). Namely, cellular proliferation of ORSK in the „mixed" sandwich significantly increased compared to that seen in the „layered" system. Moreover, no major differences were found among the effects of different media in either sandwich. In addition, similarly to that seen in medium 2, significantly less Ki67 expression in versican+ DPC was identified in the low calcium medium 3 in both sandwiches than found in medium 1.

Interestingly, the proliferation of ORSK was not affected by the serum or calcium content of the media in both systems, similarly to findings observed with

organotypic co-cultures with NHEK[193]. However, it is important to note that ORSK proliferated better in the „mixed" sandwich than in the „layered" cultures (**Fig. 8A**).

Apoptosis of DPC is highest in the presence of serum and/or under high calcium conditions, while the apoptosis of both ORSK and DPC is lowest in the "mixed" sandwich model cultured in serum-free, low calcium medium

The percentage of TUNEL positive cells in ORSK growing in the „layered" 3D system was almost identical in the three different solutions (**Fig. 8C**). Similarly to these data, no differences among the apoptosis of ORSK growing in the „mixed" sandwich in the high calcium concentration media (medium 1 and 2) were observed. However, in contrast to the above data, ORS cells of the „mixed" sandwich showed an extremely suppressed apoptosis in the low calcium serum-free medium 3 (**Fig. 8C**).

In the „layered" sandwich, the percentage of TUNEL positive DPC cells in the different media changed very similarly to that found with the expression of the proliferation marker (seen in **Fig. 8B**). Namely, the highest level of apoptosis was observed in medium 1 (high calcium, presence of serum), whereas the decrease of calcium concentration in medium 3 significantly (and the withdrawal of serum markedly yet not significantly) suppressed the number of TUNEL positive cells (**Fig. 8D**). In the „mixed" sandwich, the highest level of DPC apoptosis was also observed in medium 1, which, similarly to that seen in the „layered" system, slightly decreased by the removal of serum in medium 2, which however was remarkably suppressed when calcium was decreased in medium 3.

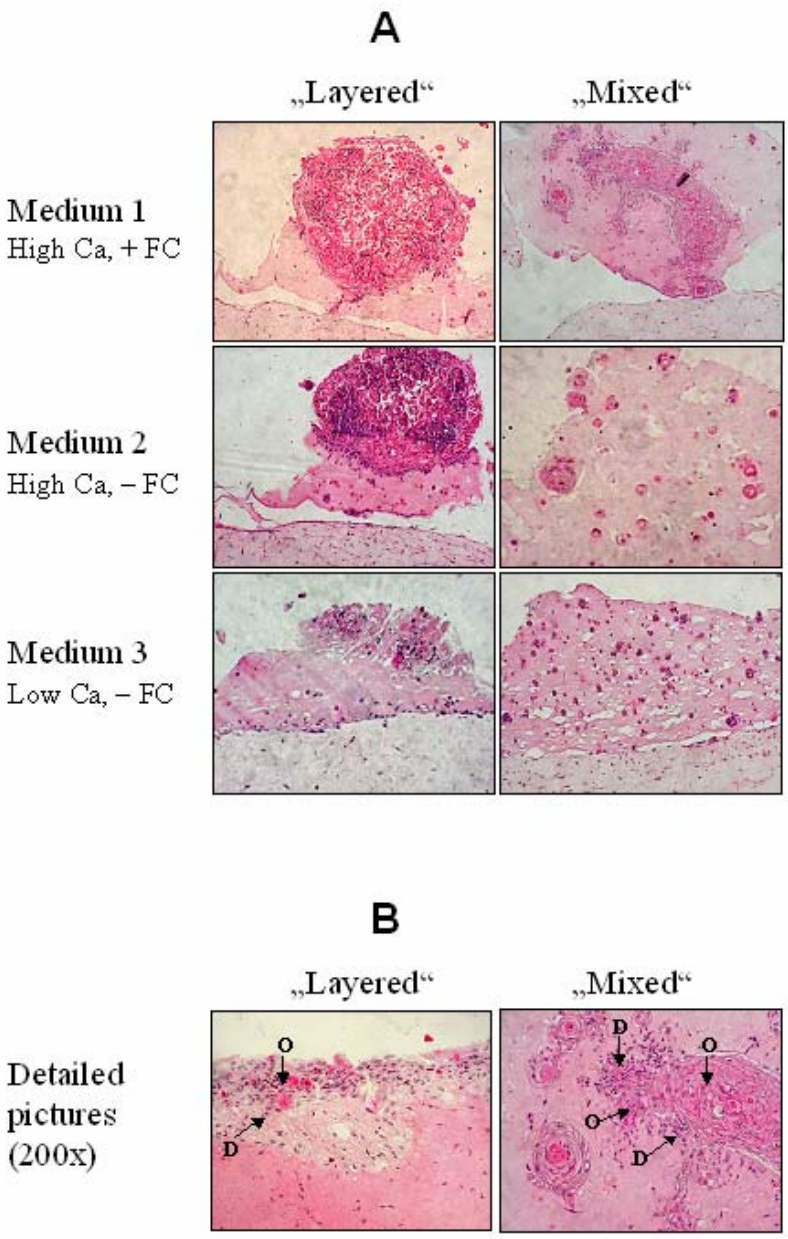


Figure 7. Histology (hematoxylin-eosin stain) of spheroid structures in the "layered" and "mixed" systems cultured in various media.

Panel A. Different 3D co-cultured "layered" and "mixed" systems were established and cultured submerged in medium 1 (high Ca concentration, with Fetal Clone, + FC), in medium 2 (high Ca concentration, without Fetal Clone, - FC), or in medium 3 (low Ca concentration, without Fetal Clone, - FC). Panel B. Outer root sheath keratinocytes (O) and dermal papilla fibroblasts (D) are in physical contact under 3D conditions. Original magnification, 100x for panel A, 200x for panel B.

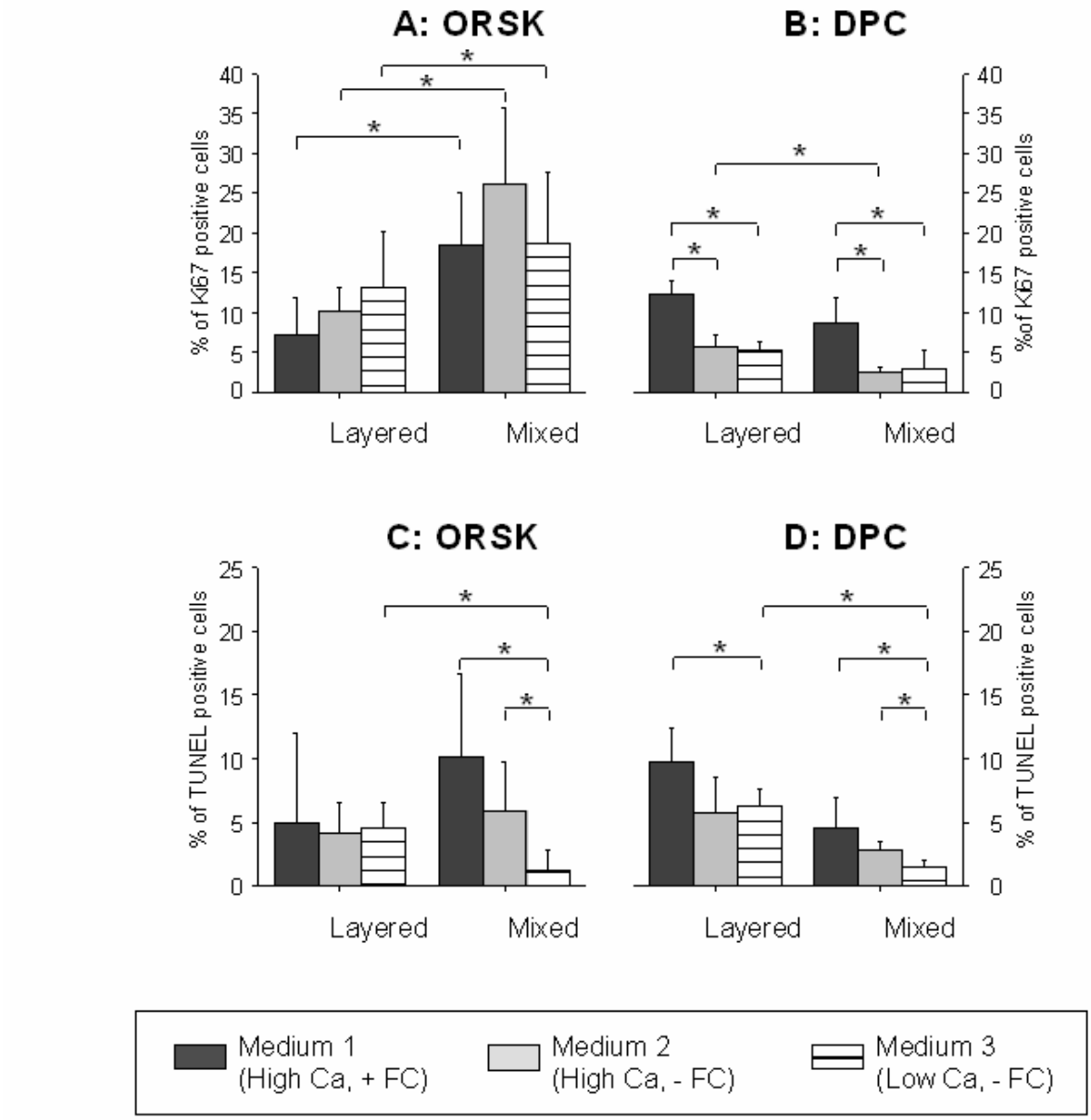


Figure 8. Quantitative analysis of proliferation and apoptosis of ORSK and DPC in the two systems cultured in various media for 14 days.

Series of double immunolabeling was performed to define the number of Ki67 positive (proliferating, panel A and B) and TUNEL positive (apoptotic, panel C and D) cells in cytokeratin-6 (CK6) expressing outer root sheath keratinoctyes (ORSK, panel A and C) or versican expressing dermal papilla fibroblasts (DPC, panel B and D) cells, 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.

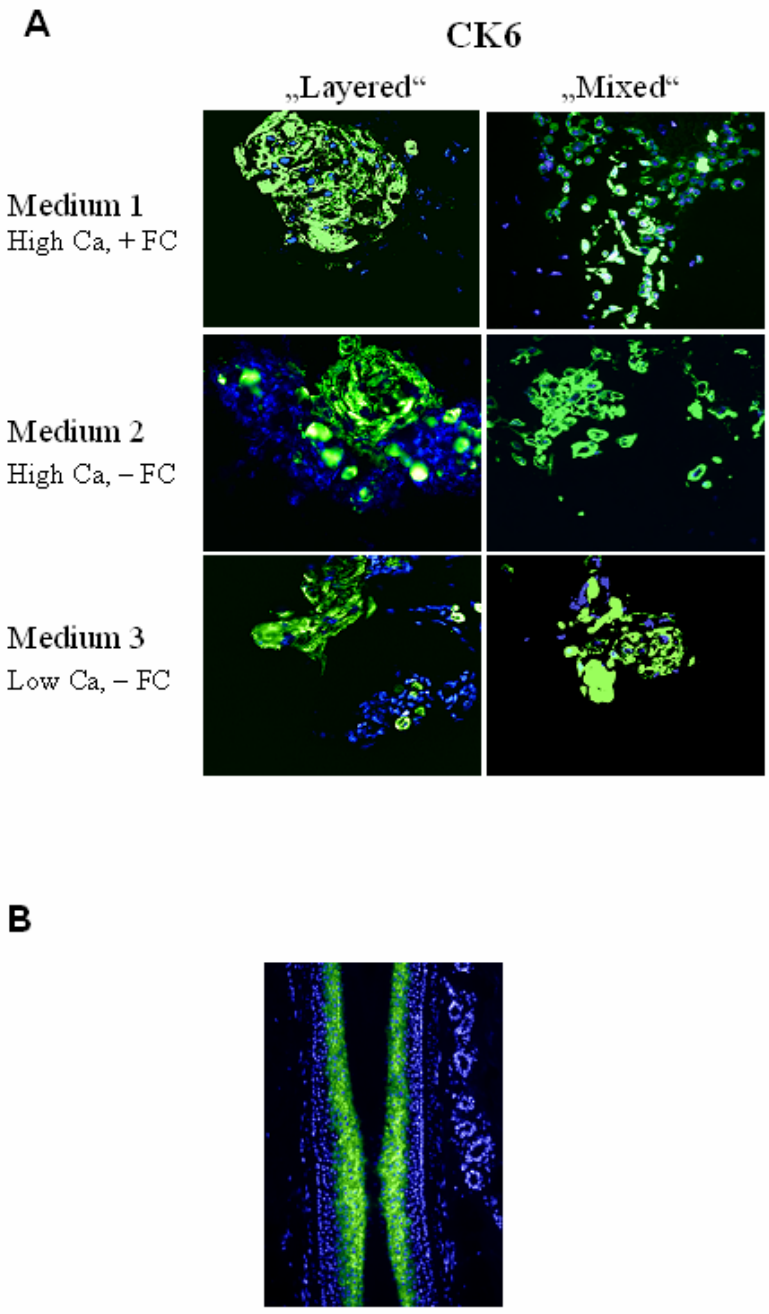


Figure 9. Immunohistochemical identification of CK6 expression in the two systems cultured in various media.

Cryosections of 3D co-cultures (panel A) and human hair follicle, which served as a positive control (panel B), were air dried, fixed in acetone, and then probed with a mouse anti-human cytokeratin-6 (CK6) to identify outer root sheath keratinocytes. Immunofluorescent labeling was employed using a FITC-conjugated secondary antibody (green fluorescence) whereas cell nuclei were labeled using DAPI (blue fluorescence). Original magnification, 250x for panel A, 100x for panel B. All figures are representatives of at least three determinations with similar results.

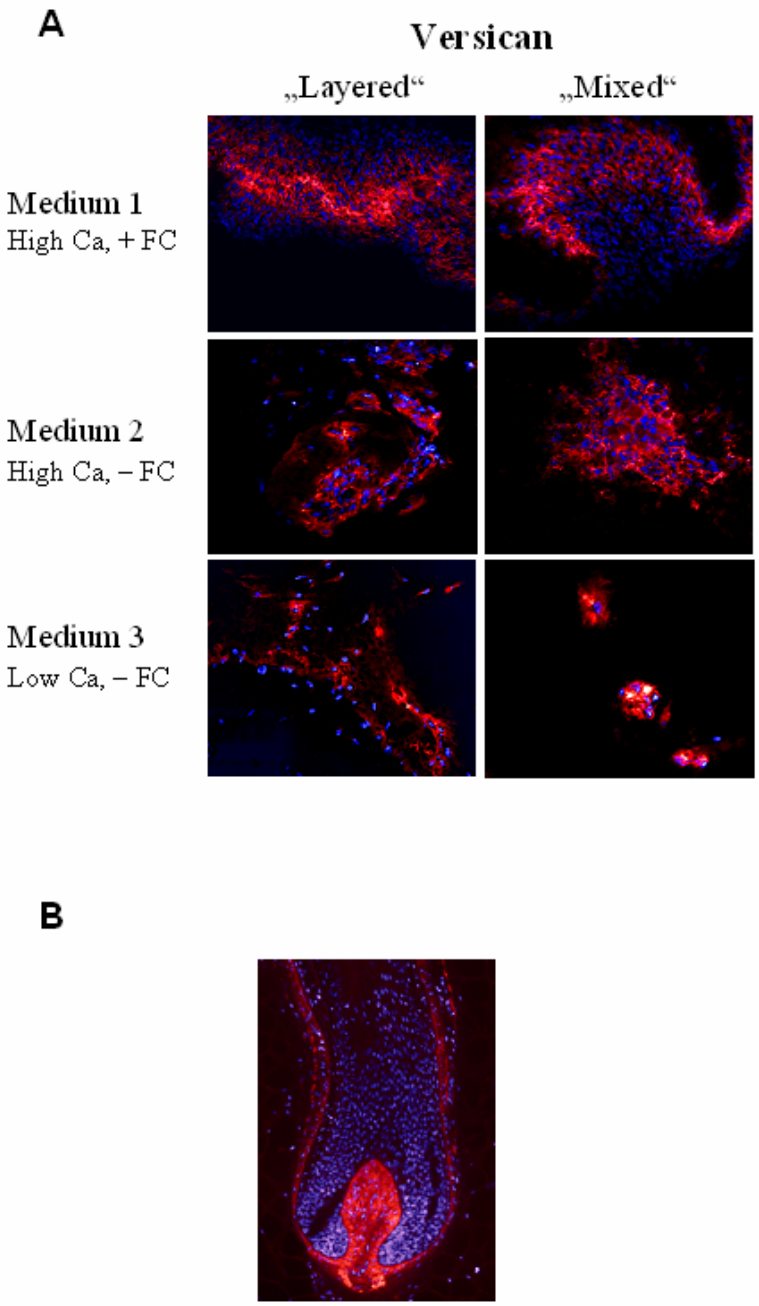


Figure 10. Immunohistochemical identification of versican expression in the two systems cultured in various media.

Cryosections of 3D co-cultures (panel A) and human hair follicle, which served as a positive control (panel B), were air dried, fixed in acetone, and then probed with a mouse anti-human versican to identify dermal papilla fibroblasts. Immunofluorescent labeling was employed using a rhodamine-conjugated secondary antibody (red fluorescence) whereas cell nuclei were labeled using DAPI (blue fluorescence). Original magnification, 250x for panel A, 100x for panel B. All figures are representatives of at least three determinations with similar results.

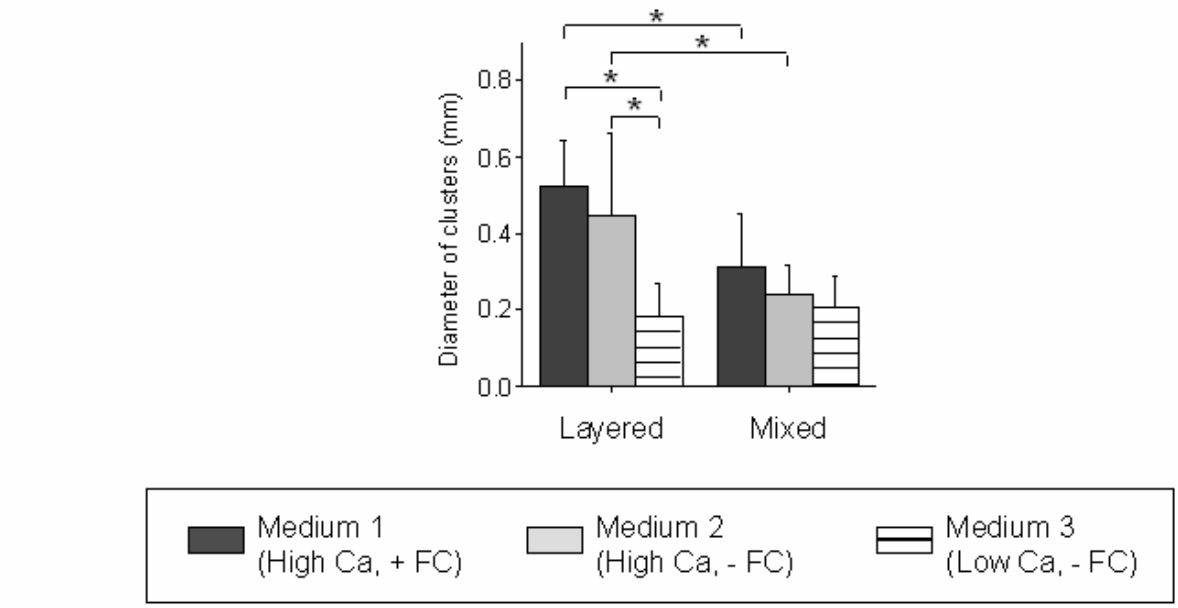


Figure 11. Quantitative analysis of the averaged diameter of multicellular spheroid clusters in the two systems cultured in various media.

On the HE stained histological sections, using a calibrated ocular eye-piece of a light-microscope, the largest diameter of the spheroids was measured on at least 75 clusters per group. The values were then averaged and were expressed as mean ± SD. Statistical analysis was performed using Mann-Whitney non-parametric test. Two types of statistical comparison were carried out. First, to reveal the differences between the different media within the given co-culture, the values obtained in the 3 different media were compared to one another either in the “layered” or in the “mixed” cultures. Then, to assess differences between the two co-cultures, values obtained in the given culturing medium in the “layered” sandwich were compared to those of the “mixed” one cultured in the same medium. Asterisks mark significant ($p < 0.05$) differences.

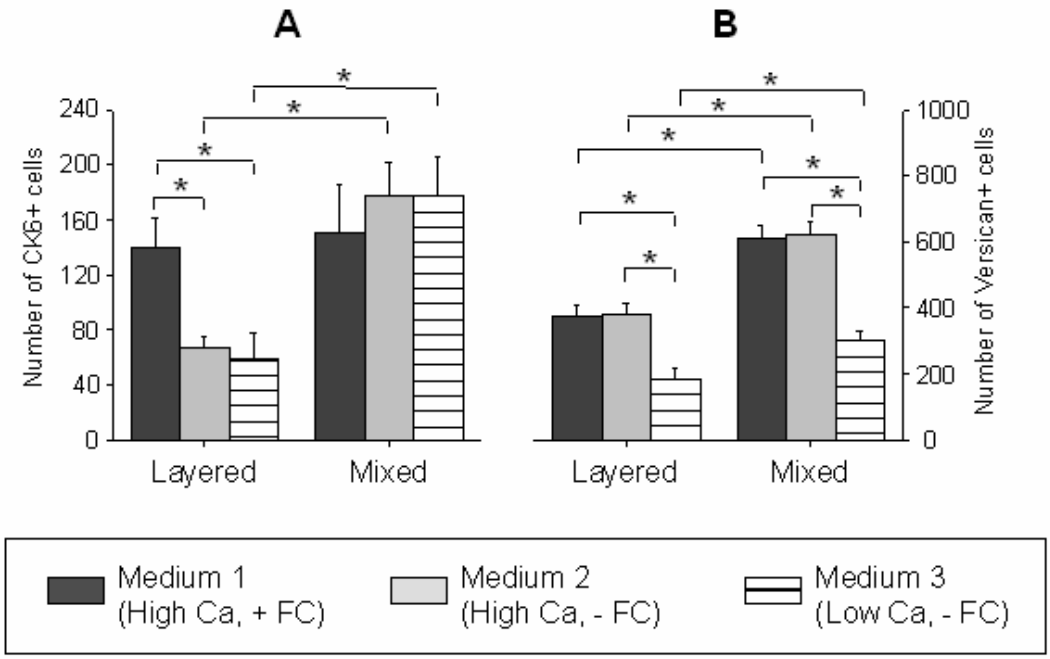


Figure 12. Quantitative analysis of the number of CK6 or versican positive cells of multicellular spheroid clusters in the two systems cultured in various media.

The number of cytokeratin-6 (CK6) (panel A) or versican (panel B) positive cells in clusters was determined by visual counting of cells on immunolabeled sections of 3D systems. Values were then averaged and shown as mean ± SD. Asterisks mark significant ($p < 0.05$) differences.

7.1.3 Discussion

The aim of the present study was to develop the first “folliculoid” 3D system that meets - as pragmatically and as economically as possible - six defined minimal criteria of a useful human “folliculoid” organotypic assay for studying and dissecting epithelial-mesenchymal interactions that approximate those occurring in a human HF. As shown in **Figs. 7-12**, both the “mixed” and the “layered” sandwich systems meet this criteria. In this respect the “mixed” system (= pseudodermis plus one layer of mixture of ORSK and DPC in Matrigel^{TM}), cultured under low calcium and serum-free conditions, was identified as optimal.

Simultaneously, the investigation was carried out in order to establish well-defined basic parameters for a qualitative and quantitative assessment of the modulatory effects of a given test agent. By assessing the effect of different media with high/low calcium conditions and/or absence/presence of serum on these parameters (number and diameter of ORSK aggregates, Ki67+ , TUNEL+, CK6+, Versican+ cells), it was shown that both “folliculoid” sandwich systems can serve as robust screening systems. These assays now can be applied to the testing of standard hair growth-modulatory agents in the human system (e.g. cyclosporin A, HGF, minoxidil, IGF-1, steroid hormones [122,145]) in order to explore whether recognized hair growth stimulators really upregulate e.g. ORSK proliferation and/or DPC secretion of hair follicle morphogens in the sandwich assays reported.

In both sandwich systems (“mixed”, “layered”), the ORSK appeared to behave essentially “*in vivo*-like” since they formed CK6+ spheroids or cells aggregates (ORSK) (**Fig. 7**). However, epidermal-like stratification was not seen in the organotypic co-cultures of ORSK, in contrast to what Limat et al. had described in his co-culture systems at the air-liquid interface [100,104] (**Fig. 6A**). This difference can be interpreted as a reflection of the – more *in vivo*-like - continuous submerged culture conditions used in this study. It has also been shown that ORSK form epidermis-like stratified epithelia, when layered to matrices without DPC[100,104]. The “layered” 3D system investigated herein showed that the presence

of an intermediary layer of Matrigel™-embedded DPC induced the overlying ORSK to aggregate in clusters, rather than into an epidermis-like structure (**Fig. 7A** left side).This confirms the dominant role of DPC in driving cultured human ORSK towards a more hair follicle-like differentiation pathway.

It has been documented extensively that the serum and/or calcium content of the culture medium exerts profound influences on the morphological and functional characteristics of NHEK-based organotypic co-cultures [6,11,19,51,137,180,188,189,193]. This study however provides the first systematic comparison of various media with differences in calcium concentration and/or serum content on basic test parameters in human “folliculoid” organotypic cultures. This comparison suggests that the “mixed” system (= pseudodermis plus one layer of mixture of ORSK and DPC co-cultured in Matrigel™), comes closest to meeting the defined criteria, if the “folliculoids” are cultured under low calcium- and serum-free conditions. As one would demand of an optimal *in vitro*-system that imitates human HF epithelial-mesenchymal interactions as closely as can reasonably be expected in vitro, DPC show only negligible apoptosis and minimal proliferation under these conditions, while the ORSK proliferate at comparatively very high levels, while displaying minimal apoptosis (**Fig. 8**).

This defined optimal growth conditions for a human “folliculoid” 3D system should greatly facilitate further optimization efforts towards the development of continuously improved organotypic systems that mimic the *in vivo* situation in the human HF.

7.2 Experimental Part II - Microsphere system

Development of a Novel Human Folliculoid Microsphere Assay as Screening and Discovery Tool for Basic and Applied Hair Research.

7.2.1 Introduction

Previously, we have defined basic criteria that all hair biology-related organotypic systems should meet in order to support the claim that they mimic the *in vivo* situation as closely as possible[64]. Briefly, these criteria are:

1. Outer root sheath keratinocytes (ORSK) and follicular dermal papilla fibroblasts (DPC) should be physically interacting.
2. The extracellular matrix through which this occurs should also contain basement membrane components.
3. The epithelial HF cells should form cell aggregates.
4. 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 and minimal apoptosis and should display specialized, HF-type secretory activities,
5. and these organotypic systems should be cultured under continuously submerged culture conditions in well defined media (i.e. under serum-free conditions and with a defined concentration of calcium ions).

Using these criteria, two novel folliculoid organotypic systems[64] “layered sandwich” and “mixed sandwich” have been developed. Comparison of these systems suggest 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[64,101,104].

However, so far 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, the goal of this study was to develop a novel, more easy-to-handle *in vitro*-assay which also:

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 best approximates the 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.

This study describes a novel and pragmatic human folliculoid microsphere (HFM) assay that meets all the basic and modified prerequisites for *in vitro* higher-throughput screening systems for pre-clinical candidate hair drug screening.

7.2.2 Results

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

First the characteristics of cells within the HFM system were investigated. We found that ORSK and DPC, forming spheroid cell aggregates, were in close physical contact in the HFM (**Fig. 13B-C**) and that the employed extracellular matrix indeed contained basement membrane components such as fibronectin (**Fig. 13D-E**). In addition, epithelial ORSK showed HF-type keratinization (i.e. CK6 expression; **Fig. 13G-H, K, L**), substantial proliferation (i.e. Ki67 expression in the CK6 positive cells, **Fig. 13K and 14A**), and a low level of apoptosis (number of TUNEL positive ORSK, **Fig. 13L and 14C**). Instead, the HF mesenchymal DPC exhibited minimal proliferation and apoptosis (**Fig. 13M and N, Fig. 14B and D**) but maintained their characteristic, specific secretory activity, i.e., they displayed strong expression of the large proteoglycan versican[191] (**Fig. 13 I-J, M, N**).

In most of experiments, in accordance with the findings in the previously developed "sandwich" 3D systems [64], serum-free culturing media that contained low concentrations of calcium (0.15 mM) was used. However, the biological features of the cells in the HFM cultured in high calcium solutions were also determined. During the 10 day culture period no difference in proliferation and apoptosis of ORSK in the two different media was found (**Fig. 14A and C).** In contrast, both the proliferation and the apoptosis of DPC significantly increased in high-Ca medium compared to low-Ca solution (**Fig. 14B and D**).

Furthermore, the processes of possible necrosis were also investigated by measuring the LDH release during the 10 day culture period in low-Ca media. The maximum amount of LDH (20 U/L) was seen on day 3 when the system was (most probably) still under the "stress" caused by the preparation of the HFM. On days 7 and 10, the LDH levels returned to normal, which suggests a full recovery of the cells in the HFM (**Fig. 14E**).

It was therefore concluded, that the HFM meet all the basic criteria of an organotypic folliculoid system and that they can be successfully cultivated under continuously submerged culture conditions (most efficiently in low-Ca media) remaining 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 micropheres:

1. requires lower number, yet higher density of cells (especially DPC) per test unit. Although the density of ORSK was essentially the same (1 x 10^6 cells mL^{-1}) both in the "sandwich" and HFM systems, double DPC density (2 x 10^6 cells mL^{-1}) in the HFM was achieved. In addition, the remarkably higher number of test units that can be obtained using 1 ml of cell suspension in the HFM system (i.e. 30-50 HFM 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 HFM was only a few hours compared to the approximately 5 day-long preparation time at best needed for any of the two "sandwich" systems presented before[64]
3. is easily repeatable with higher number of test units, which allows a higher degree of standardization and greatly facilitates automation. In addition, the HFM system allows for the preparation of test units with variable sizes, depending on specific experimental needs; and
4. due to the dramatic decrease in culture time and consumables, is less expensive.

Negative regulators of HF growth differentially inhibit proliferation and induce apoptosis in cells growing in the HFM.

In the next phase of these experiments, the novel HFM system was assessed in terms of whether is also suitable to analyze the actions of recognized hair growth-modulatory drugs (the effects of all agents examined are summarized in **Table 1 and 2**). At first the effects of the potent HF growth inhibitory agents were investigated, i.e. 10^{-6}M tretinoin [50], 25 ng/ml transforming growth factor-β2 (TGFβ2) [69,192], and 10^{-7} M corticotropin-releasing hormone (CRH) [78,187] (**Table 1**). As expected, all agents significantly decreased the number of Ki67 positive (hence proliferating) cells in the CK6 positive ORSK (**Fig. 15A, 16A, Table 1A**). In addition, tretinoin (**Fig. 15C**) and TGFβ2 (**Fig. 16C**), but not CRH (**Table 1B**), increased the number of TUNEL positive ORSK, suggesting stimulation of apoptosis. Furthermore, TGFβ2 (**Fig. 16B**) suppressed the number of Ki67 positive DPC, whereas the other two agents did not affect the proliferation of these cells (**Fig. 15B and Table 1A**). Finally, tretinoin (**Fig. 15D**) and TGFβ2 (**Fig. 16D**) stimulated apoptosis in DPC as well, while CRH, similar to its lack of action as seen on ORSK, did not affect the process (**Table 1B**).

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				
	Ki67-positive cells in ORS (% of control)		**Ki67-positive cells in DPC (% of control)**	
Days of culture	**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 was 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; ↑ increase; ↓decrease) 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				
	Ki67-positive cells in ORS (% of control)		**Ki67-positive cells in DPC (% of control)**	
Days of culture	**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 was 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 (* $p<0.05$; ** $p<0.01$; ↑ increase; ↓decrease) differences.

Positive regulators of HF growth stimulate proliferation of ORSK and DPC in the HFM without affecting apoptosis.

The effect of recognized HF growth-promoting agents was investigated next, i.e. insulin-like growth factor-I (IGF-I) [160], 1α,25 dihydroxyvitamin D3 (1,25(OH)2D3, calcitriol) [63,183,202], cyclosporin A (CsA) [91], and hepatocyte growth factor (HGF)[83] (**Table 2**). Applications of 100 ng/ml of IGF-I, 10^{-8} M calcitriol, 10 ng/ml CsA, and 10 ng/ml HGF resulted in very similar modifications in the functions of the cells in the microspheres (**Table 2**). Namely, all agents increased proliferation (elevated the number of Ki67 positive cells) both in the ORSK (**Fig. 17A and C, Fig. 18A and C**) and DPC (**Fig. 17B and D, Fig. 18B and D**) populations without exerting any measurable effect on apoptosis of these cells (**Table 2B**).

The effects of CsA and HGF are modified by the alteration in the calcium concentration of the culture media.

The above data, as mentioned before, was obtained in experiments where low-Ca media was used to culture the HFM. Previous studies, however, suggest that the effects of CsA [197] and HGF [181] on proliferation of keratinocytes are strongly affected by the calcium content within the culture media. Therefore, these molecules were also tested on HFM cultured under high-Ca conditions. In marked contrast to data obtained in the low-calcium medium, 10 ng/ml CsA in high-calcium 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) 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 findings unambiguously argue for the careful selection of which calcium concentration to use for the culture media.

The HFM are suitable for the investigation of protein and gene expression of cytokines and growth factors involved in HF biology.

The question whether the HFM system can operate as a discovery tool for identifying new target genes (and their protein products) for candidate hair growth modulatory agents was investigated as well. Therefore the expression of such biological markers (on protein and mRNA level) in the HFM, which were previously described as important regulators in HF biology *in vivo* were measured. Using immunofluorescence, the expression of TGFβ2 (negative regulator of hair growth) and stem cell factor (SCF, hair growth stimulator [195,153]) in the cells of the microspheres could clearly be shown (**Fig. 19A-D**). In addition, employing RT-PCR on RNA isolated from the HFM, we could also detect the mRNA transcript of these molecules (**Fig. 19E-F**).

Positive and negative regulators of hair growth may not only directly affect the biological processes of the cells of the HF but, very often, significantly modify the complex cytokine/growth factor network of the mini-organ. Investigation, using RT-PCR, was used in order to highlight 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 the HFM (see above).

As shown in **Fig. 19G**, 25 ng/ml "exogenous" growth inhibitor TGFβ2 (**Fig. 16 and Table 1**) remarkably up-regulated 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, **Fig. 15 and 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, it was shown that the CsA (a positive regulator of HF growth, **Fig. 18A and B**), significantly suppressed the expression of the growth-inhibitory TGFβ2 without affecting the level of SCF.

The HFM are also suitable for the investigation of global gene expression profiles.

Finally, the novel model system was further analyzed in order to see whether it could determine changes of global gene expression profiles upon experimental manipulations. Therefore, in a pilot study, HFM were treated with certain hair growth stimulatory agents (such as HGF, calcitriol, IGF-I, and 17β Estradiol)[24,133,162,198] for 7 days, then a Micro-Array analysis that represented the whole human genome (Agilent®, 44K, G4112A) was performed to follow gene expression alterations. As seen in **Table 3** (showing changes of only selected genes), treatment of the 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 **Fig. 19**) further indicate that the novel HFM system was indeed a suitable model for studying HF biology since, similarly to those seen in the HF, agents affecting proliferation and apoptosis of cells in the HFM may also significantly alter the global gene (and protein) expression profile of e.g. molecules of cytokine and growth factor signaling.

Table 3. Alterations in the expression of selected genes expressed in HFM after 7 days of incubation with various recognized hair growth stimulators (HGF, calcitriol, IGF-I, 17-ß estradiol).

The genes shown have been selected on the basis of established biological functions, relevant expression levels or relatedness to factors known in hair follicle biology.

HGF (10 ng/ml)

Gene	*Expression change (fold)*	*Recognized function in HF biology*	*Comments*	*Selected references*
Ephrin-A5 (NM_001962)	↑, **6.5**	Ephrin A3 gene was down-regulated in androgenic alopecia. In addition, ephrin A3 raised the proliferation rate of the outer root sheath keratinocytes (ORSK) and induced gene expression in acidic hair keratin 3-II suggesting that ephrin A3 functions as hair growth promoting factor in the hair cycle.	No information on Ephrin-A5 in skin available.	126
Insulin-like growth factor-II mRNA-binding protein-1 (AK022617)	↑, **3.5**	Not known. However, IGF is a potent hair growth stimulator.	Implicated in mRNA localization, turnover, and translational control. Stimulates cell proliferation. In deficient mice, significant retardation of growth is observed.	62,99
Lysyl oxidase (NM_002317)	↑, **3.1**	Progressive hair loss in rough coat (rc) mice which show reduced lysyl oxidase-like protein.	Extracellular matrix regulator.	65
Retinol binding protein-4 (NM_006744)	↑, **2.5**	Stimulation of RA receptors inhibits hair growth.	Potential hair growth inhibitor.	50
Sex hormone-binding globulin (SHBG) (NM_001040)	↑, **2.2**	Decreased protein levels in patients with isolated hirsutism.	Limits the levels of circulating free sex hormones (especially androgens).	200
Ectodysplasin A (NM_001399)	↑, **2.2**	Critical for normal HF development.		184,185
Angiopoietin-2 (NM_001040)	↑, **2.1**	Modulation of perifollicular vasculation (hair cycle-dependent vascular remodeling).		147

Calcitriol (10^{-8} M)

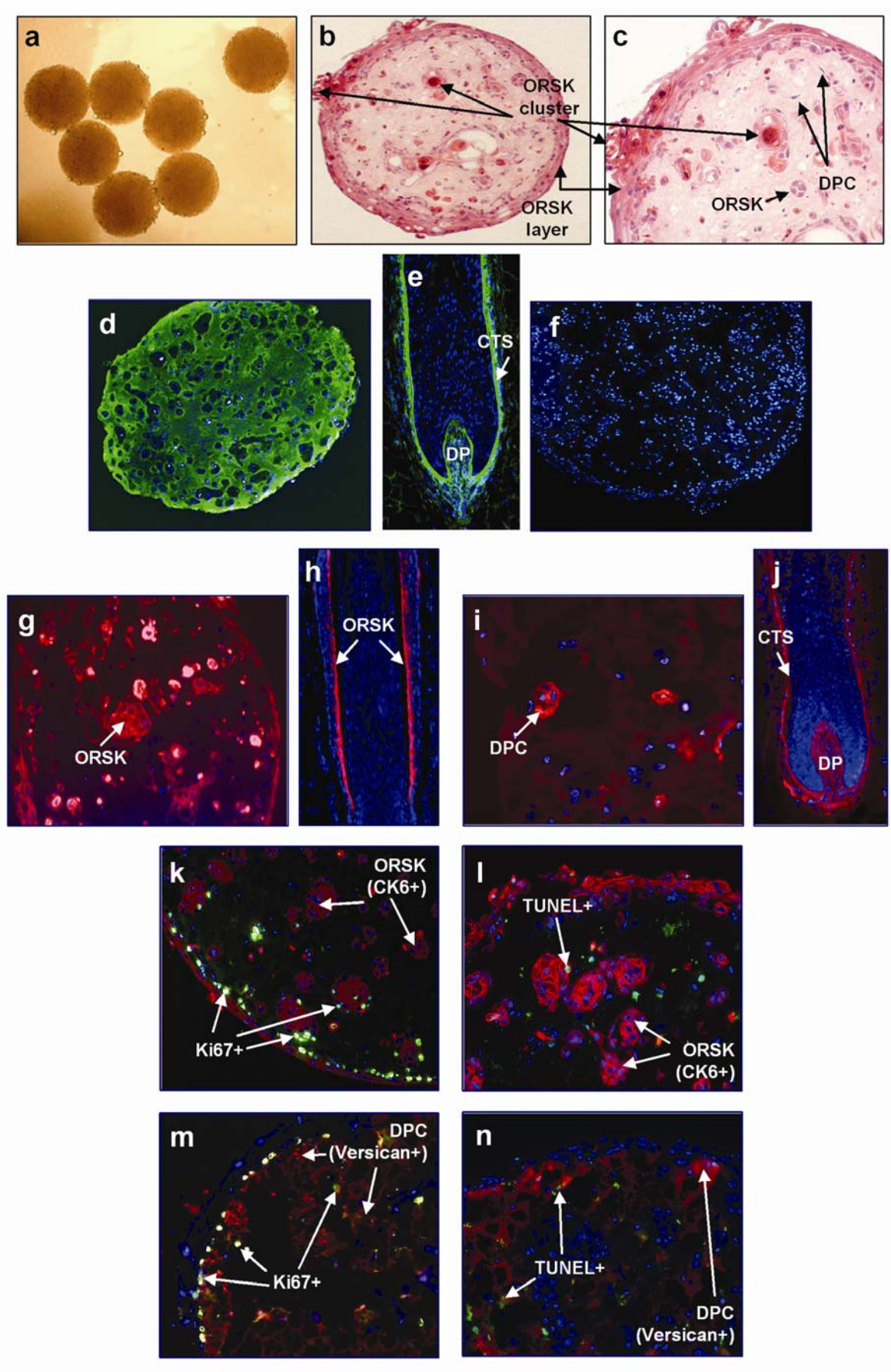
Gene	*Expression change (fold)*	*Recognized function in HF biology*	*Comments*	*Selected references*
Delta- and Notch-like Epidermal Growth Factor-related receptor (NM_139072)	↑, **4,9**	The Notch pathway (and its receptor, Delta-1) is a central regulator of hair follicle growth and development and inter-cellular communication mechanisms.		168
Interleukin 1 receptor, type II (NM_004633)	↑, **2.9**	Unknown.	Antagonizes interleukin 1 activity.	71
Insulin-like growth factor binding protein-3 (S56205)	↑, **2.1**	Regulates activity of IGF, a potent hair-growth stimulator.	Potential hair growth inhibitor.	68
Transforming growth factor β_1 (NM_000660)	↑, **2.1**	Potent hair growth inhibitor.		69,192

IGF-I (100 gn/ml)

Gene	*Expression change (fold)*	*Recognized function in HF biology*	*Comments*	*Selected references*
Growth hormone 2 (NM_173841)	**↑, 4.5**	Unknown.	In peripheral tissues, activates the hair growth promoting insulin-like growth factor-I signaling.	161
Interleukin 1 receptor antagonist (NM_173841)	**↑, 4.2**	Hair growth promoter by inhibition of IL-1.		162,163
Frizzled homologue 5 (NM_003468)	**↑, 4.0**	The Frizzled gene family plays a key role in hair follicle development.		172
WNT-6 (NM_006522)	**↑, 3.1**	WNT-receptors are important for hair development.		127,185
Vascular endo-thelial growth factor (AK098750)	**↑, 3.1**	Key promoter of anagen-associated angiogene-sis.		209
Keratin 5b (NM_173352)	**↑, 2.9**	Proliferation-associated keratin.		93,94

17β Estradiol (10^{-6} M)

Gene, (Accession number)	*Expression change (fold)*	*Recognized function in HF biology*	*Comments*	*Selected references*
Ephrin-A5 (NM_001962)	**↑, 6.3**	Ephrin A3 gene was down-regulated in androgenic alopecia. In addition, ephrin A3 raised the proliferation rate of ORSK and induced gene expression in acidic hair keratin 3-II suggesting that ephrin A3 functions as hair growth promoting factor in the hair cycle.	No information on Ephrin-A5 in skin available.	126
Insulin-like growth factor-II mRNA-binding protein-1 (AK022617)	**↑, 5.5**	Not known. However, IGF is a potent hair growth stimulator.	Implicated in mRNA localization, turnover, and translational control. Stimulates cell proliferation. In deficient mice, significant retardation of growth is observed.	62,99
Alkaline phosphatase (NM_001631)	**↑, 4.3**	Expressed in DP, activity linked to normal hair matrix – papilla interaction.	Marker of inductive potential of DPCs.	59
Protein kinase Cβ1 (AJ002788)	**↑, 3.6**	PKCβ pseudosubstrate (as well as the general PKC inhibitor GF109203X) was shown to reduce skin and hair pigmentation; hence, it is a potential hair growth stimulator.	PKCβ, overexpressed in HaCaT cells, stimulates proliferation/tumor growth, suppresses differentiation and apoptosis.	141,142
Retinol binding protein-4 (NM_006744)	**↑, 3.2**	Stimulation of RA receptors inhibits hair growth.	Potential hair growth inhibitor.	50
Ectodysplasin A (NM_001399)	**↑, 2.8**	Critical for normal HF development.		185,186
Insulin-like growth factor binding protein (NM_004970)	**↑, 2.5**	Regulates activity of IGF, a potent hair-growth stimulator.	Potential hair growth inhibitor.	68
Sex hormone-binding globulin (NM_001040)	**↑, 2.3**	Decreased protein levels in patients with isolated hirsutism.	Limits the levels of circulating free sex hormones (especially androgens).	200
Glial cell-derived neurotrophic factor (NM_000514)	**↑, 2.2**	Catagen inhibitor.		16


a
b
ORSK
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ORSK
layer
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DPC
ORSK
d
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CTS
DP
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i
DPC
j
CTS
DP
k
ORSK
(CK6+)
Ki67+
l
TUNEL+
ORSK
(CK6+)
m
DPC
(Versican+)
Ki67+
n
TUNEL+
DPC
(Versican+)

Figure 13. Microscopic photography and histology of the microspheres and the expression of certain markers in cells and matrix of the HFM

A) Photomicrographs of HFM 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 the HFM. **D-E)** Expression of fibronectin (as revealed by fluorescein isothiocyanate, FITC, immunostaining, green fluorescence) in the HFM (**D**) and in microdissected HF used as a positive control (**E**). **F)** Negative control, the primary antibody was omitted. **G-H)** Expression of the ORSK marker CK6 (as revealed by rhodamine immunostaining, red fluorescence) in the HFM (**G**) and in microdissected HF (**H**). **I-J)** Expression of the large proteoglycan versican (DPC marker) (as revealed by rhodamine immunostaining, red fluorescence) in the HFM (**I**) and in microdissected HF (**L**). **K-L**) Double immunolabeling of the ORSK marker CK6 (as revealed by rhodamine immunostaining, red fluorescence) with the proliferation marker Ki67 (**K**) or with 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 proliferation marker Ki67 (**M**) or with the apoptosis marker TUNEL (**N**) (in both cases, FITC immunostaining, green fluorescence). Original magnification, 40x for **A**; 100x for **B**, **D-F**, **H**, **J**; 250x for **C, G**, **I, K-N**. For **D-N**, nuclei were counterstained with DAPI (blue fluorescence).

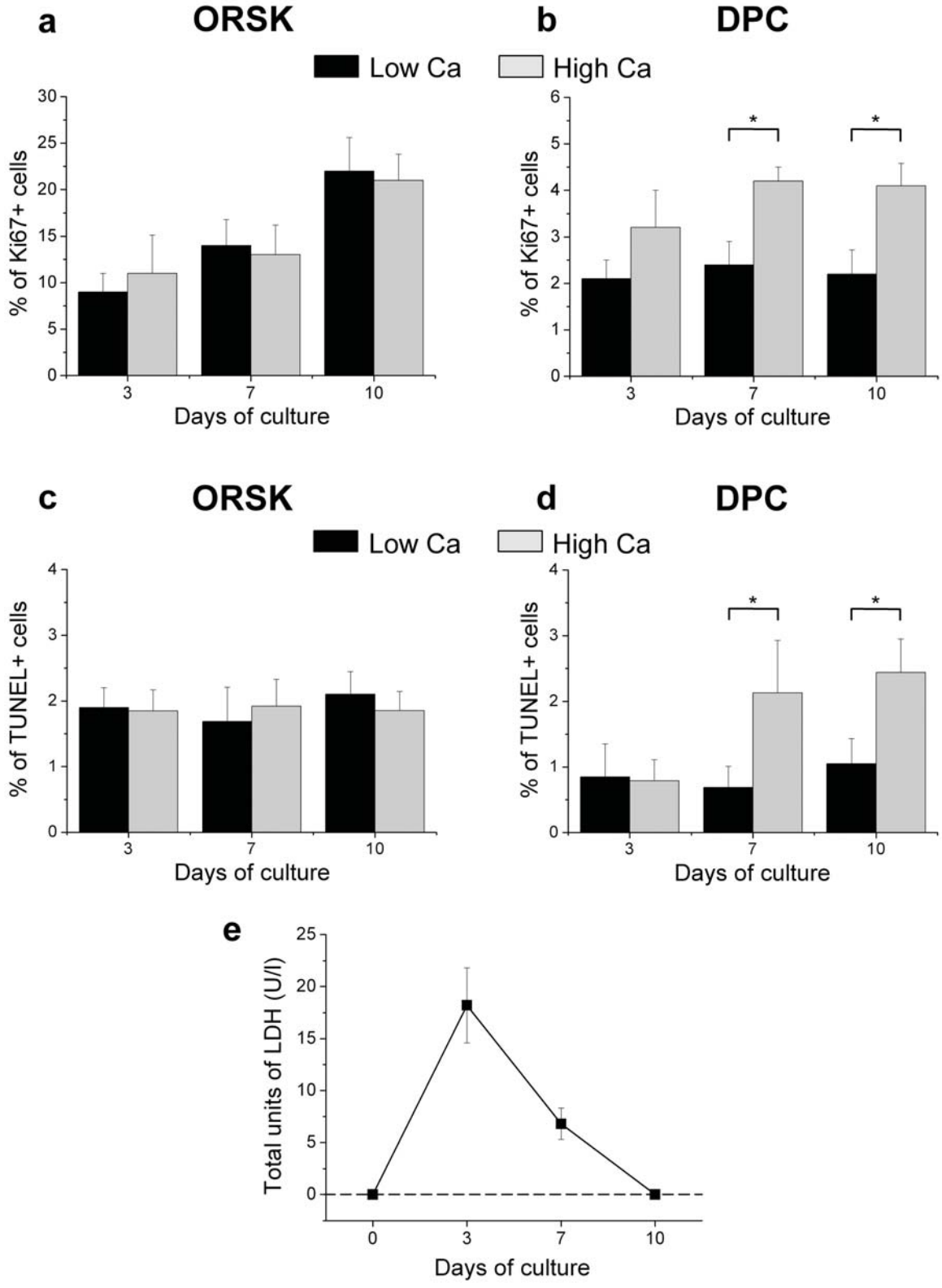
a
ORSK
Low Ca
High Ca
% of Ki67+ cells
Days of culture
b
DPC
% of Ki67+ cells
Days of culture
c
ORSK
% of TUNEL+ cells
Days of culture
d
DPC
% of TUNEL+ cells
Days of culture
e
Total units of LDH (U/l)
Days of culture

Figure 14. Quantitative analysis of proliferation and apoptosis of ORSK and DPC in the HFM cultured in various media for 10 days

Series of double immunolabeling was 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.

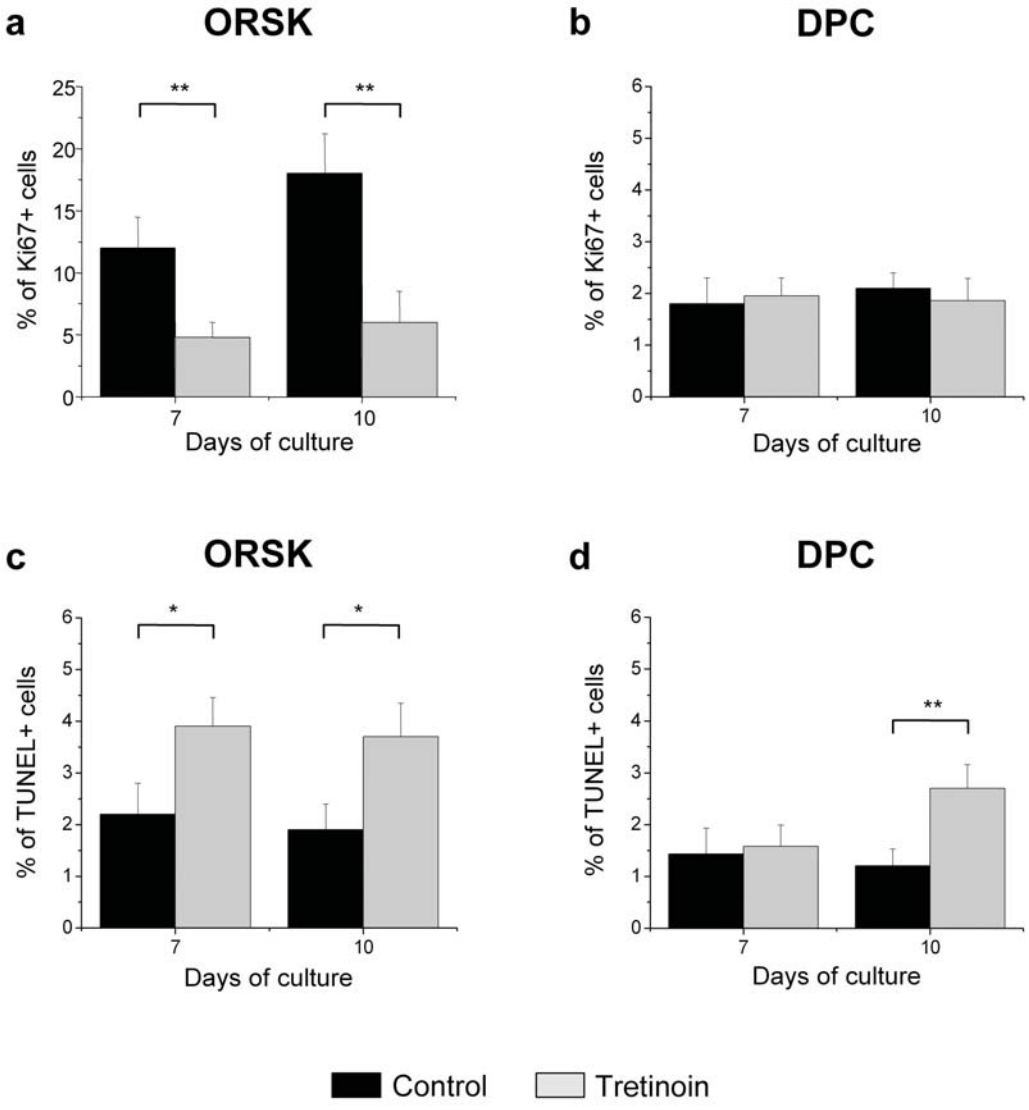

Figure 15. Effects of tretinoin on proliferation and apoptosis of ORSK and DPC in the HFM

HFM were treated with vehicle (Control) or with 10^{-6} M tretinoin for up to 10 days, then a series of double immunolabeling was performed in order 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.

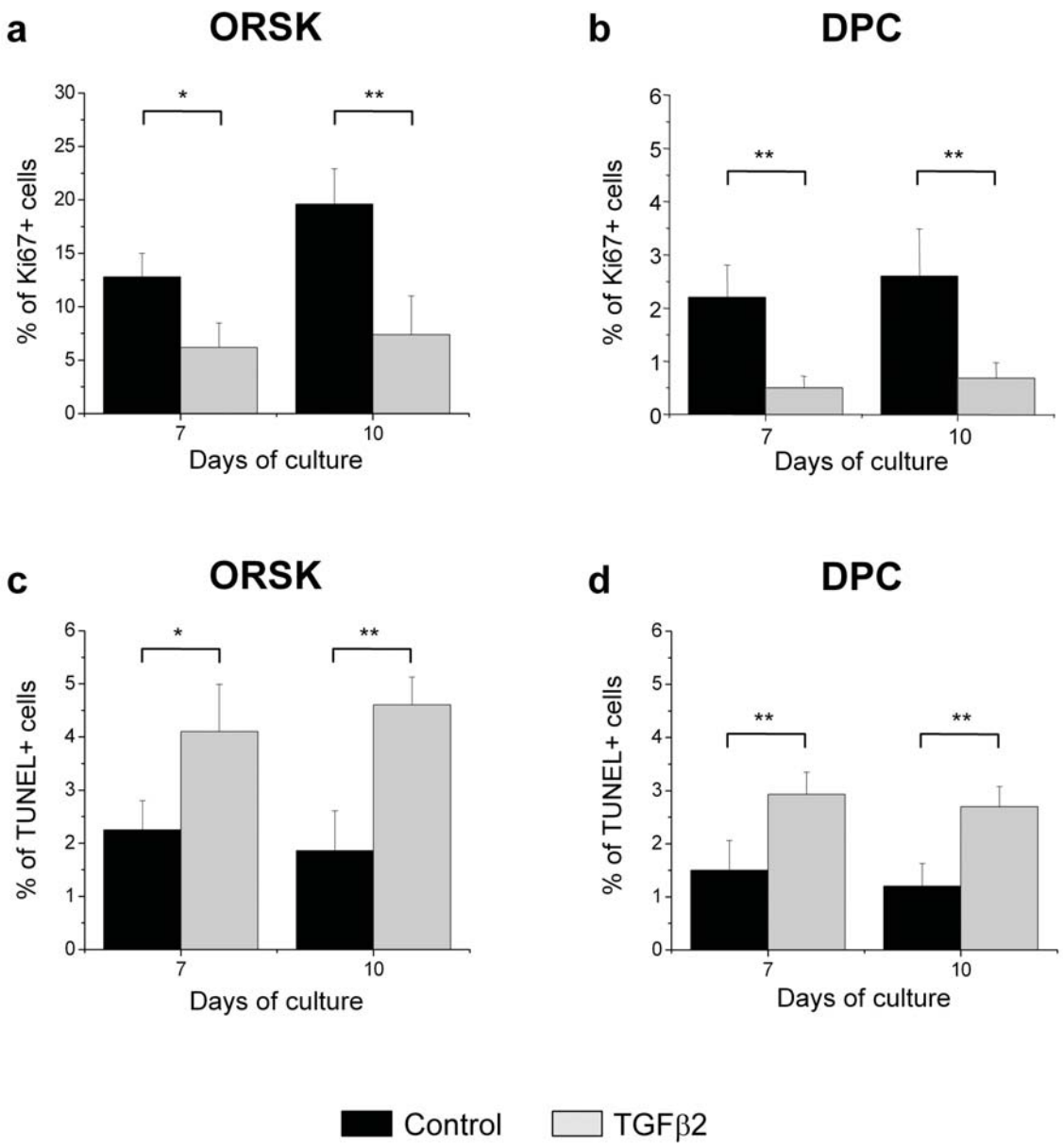

Figure 16. Effects of TGFβ2 on proliferation and apoptosis of ORSK and DPC in the HFM

HFM were treated with vehicle (Control) or with 25 ng/ml TGFβ2 for up to 10 days, then a series of double immunolabeling was performed in order 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.

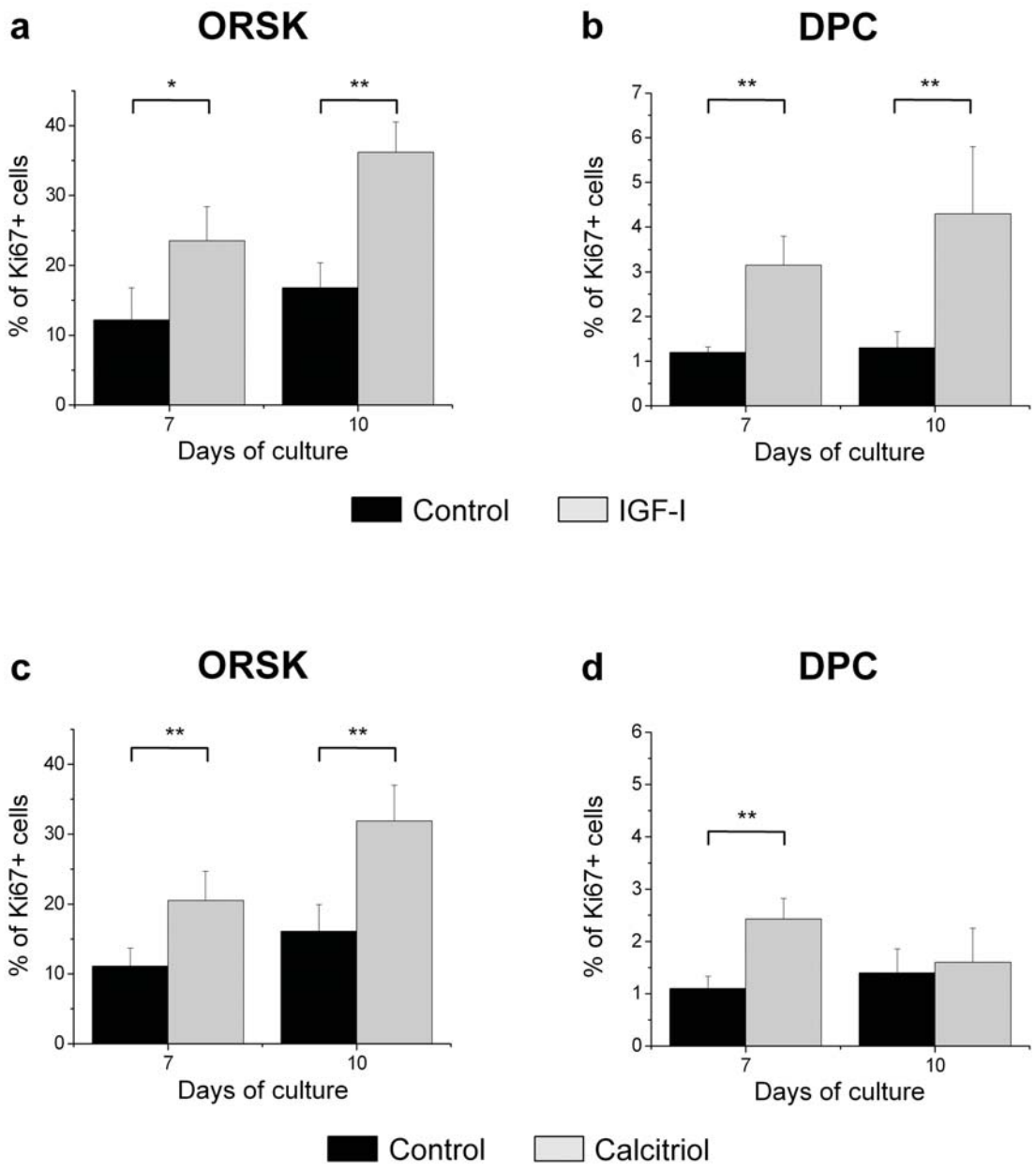

Figure 17. Effects of IGF-I and calcitriol on proliferation of ORSK and DPC in the HFM

HFM were treated with vehicle (Control), with 100 ng/ml of IGF-I (**A and B**), or with 10^{-8} M calcitriol (**C and D**) for up to 10 days, then a series of double immunolabeling was 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.

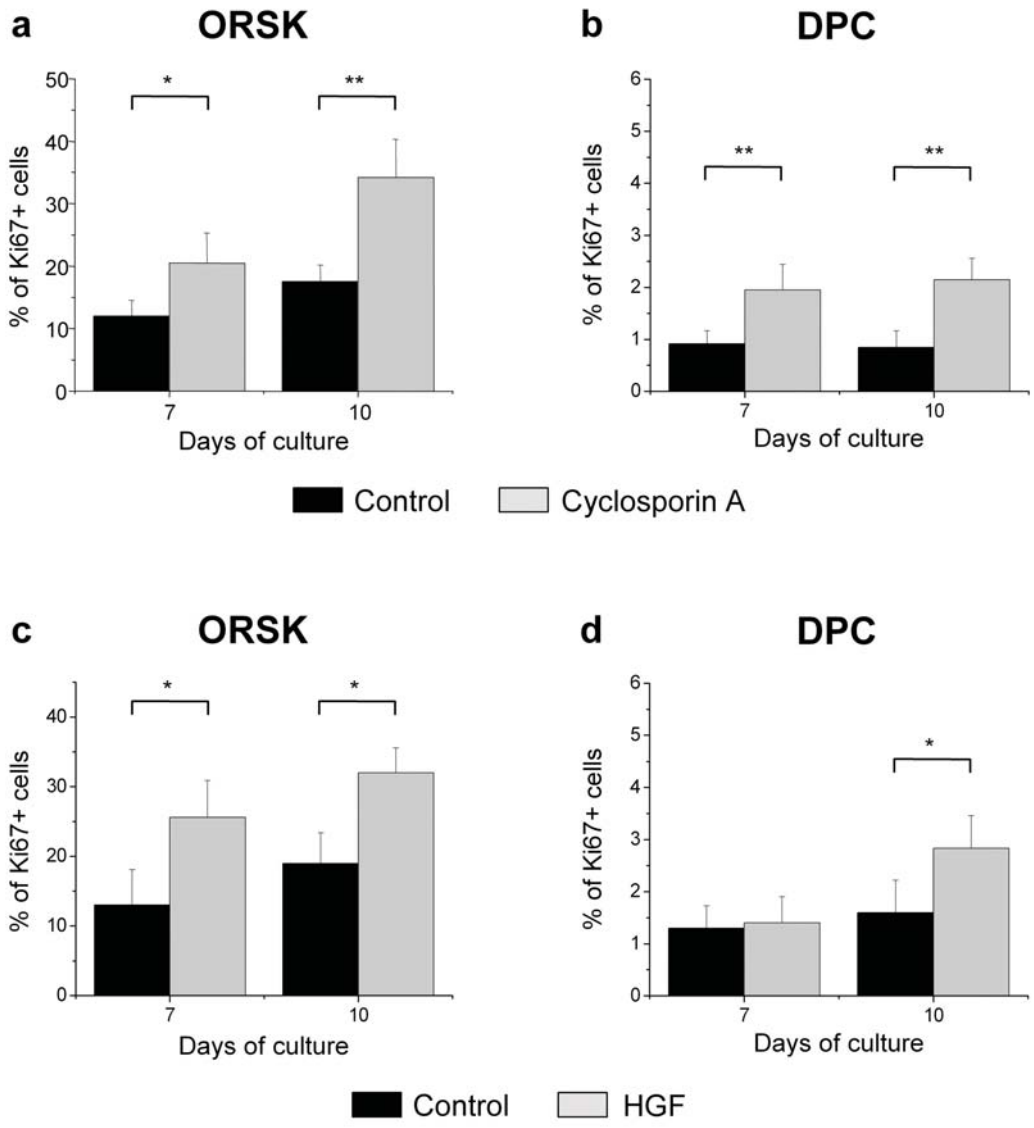

Figure 18. Effects of Cyclosporin A and HGF on proliferation of ORSK and DPC in the HFM

HFM were treated with vehicle (Control), with 10 ng/ml Cyclosporin A (**A and B**), or with 10 ng/ml HGF (**C and D**) for up to 10 days, then a series of double immunolabeling was 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.

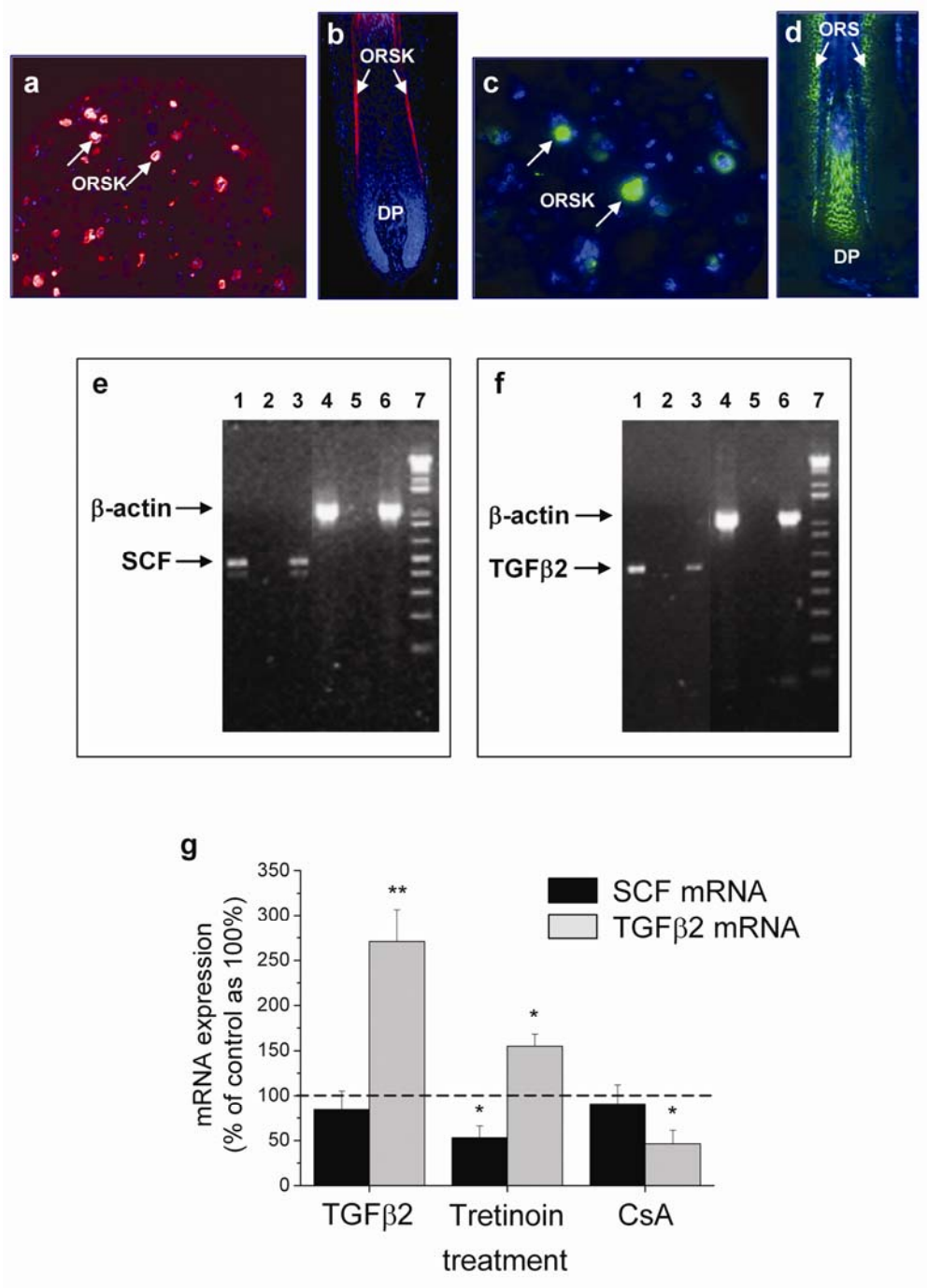
a
ORSK
b
ORSK
DP
c
ORSK
d
ORS
DP
e
1 2 3 4 5 6 7
β-actin
SCF
f
1 2 3 4 5 6 7
β-actin
TGFβ2
g
mRNA expression
(% of control as 100%)
350
300
250
200
150
100
50
0
**
*
*
*
SCF mRNA
TGFβ2 mRNA
TGFβ2
Tretinoin
CsA
treatment

Figure 19. Expression of TGFβ2 and SCF in the HFM, RT-PCR analysis of SCF and TGFβ2

A-B) Expression of TGFβ2 (as revealed by rhodamine immunostaining, red fluorescence) in the HFM (**A**) and in microdissected HF used as a positive control (**B**). **C-D)** Expression of SCF (as revealed by FITC immunostaining, green fluorescence) in the HFM (**C**) and in microdissected HF (**D**). Original magnification, 100x for **A**, **B**, **D**; 250x 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 the HFM and in microdissected HF (used as positive controls). Lanes, *1 and 4*, HF (positive control);*2 and 5*, reaction without template (negative control); *3 and 6*, HFM. **G**) HFM were treated with 25 ng/ml TGFβ2, 10-6 M tretinoin or 10 ng/ml 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 3 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.

7.2.3 Discussion

Studies of the epithelial-mesenchymal interactions in the HF have been limited due to a lack of suitable *in vitro* screening systems that sufficiently mimic conditions as they occur in human HF. So far, there are no available systems, which allow a higher-throughput screening of candidate hair drugs than can be obtained by using histoculture techniques or by the classical organ-culture of microdissected, amputated human scalp HF in the anagen VI phase of the hair cycle [115,159]. 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 [50,152,192], the very limited number of human HF available severely restricts the number of agents that can be screened. Also, while our understanding of epithelial-mesenchymal interactions in murine HF has recently progressed substantially[173], our understanding of these interactions in human HF is still very rudimentary.

Some HF-like *in vitro* models derived from human follicular cells have been described [64,89,101,104,193]. However, so far none of them are easily prepared in large amounts to allow high-throughput screening and to properly investigate epithelial-mesenchymal interactions. The aim of this study was, therefore, to develop a 3D *in vitro* system that allows a more detailed examination of the basic molecular processes involved in HF growth and development with a robust reproducibility.

In this study, a novel and very pragmatic organotypic assay, which imitates human HF-like epithelial-mesenchymal interactions and is prepared with comparative ease has been described. Studying the expression of a number of different markers (e.g., proliferation, apoptosis, CK6 and versican expression), it was shown that, under culture conditions with a serum-free, low-Ca medium, these HFM retain several essential characteristic for human scalp HF.

The novel technique of the microsphere preparation using a gelling 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 hair follicle mesenchyme. The low number of TUNEL positive cells in later phases of the culture with normalized LDH level suggests the optimal nutrient supply to the HFM 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, as thousands of microspheres were generated in past 10 experiments.

The technique of the HFM preparation also allows easy cell migration within the microsphere compared to other spheroid systems. Nevertheless, although a mixture of isolated ORS and DPC with HF inductive potential[175] was used in our experiments and the cell migration and matrix reorganization within the microsphere was indeed observed, the formation of HF unit did not occur. This suggests that the interaction and signaling of the follicular cells with surrounding dermal micro-environment are crucial for morphogenesis and development of the HF. However, the main aim of this study was to develop a simplified hair follicle-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 the HFM system in a manner that suggests that HFM allow the standardized pre-clinical assessment of test agents on relevant human hair growth markers under substantially simplified *in vitro* conditions that approximate the *in vivo* situation. It was further shown using a DNA microarray that HFM also offers a useful discovery tool for the identification of novel target genes for candidate hair drugs. Evidently, the HFM method still has limitations, especially in the rapid analysis of various readout parameters. These shortcomings will hopefully be overcome in the near future with the “automation” of the evaluation process by employing e.g. staining “robots”, automated image analyzer software packages, and high-content screening de-

vices. Nevertheless, the novel assay system described 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 automation.

In HFM, we were also able to identify several other markers important for the HF development and differentiation (e.g. 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[93,94], 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 (e.g. wounded, inflamed or UV-irradiated) interfollicular epidermal keratinocytes and in the companion layer of HF[94]. The latter is unproblematic, since 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 ORS keratinocyte cultures were generated (as confirmed by negative IRS markers).

In conclusion, the presented data suggest that HFM represent a valuable system to study epithelial-mesenchymal interactions and their changes in response to treatment with various candidate hair-drugs. HFM, therefore, do not only offer a novel and pragmatic basic screening tool, but also an instructive new experimental system for basic and applied pre-clinical hair research in the *human* system.

8 Conclusions from the study

- Three new organotypic folliculoid systems (“layered sandwich”, “mixed sandwich”, microsphere system) for the exploration of molecular processes involved in HF growth were developed.

- All newly developed systems meet all basic criteria for „folliculoid” organotypic systems that imitate human hair follicle epithelial-mesenchymal interactions.

- Outer root sheat keratinocytes proliferate in all systems, regardless of the calcium or serum content of the media, whereas the proliferation and apoptosis of dermal papilla cells, in all systems, strongly depends on the amount of serum and calcium in culture media.

- The human folliculoid microsphere system (HFM), selected as the best optimized system, proved to be highly reproducible organotypic assay, which under culture conditions with a serum-free, low-Ca medium, retains several essential characteristic for human scalp HF.

- HFM allow the standardized pre-clinical assessment of test agents on relevant human hair growth markers under substantially simplified *in vitro* conditions that approximate the *in vivo* situation.

- HFM also offers a useful discovery tool for the identification of novel target genes for candidate hair drugs.

- The HFM therefore offer a novel and pragmatic screening tool and an instructive new experimental system for basic and applied pre-clinical hair research in the human system.

9 Summary

The search for more effective drugs for the management of common hair growth disorders remains a top priority, both for clinical dermatology and the industry. Human hair growth can currently be studied *in vitro* by the use of organ-cultured scalp hair follicles. However, simplified organotypic systems are needed for dissecting the underlying epithelial-mesenchymal interactions and as screening tools for candidate hair growth-modulatory agents.

In this study, three novel organotypic human folliculoid assays for basic and applied hair research were developed. Two organotypic “sandwich” systems consist of a pseudodermis (collagen I mixed with and contracted by human interfollicular dermal fibroblasts) on which one of two upper layers is placed: either a mixture of Matrigel™ and follicular dermal papilla fibroblasts (DPC), with outer root sheath keratinocytes (ORSK) layered on the top (“layered sandwich” system), or a mixture of Matrigel™, DPC and ORSK (“mixed sandwich” system). Third, the patented new technique generating human folliculoid microspheres (HFM), consisting of human DPC and ORSK within an extracellular matrix was established.

Studying a number of different markers (e.g. proliferation, apoptosis, cytokeratin-6, versican), it was shown that these folliculoid systems, 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 all developed organotypic systems allow the standardized pre-clinical assessment of test agents on relevant human hair growth markers under substantially simplified *in vitro* conditions that approximate the *in vivo* situation. Furthermore, by using immunohistochemistry, RT-PCR, and DNA microarray techniques it was shown that the folliculoid systems also offer a useful discovery tool for the identification of novel target genes and their products for candidate hair drugs.

Organotypic folliculoid systems thus represent an instructive new experimental and screening tool for basic and applied hair research in the human system.

10 References

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11.3 Publication of abstracts

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Commentary to the article Havlickova et al., JID 2009

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

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Building Complex Tissues: High-Throughput Screening for Molecules Required in Hair Engineering

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A fully functional model of hair reconstitution remains elusive because of the complexity of cellular organization and the number of molecular interactions that must be approximated. In this issue, Havlickova *et al.* (2009) report a significant contribution to hair engineering with their human folliculoid microsphere assay.

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