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Human Skin Cell Culture and its Impact on Dermatology

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Introduction

Initial attempts to grow skin and its cellular components centered on the use of organ cultures and explants cultures, where whole pieces of skin were kept alive and growth was confined to the epipole around the piece or onto the plastic around the explants. These cultures have a short life span and limited applications, as mixed cultures of keratinocytes, melanocytes, Langerhans cells, fibroblasts, Merkel's cells, nerve cells and glandular cells are obtained [1].

To understand the function and dysfunction of this complex structure, it is necessary to study partial aspects in models of manageable size and to reintegrate the results back into context, i.e., establishment of pure cell culture of each cellular component of the skin. During the past three decades selective cultivation of cell components of skin has been achieved and considerably added to our understanding of their biology in health and disease. Furthermore, cultivated cells could be reassembled according to experimental design and indications of their use.

Pure cell culture and reconstructed human skin equivalents as an alternative to animal experimentation offer, not only a way to concede to demands of regulatory authorities, animal welfare organizations, consumers and scientists, but also provide a means to improve and extend our knowledge of biological processes in the skin. Molecular definition of disease at the level of the gene and advances in recombinant DNA technology suggest that many diseases are amenable to correction by genes not bearing the defective elements that result in disease. By the use of cell cultures dermatopharmacology has also been enormously stimulated. In addition, modern molecular genetics has been utilized to distinguish between benign and malignant cells providing a mean of targeting the abnormal mutant gene by very specific and precise

therapeutic modality, e.g., gene and antisense therapy [2]. In this regard, tissue culture models are ranging from simple monolayer cultures of one cell type to co-cultures of epithelial and mesenchymal cells and finally to three dimensional skin equivalents-organotypic cultures-that form a highly organized epithelium under the control of co-cultured fibroblasts [3]. In skin equivalents, epidermal keratinocytes grow exposed to air on a matrix of type I collagen in which either primary dermal fibroblasts or heterologous mouse fibroblast lines and possibly other cells are embedded, giving rise to almost normal tissue architecture [4]. Presently, various skin reconstructs are available composed either of the epidermal compartment only or of both the epidermal and dermal compartments. Within each compartment various types of cells can be incorporated, including keratinocytes, melanocytes and Langerhans cells in the epidermal, and fibroblasts and endothelial cells in the dermal compartment. The quality of the human skin equivalents has reached a point that their suitability for skin toxicity testing will make great progress. Next to the field of toxicity and safety standards, skin equivalents offer a well-characterized model for studies of the basic skin biology, wound repair, regulation of melanogenesis, pathogenesis of skin diseases and skin cancer [5].

Melanocyte Culture

Melanocytes are highly differentiated neural crest derived cells responsible for synthesizing melanin which is transferred in the melanosomes through dendritic processes to surrounding epidermal keratinocytes. The problem of overgrowth of other epidermal and dermal cells, such as keratinocytes and fibroblasts in culture has been solved and in the past few decades, selective cultivation of human melanocytes from newborn and adult skin has been achieved [6].

Tetradecanoyl phorbol acetate (TPA), cholera toxin and serum containing Dulbecco's minimal essential medium (DMEM) was the first described melanocyte growth medium. Obtained melanocytes appear slender with a central bulge containing their vesicular nuclei. On both poles, small globular swellings are always observed that represent melanosomes [7]. It has been shown that melanosomes are phagocytosed by surrounding keratinocytes, a process that is controlled by keratinocyte related protease-activated receptor-2 [8]. Newborn melanocytes usually display two polar dendrites, whereas adult melanocytes frequently have several branched dendrites (Fig. 1) [7].

Because of the presence of serum, melanocytes grown under these conditions are not optimal for examination of factors that may influence melanocyte function as serum may contain substances having functional similarities with those being tested and hence their effects could be masked. Also, the presence of TPA, a potential carcinogenic agent, renders melanocytes grown under these conditions not suitable for therapeutic purposes. Nevertheless, this medium has also several advantages. For example, pure melanocyte cultures are obtained within a few days due to the toxic effects of phorbol esters on keratinocytes. Furthermore, melanocytes can be passaged up to 12-14 passages, enabling reproducibility of the experimental trials, thus posing additional validity to the results obtained in these experiments [7]. In order to circumvent these drawbacks, a serum free and hormone supplemented medium has been successfully used for melanocyte cultivation. The used medium (MCDB 153) supports growth of both keratinocytes and melanocytes, but it is not optimal for growth of fibroblasts. Crude bovine pituitary extract (BPE), cholera toxin, and basic fibroblast growth factor (bFGF) were found essential for melanocyte proliferation. Addition of fetal calf serum (2%) to cultures at time of inoculation and with every passage enhances attachment of the cultivated cells to plates and stimulates their growth. Serum is essential for attachment of adult melanocytes, but not for those of newborn origin, though, melanocyte yield in the absence of serum is low. Keratinocytes and melanocytes grow usually together, however, after 3-4 weeks in adult cultures and 5-7 weeks in newborn cultures the keratinocytes differentiate and separate; whereas, the melanocytes continue to grow and pure melanocyte cultures are obtained (Fig. 2).

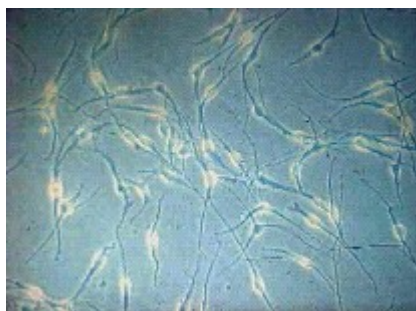


Fig. 1: Melanocytes grown in TPA and serum. Cells are slender showing dendrites with terminal swellings

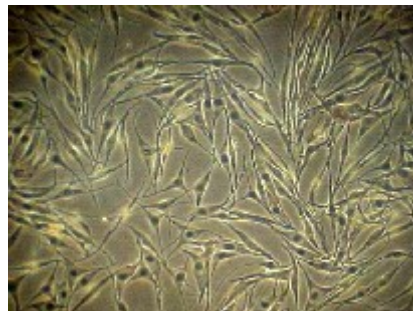


Fig. 2: Melanocytes grown in a serum free medium showing fibroblast-like morphology

Newborn melanocytes were found to be bipolar, slender and tended to be less dendritic in comparison with those of adults; both have a fibroblast-like morphology. Moreover, melanocytes obtained from newborn with dark skin showed strong proliferative capacity, were more dendritic and contained more melanin. Newborn melanocytes could be passaged up to 5-6 passages, whereas those of adults up to 3-4 passages only. The identity of the cultured melanocytes is confirmed by being DOPA+, S.100+, HMB 45+ and K.1.2.58+ and by being negative for the other epithelial cell markers, such as CK1, CK10 for keratinocytes and CD1a for Langerhans cells [6, 9].

Melanocytes were almost always seen in contact with the adjacent keratinocyte colonies with dendritic projections and bridged space between colonies. Interestingly, addition of viable keratinocytes to melanocyte cultures showing senescence resulted in restoration of their viability and proliferative activity. It has also been shown that keratinocyte conditioned medium induced melanocyte proliferation and melanization. These observations indicate that keratinocytes synthesize and release factors that modulate melanocyte function. Even *in vitro*, a close anatomical and functional relationship between melanocytes and keratinocytes exists, thus posing additional validity on the concept of epidermal melanin unit [10]. Cultivation of nevus cells confirmed its melanocytic origin and that the morphologic and behavioral differences observed between epidermal melanocytes and nevus cells in the skin are the result of local environmental influences rather than intrinsic cellular differences [11].

Other factors that have been found to induce melanocyte proliferation are α -MSH, endothelins, leukotrienes, prostaglandins and several others released from adjacent keratinocytes and fibroblasts. In addition, ultraviolet light was found to induce melanocyte melanization and a slight inhibition of melanocyte proliferation. Ultraviolet A (UVA) irradiation did not substantially affect melanocytes whereas ultraviolet B (UVB) irradiation directly activated cultivated melanocytes. On the other hand, IL-1 α , IL-6, TNF- α and interferon β inhibit both melanocyte proliferation and melanin synthesis. These cytokines are released from epidermal and dermal cells in several inflammatory skin conditions and it has been suggested that postinflammatory hypo- and/or hyperpigmentation may result from the effect of these factors on epidermal melanocytes and or epidermal melanin unit. The epidermal melanin unit has now been extended to include not only melanocytes and neighboring keratinocytes, but also Langerhans cells, trafficking lymphocytes and dermal fibroblasts (Table. 1) [12-14].

Table (1): Growth factors and their characteristics [12-14].

Growth factor	Site	Biologic effect	Family
EGF	Almost all body fluids, platelets	Mitogenic for most epithelial tissues, fibroblasts, endothelial cells.	The EGF family includes EGF, TGF- α , VEGF and HBEGF.
TGF-α	DC, eosinophils	Similar to EGF, but more potent angiogenesis factor	
HBEGF	DC	Mitogenic for keratinocytes.	
TGF-β	DC, lymphocytes, fibroblasts, keratinocytes, platelets	Inhibits replication of most cells in vitro (keratinocytes, endothelial cells, lymphocytes, macrophages) may inhibit or stimulate fibroblasts.	TGF- β family (TGF- β_{1-5}) Only TGF- β_{1-3} are found in mammalian cells and have similar effect.
IGF-1	Most tissues, fibroblasts, DC	Mitogenic for fibroblasts, bone cells, neural tissues, haematopoietic cells, endothelial cells.	The IGF-1 family (IGF-I and IGF-II). Both have similar structures and effects.
PDGF	Endothelial cells, platelets, DC, fibroblasts	Mitogenic for vascular smooth muscles, fibroblasts.	The PDGF family consists of PDGF and VEGF
VEGF	Pituitary cells	Mitogenic for endothelial cells but not keratinocytes, smooth muscles or fibroblasts.	
FGF (acidic and basic)	Fibroblasts, astrocytes, endothelial cells, smooth muscles	Mesenchymal and neural tissue mitogen	The FGF family (aFGF, bFGF, FGF-5, FGF-6). Only the first 3 have been extensively studied
KGF	Fibroblasts	Mitogenic for epithelial cells but not fibroblasts or endothelial cells	

DC = dendritic cells, EGF= epidermal growth factor, FGF= fibroblast growth factor, HBEGF= heparin-binding EGF, IGF= insulin growth factor, KGF= keratinocyte growth factor, PDGF= platelet-derived growth factor, TGF= transforming growth factor, VEGF= vascular endothelial growth factor.

By the year 2004, melanogenic paracrine and autocrine networks have been elucidated between melanocytes and other types of skin cells. These include endothelin (ET)-1, granulocyte macrophage colony stimulating factor (GM-CSF), membrane type stem cell factor (SCF) and growth-related oncogene- α (GRO α) for interactions between keratinocytes and melanocytes, and hepatocyte growth factor (HGF) and soluble type SCF for interactions between fibroblasts and melanocytes [15]. These networks are also associated with corresponding receptors expressed on melanocytes, including ET B receptor and the SCF receptor, c-KIT. Consistent with in vitro findings on the melanogenic paracrine or autocrine cytokine networks, it has been found that the up- or down-regulation of such networks is intrinsically involved in vivo in the stimulation of melanocyte functions in several epidermal hyper- or hypo-pigmentary disorders. These are ET-1/ET B receptor as well as membrane type SCF/c-KIT for ultraviolet B-melanosis, GM-CSF for ultraviolet A-melanosis, ET-1/ET B receptor as well as membrane type SCF for lentigo senilis, GRO α for Riehl's melanosis, sphingosylphosphorylcholine for hyperpigmentation in atopic dermatitis, ET-1 for seborrheic keratosis, soluble type SCF as well as HGF for dermatofibroma and cafe' -au-lait macules, and c-KIT for vitiligo vulgaris. These unveiled regulatory mechanisms involved in the abnormal increased or decreased levels of lesional melanocyte function provide new insights into therapeutic tools utilizing blockage of responsible cytokine networks [16].

Cultivated melanocytes have also enriched our knowledge about the pathogenesis of several diseases. Sera of active vitiligo patients exerted cytotoxic effect directly and via the classical pathway of complement activation on melanocytes in vitro, a finding that has been refuted by others. Immunofluorescence studies using living cultivated melanocytes as a substrate revealed deposition of immunoglobulins not only on surface of cultivated melanocytes but also on cultivated keratinocytes and fibroblasts. At the same time, an antibody dependent cellular cytotoxicity (ADCC) and specific melanocyte-specific cytotoxic T cells have been detected. This observation further supports the involvement of the immune system in melanocyte damage in vitiligo [16]. On the other hand, sera of alopecia areata failed to exert any cytotoxic effect on cultured melanocytes casting doubt on involvement of these cells in the pathogenesis of alopecia areata [18]. Western blotting analysis using antigens obtained from cultivated melanocytes in both diseases was also inconclusive [16].

The effect of various agents and drugs has also been examined on cultured melanocytes. It has been shown that nitric oxide (NO), released by inflammatory cells and Langerhans cells is toxic to melanocytes which may play a role in disorders associated with loss of pigmentation [19]. Retinoids reduce tyrosinase activity and therefore are used to regulate pigmentation and lighten hyperpigmented skin. All-trans-retinoic acid (tretinoin) enhanced the toxic effect of hydroquinone on melanocytes [20]. Other inhibitors of melanogenesis by competitive inhibition of tyrosinase are arbutin, kojic acid and possibly niacinamide [21]. Ultraviolet light induced melanocyte cytotoxicity but in vivo it results in melanocyte proliferation and melanization which are due to a paracrine effect. Sera obtained from psoralen plus ultraviolet A (PUVA) responding vitiligo patients induced a significant melanocyte and dermal fibroblasts proliferation indicating that this therapy may have a systemic effect [22]. Reconstructed melanocyte bearing skin is currently used in evaluation of sun screening agents [23].

Normal human melanocytes provided the proper control of melanoma cell research. IFN- α inhibited melanoma cell proliferation in vitro and it is currently an established adjuvant therapy for advanced cases of melanoma. In addition, several oncogenes, e.g., B-raf gene, and adhesion molecules, e.g., E- and N-cadherins, are currently evaluated for being potential targets for therapy and prevention of melanoma progression [24].

Because of the absence of serum and tumor promoting factors, the serum free and hormone supplemented medium could be suitable not only for investigative but also for therapeutic purposes, such as transplantation

procedures in patients with vitiligo, piebaldism and other depigmentary disorders. Indeed, several trials have been carried out with successful results and melanocyte transplantation has become one of the established lines of treatment of several recalcitrant hypopigmentary and depigmentary disorders. In the transplantation procedure, either pure melanocytes or epidermal sheet-bearing melanocytes could be utilized [25].

Keratinocyte Culture

Keratinocytes have been successfully cultivated in 1975 when Rheinwald and Green reported the ability to grow pure keratinocytes using a serum containing DMEM/Ham's F12 medium and a feeder layer (irradiated well-defined transfected mouse 3T3 cells). Shortly thereafter the culture cell yield has been improved by the incorporation of keratinocyte mitogens, such as epidermal growth factor (EGF) and cAMP-elevating agent, the cholera toxin [26].

In cultures, keratinocytes attach as single cells or small clusters and then grow at the periphery of the colony whilst stratifying in the center. The cells form intercellular desmosomes and so grow as coherent colonies until confluent stratifying multilayered sheets are obtained. These have poorly formed squames and do not show normal skin morphology, with cells being flattened, attenuated and forming no stratum granulosum or stratum corneum. Membrane coating granules and keratohyaline granules are sparse and the cultures show the same phenotype as regenerating epidermis [27].

Since the fundamental breakthrough of keratinocyte culture, other ways trying to avoid serum and mouse transfected feeder layer (3T3 cells) in growing keratinocytes have been developed. The search for a defined serum-free medium has resulted in a low-calcium MCDB 153 medium containing several growth factors, which is now commercially available. In low calcium medium (<0.06 mM), the cells fail to form desmosomal interconnections and are spaced out as monolayer (Fig. 3). Although the keratinocytes fail to stratify, they commence terminal differentiation with the expression of involucrin in the larger cells which move suprabasally when calcium levels are restored (to 1.2 mM) (Fig. 4) [28]. The cell cycle is around 22 h with a growth fraction of 60-70%. These systems are therefore heavily weighted towards hyperproliferation [29]. In low Ca⁺⁺ medium keratinocytes expressed cytokeratins of germinative basal epidermis, namely CK 5 and 14, whereas in high Ca⁺⁺ medium they expressed cytokeratins of differentiated epidermis, namely CK 1 and 10. BPE was an essential mitogen for keratinocyte proliferation and cells obtained from old individuals showed weak proliferative capacity that hampered their use in autografting [30]. In contrast to epidermal keratinocytes, those obtained from the outer root sheath of hair follicles have extensive proliferative capacity irrespective of donor age, which probably depends on the residing pluripotent epithelial stem cells. Interestingly, autologous outer root sheath keratinocytes are successfully used in grafting of recalcitrant chronic leg ulcers. The use of autologous keratinocytes for grafting depends on mechanical stability of the graft that can be improved in hybrid models. Hyaluronic acid membranes, also known as keratinocyte delivery system, have been proved to be extremely helpful and textile spacers are under investigation for scaffolding cultivated skin structures with plasticity and ease of degradability [31].

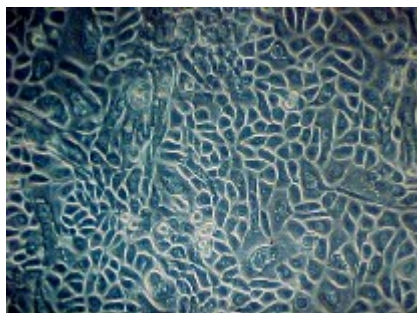


Fig. 3: Keratinocytes grown under low Ca^{++} level. Cells appear as polygonal and monolayer with prominent nuclei.

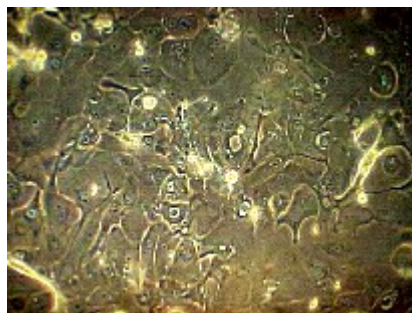


Fig. 4: Keratinocytes grown under high Ca^{++} level. Cells stratify and show evidence of differentiation.

In skin equivalents, keratinocytes tend to form a more differentiated epidermis, with stratum corneum and granulosum including keratohyaline and membrane coating granules. Although these complex cultures are useful for pharmacological experiments, they provide little tissue expansion, whereas in monocellular cultures one cm of skin can generate 1 m culture area within 6 weeks. This huge population expansion has permitted the use of keratinocyte cultures for skin grafting and keratinocyte grafting. This has been applied to burns, difficult to heal chronic leg ulcers, excision sites of giant melanocytic nevi and correction of hypospadias [32-34]. Both autologous keratinocytes and allogenic keratinocytes have been used, i.e., keratinocytes autografting and keratinocyte allografting. Although allografts do not survive transplantation, they provide a biological dressing and produce wound healing via various cytokine releases [35]. Melanocyte-containing epidermal grafts are an established method for surgical management of leukodermas, e.g., piebaldism [36].

The development of keratinocyte culture has also led to an increased understanding of factors influencing keratinocyte growth and differentiation. Keratinocyte growth factor (KGF) and GM-CSF released by dermal fibroblasts under the effect of IL-1 α and β exert a paracrine effect on keratinocytes and induce their proliferation and differentiation [37]. The keratinocyte has been shown to play an active part in the skin associated lymphoid tissue (SALT) both in cellular interactions with Langerhans cells and epidermotropic T lymphocytes and in the production of several growth factors and cytokines (Table 1). Keratinocytes express Fc γ R and this expression is upregulated by IFN γ [38]. Cytokines produced by keratinocytes include interleukin (IL)-1, -6, -7, -8, -10, -12, -15, -18, and -20 and TNF- α . Keratinocyte-derived IL-7 and -15 are considered to be significant in T-cell trafficking, possibly even in the pathogenesis of cutaneous T-cell lymphoma. IL-15 inhibits keratinocyte apoptosis and plays a role in psoriasis. Immunomodulatory IL-10 and -12 originating from keratinocytes are considered to be responsible for systemic effects, and IL-18 perhaps has a similar action. In addition, IL-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair. Keratinocytes were fairly recognized as being source or target of other IL-10 family members like IL-20 and IL-24 and the role of these cytokines in specific diseases is under investigation. In addition, a variety of cytokine receptors are present on keratinocytes like those for IL-4, -13, and -17 and to lesser degree IL-2. [39]. NO is produced in high quantities by epidermal keratinocytes in psoriatic skin in response to IFN- γ and TNF- α . Based on these observations several biological agents are introduced in the management of inflammatory skin conditions. Etanercept (Enbrel), infliximab (Remicade) and adalimumab (Humira) as TNF- α antagonists and anakinra (Kineret) as IL-1 blocker have been recently approved for management of severe psoriasis, psoriatic arthropathy and several other inflammatory diseases [40].

Keratinocytes can be transformed by virus exposure, e.g., Simian virus 40 (SV40), human papilloma virus and by oncogene transfections, which have illuminated the process of skin carcinogenesis and antisense

therapy to knock out the mutant genes that are responsible for malignancy and metastases. Keratinocytes have been transfected with human growth hormone genes and have been shown to release human growth hormone; a procedure that can be used in delivery of gene products by genetically manipulated grafts [41]. In this regard, this might form a basis for the use of keratinocytes in gene therapy, perhaps to correct genetic defects such as type VII collagen abnormalities in recessive dystrophic epidermolysis bullosa. There is also a growing interest in genetic manipulations *ex vivo* of epidermal keratinocytes as a curative therapy for otherwise untreatable hereditary dermatoses, such as mechanobullous disorders and xeroderma pigmentosum [42]). Cultivated keratinocytes have also been used to precisely localize and define antigens targeted by various autoantibodies of autoimmune bullous disorders by immunofluorescence, immunoelectronmicroscopy and immunoblotting [43]. Nicotine induced keratinization and cornification of cultured keratinocytes, thus explaining leukokeratosis nicotinic palati [44]). Human leukocyte elastase induces keratinocyte proliferation *in vitro*, a finding that may explain keratinocyte hyperproliferation in psoriasis [45]).

In keratinocytes, retinoids induce proliferation, resulting in epidermal hyperplasia and modulate epidermal differentiation [46]. Antineoplastic compounds exerted growth inhibitory effect on malignant keratinocytes *in vitro*. For example, perifosine, a novel alkylphospholipid, induces p21 (WAF1) expression in squamous carcinoma cells through a p53-independent pathway, leading to loss in cyclin-dependent kinase activity and cell cycle arrest [47]). PUVA treatment of keratinocytes reduced ICAM-1 and HLA-DR expression induced by IFN γ and IL- α [48]. Vitamin D3 and its analogues inhibited keratinocyte proliferation, which explains the efficacy of these compounds in treatment of hyperproliferative skin disorders, such as psoriasis [49].

It is now realized that the keratinocyte is not simply a passive cell awaiting terminal differentiation but is an active secretory cell with important biochemical and immune functions.

Sebaceous Gland Cell (Sebocyte) Culture

Selective cultivation of normal human sebaceous gland cells (sebocytes) has helped in investigating disorders of the pilosebaceous units and served as a model for better understanding of drug pharmacokinetics, such as retinoids, androgens and toxic agents with special affinity to lipids [50].

Sebocytes are specialized epithelial cells committed to synthesize lipid rich sebum. Most of the available data of the physiology and pathophysiology of the sebaceous gland originates from hamsters [51]. However, several years ago *in vitro* cultivation of pure adult human sebocytes has been achieved and well characterized in MCDB 153 medium enriched with serum, cholera toxin, crude BPE, bFGF, hydrocortisone and insulin. Cells grew from the periphery of the lobules to form a single layer sheet of polygonal cells resembling basal keratinocytes (Fig. 5) that later undergo differentiation with increase in cell volume, accumulation of lipids and finally nuclear degeneration and cell death (Fig. 6). Sebocytes *in vitro* show similar features to keratinocytes being polygonal in shape and synthesize cytokeratins, nevertheless they express specific epidermal membrane antigen that has been used immunohistochemically in their identification [52]. Analytical thin-layer chromatography revealed that the cells synthesized a large amount of sebum-specific lipids, squalene and wax esters *in vitro*. As the lobules are obtained from adults, sebocyte yield is usually low despite the several methods described for sebocyte cultivation [53]. A modified technique moderately improved the sebocyte yield, but obviously the problem of the donor age and the necessity of serum supplementation were unavoidable obstacles. To overcome the yield problem, sebocytes were transfected by SV 40 large T antigen and the immortalized sebaceous gland cell line SZ95 has been obtained that has been shown to preserve the major functional and behavioral characteristics of normal human sebocytes [54].

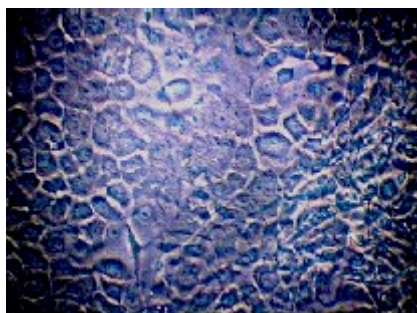


Fig. 5: Sebocytes in culture in the proliferative phase showing features of basal keratinocytes.

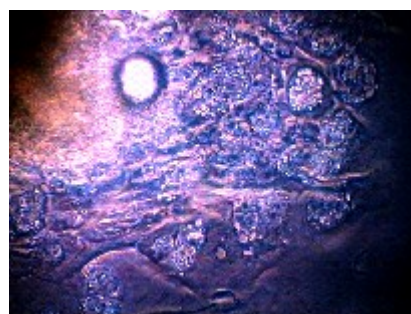


Fig. 6: Sebocytes in culture showing evidence of differentiation with refractile lipid vacuoles

It has also been shown that growth hormone stimulates differentiation of sebocytes. Insulin-like growth hormone (IGF-1) increases DNA synthesis and cell proliferation that may contribute to the increase in sebum production during puberty and in acromegaly [55]. Substance P, which can be elicited by stress, promotes the development of cytoplasmic organelles in sebaceous cells, stimulates sebaceous germinative cells and increases the size of individual sebaceous cells and the number of sebum vacuoles for each differentiated sebaceous cell. Therefore, it seems that substance P promotes both the proliferation and the differentiation of sebaceous glands. It has been suggested that this may be responsible for the stress-induced exacerbation of acne [56]. The powerful sebosuppressive effect of some retinoids, such as 13-cis-retinoic acid (isotretinoin), demonstrates that sebocyte differentiation is altered by retinoids. Retinoids inhibit proliferation and lipid synthesis in cultured human sebocytes and alter their keratin expression [57]. Later it has been shown that in addition, 13-cis-retinoic acid (isotretinoin), 3, 4-didehydroretinoic acid, and 3, 4-didehydroretinol, but not all-trans-retinoic acid (tretinoin) or the synthetic retinoids acitretin and adapalene, were potent competitive inhibitors of the oxidative 3α -HSD activity of RoDH-4. As a result, they reduce the formation of dihydrotestosterone and androstandione in vitro that might explain the unique sebosuppressive effect of isotretinoin when treating acne [58]. Both testosterone and 5α -dihydrotestosterone significantly stimulated the proliferation of the sebocytes, a finding that has been completely abolished by spironolactone. Cultured sebocytes strongly expressed type 1 5α -reductase and metabolized testosterone to androstenedione, 5α -androstenedione, 5α -dihydrotestosterone, androsterone and 5α -androstenediol. Stimulation of sebocyte proliferation by insulin, thyroid-stimulating hormone and hydrocortisone indicates that the hormonal control of the sebaceous gland could be a complex mechanism [53, 59]. Incorporation of sebocytes and/or sebaceous glands in skin reconstructs has not been achieved yet.

Langerhans Cell Culture

Langerhans cells (LCs) are dendritic cells present in the epidermis, bronchi and mucosae and are responsible for antigen presentation. The essential role of LC in the induction of contact allergic skin reactions and skin transplant rejection is well established. They have also been cultivated in vitro; however, they usually do not survive more than few weeks as they are considered nearly end differentiated cells and they are few in number. Furthermore, properties of LC, such as phenotype, morphology and the stimulatory potential to activate T lymphocytes, are dependent on the local microenvironment in which the LCs reside, thus correlation between in vitro and in vivo findings is expectedly irrelevant. The initial use of culture medium containing serum resulted in undue LCs activation by serum antigens that further invalidated in vitro findings [60].

Nevertheless, in vitro generation of dendritic cells (DCs) with the typical molecular, morphological and functional features of LC from purified CD34+ progenitor cells of peripheral blood under defined serum-free culture conditions has been achieved. TGF- β 1 was found to be an absolute requirement for in vitro LC development under serum-free conditions upon stimulation with the classical DC growth and differentiation factors GM-CSF, TNF- α and SCF (Table 1). The recently identified cytokine FLT3 ligand further dramatically enhanced in vitro LC development and even allowed efficient in vitro generation of LC colonies from serum-free single cell cultures of CD34+ hemopoietic progenitor cells (Fig. 7, 8) [61].



Fig. 7: Langerhans cells cultivated in the absence of serum.



Fig. 8: Langerhans cells grown in presence of serum showing higher number of dendritic cells.

The procedure of LC cultivation was further refined by isolation of immature epidermal LC in sufficient numbers mechanically by three-step Ficoll floatation, a method that was found to be superior to the well-known positive immuno-magnetic separation by anti-CD1a-coated beads. This has enabled easy isolation of LCs not only from peripheral blood but also from the epidermis and mucosa. The medium used is RPMI supplemented with HEPES, L-glutamine, non-essential amino acids and sodium pyruvate. Both epidermal and circulating LC appear rounded cells but with multiple small dendrites [62].

Not only epidermal but also mucosal LCs have been cultivated in vitro and it has been shown that LCs in human oral epithelium are more efficient at stimulating T cells than those of skin which may be of value as both cells meet different antigenic challenges [63]. LCs could be successfully kept viable after cryopreservation and it has been shown that cryopreserved LCs expressed high levels of HLA-DR and CD1a antigens and stimulated autologous T cells to an extent almost identical to that obtained from fresh LCs. These findings indicate that the cryopreservation of human LCs could lead to a breakthrough in various experiments dealing with these cells [64].

In atopy, Fc epsilon RI-activated LCs release chemotactic signals and increase the migratory capacity of naive T cells in vitro. Furthermore, they produce high amounts of proinflammatory cytokines and chemokines, prime naive T cells into IFN- γ -producing T cells and release IL-12 and IL-18, which together might lead to the switch of the initial T (H) 2-type immune response into a response of the T (H) 1 type in vivo [65]. The role of LCs in contact dermatitis has also been tested in vitro and it has been found that contact sensitizers, such as dinitrochlorobenzene, caused an increase of the co-stimulatory molecule CD86, of intercellular adhesion molecule CD54 and of the HLA-DR antigen, whereas the irritant sodium dodecyl sulphate and the vehicle dimethyl sulfoxide had no effect on LCs [66].

Recently, LCs have been used to test a variety of immunomodulating agents in vitro. The topical immunomodulators tacrolimus and pimecrolimus and steroid ointment induced a selective depletion of

inflammatory dendritic cells in the epidermis of atopic dermatitis patients and reduced the expression of the co-stimulatory molecules CD80 and CD86 in vivo. However, tacrolimus did not increase the rate of apoptotic LCs whereas steroid did so in vitro [67]. Assessment of cellular parameters related to UV-induced immunosuppression and the effect of sun screening agents is performed using the reconstructed epidermis containing LCs. Exposure to solar-simulated radiation provokes morphological alterations, reduces surface antigen expression and reduces the numbers of LCs cells within the exposed epidermis [68].

At the present time there is a growing interest in the use of cultivated LCs in the management of allergic contact dermatitis and malignancy. Following activation by haptens, LCs migrate to regional lymph nodes to activate effector T cells. This step can be prevented in manipulated epidermal LC by inhibitory peptide, 1 an apoptotic gene that binds and blocks the function of hyaluronic acid (hyaluronan), which is known to serve as an adhesive substrate for LC migration. Furthermore, creating a "killer" LC clone by introduction of CD95L cDNA, either before or after sensitization, results in marked suppression of contact hypersensitivity by only eliminating the pathogenic T cells [69]. The mitogenic effect of TNF- α and GM-CSF on LCs in vitro has encouraged physicians to use them systemically as an adjuvant therapy to tumor vaccination [70]. Anti-melanoma immunization therapy is currently tried in patients, through dendritic cells charged with melanoma antigens with, so far, limited side effects. Injections of antigenically charged dendritic cells were performed subcutaneously, intravenously or in the lymph nodes. Interestingly, positive clinical responses were obtained with complete remission of the metastasis in some cases [71].

Dermal Fibroblast Culture

Fibroblasts are the most numerous cells found in the loose connective tissue and they are responsible for the manufacture of all its elements or their precursors. Fibroblasts are the easiest cells to cultivate in vitro, as their growth requirements are minimal in comparison to other human cell types. Indeed, their isolation and propagation have helped not only in understanding the biology of these cells but also the biology of other cells by serving as control cells for almost all investigative work done so far. They have also been used extensively in pharmacological tests and skin reconstruction. Fibroblasts are grown in DEMEM medium supplemented with L glutamine and 10% fetal calf serum. They can also be adapted to grow in serum free medium. When cultured in monolayer, dermal fibroblasts have an elongated spindle shape with a prominent nucleus, are small in diameter and grow at a high rate (Fig. 9) [72].

Fibroblasts obtained from newborn generally have greater mitogenic responsiveness than adult fibroblasts and that age-associated loss of growth factor responsiveness may contribute to the decreased proliferative capacity of old-donor cells. It has been shown that dermal fibroblasts possess a finite replicative capacity of 50 to 100 doublings, and then cease replication in response to growth factors. Cells cultivated to the end of their replicative life span in vitro overexpress metalloproteinase activities that may explain the age-related progressive destruction of the collagenous and elastic components of the extracellular matrix [73]. In contrast to dermal fibroblasts, wound fibroblasts grew slowly and were large, star shaped and had cytoplasmic stress fibres. Smooth muscle α actin was detected in the cytoplasm of most wound fibroblasts. Wound fibroblasts also contract collagen gels during the first days more strongly than dermal fibroblasts. These results show that, in vitro, wound fibroblasts have greater contractile capacity than dermal cells. The significant proportion of wound fibroblasts containing α -smooth muscle actin suggests that α -smooth muscle actin ratio may be related to wound contraction [74].

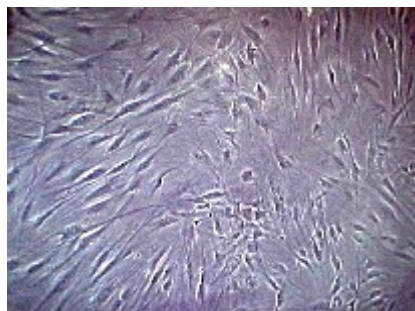


Fig. 9: Fibroblasts in culture appear as spindle shaped cells with indistinct cell boundaries.

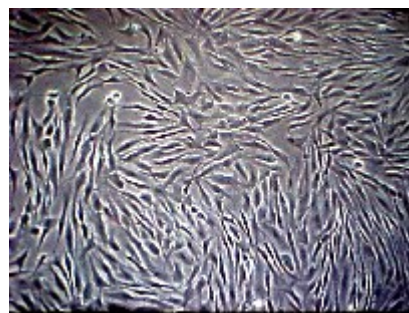


Fig.10: Endothelial cells in culture are similar to fibroblasts but with distinct cellular boundaries

Dermal fibroblasts are the source of collagen and elastin of the extracellular matrix (ECM). Procollagens are secreted through the Golgi apparatus in the extracellular space where the N-terminal and C-terminal propeptides are cleaved by specific proteases. The mature processed collagen molecules aggregate to form larger collagen fibrils and help to form the ECM with other components. Therefore, normal structural and functional type I collagen production and deposition to make normal physiological connective tissue needs regulation at several steps. Abnormality in any step may cause hypo-, hyper-, or defective synthesis and accumulation of collagen in ECM, which in turn causes different diseases in humans, such as osteogenesis imperfecta, scurvy, scleroderma or systemic sclerosis, keloids, and others [75]. Elevated level of type I collagen in scleroderma skin fibroblasts is primarily due to the increased rate of collagen gene transcription. Increasing evidence suggests that TGF- β plays a significant role in fibrosis [76]. Dermal fibroblasts are also an important source of several cytokines, e.g., IL-1 and 6, chemokines, e.g., cyclooxygenase 2 (COX-2) and growth factors, e.g., FGF, IGF-1 α that have significant autocrine and paracrine effects (Table 1)[77]. Interferons α , β and γ suppress collagen synthesis by dermal fibroblasts. In particular, IFN- γ inhibits the constitutively increased collagen synthesis characteristic of fibroblasts derived from lesions of patients with scleroderma. Inhibition of collagen synthesis by IFN- γ is associated with a coordinate inhibition of transcription for types I and III collagen. Animal studies demonstrated that IFN- γ inhibits the collagen synthesis associated with the fibrotic response to an implanted foreign body, bleomycin-induced pulmonary fibrosis, and the healing response to cutaneous thermal burns. Interferon α can decrease the size of keloids of recent onset [78].

Hyaluronan is a ubiquitous extracellular matrix component, and is present at high concentrations in skin, joints and cornea. In the skin, it is synthesized primarily by dermal fibroblasts and to a lesser extent by epidermal keratinocytes. It is commonly used in cosmetics and skin care preparations [79].

Dermal fibroblasts play also a significant role in inflammation. They, besides neutrophils and macrophages, can elaborate the proinflammatory matrix metalloproteinases 1, 3 and 9 in response to injurious agents, such as immune complexes, lipopolysaccharides and UVB radiation both directly and indirectly by IL-1 α and 6 released by keratinocytes [80]. Dermal fibroblasts may contribute to the epidermal hyperplasia of psoriasis by promoting keratinocyte proliferation through IGF-1, the secretion of which could be modulated by inflammatory cytokines, such as IFN- α (Table. 1) [81].

Several drugs have been tested with dermal fibroblasts. In vitro exposure of fibroblasts to 8-methoxypsoralen and UVA resulted in growth inhibition with morphological and functional changes reminiscent of replicative senescence that also occur in vivo upon exposure to sublethal stressors, i.e.,

hyperoxia, hydrogen peroxide, and ethanol [82]. Heparin inhibits dermal fibroblast proliferation and collagen production but significantly stimulates bFGF and TGF β 1 by normal, fetal and keloid fibroblasts which may play a role in wound healing [83]. Although minocyclin has been used to treat fibrosis, it did not alter dermal fibroblast proliferation or collagen synthesis [84]. Retinoids stimulate dermal fibroblasts to produce extracellular matrix proteins, particularly when the skin is damaged by wounding, ultraviolet radiation or glucocorticoids [85]. Inhibition of dermal activity by cortisol in culture was partially reversed by testosterone and dihydrotestosterone, whereas ACTH and the androgen precursor dehydroepiandrosterone sulphate did not show such antagonistic effect. These results suggest that increased production of adrenal androgens during ACTH therapy may account for the relative absence of 'skin-thinning' and 'steroid-bruising' which are common side-effects of corticosteroid therapy [86]. On the other hand, triamcinolone acetonide has been shown to decrease both cellular proliferation and collagen production by dermal fibroblasts and increases the production of bFGF and decreases production of TGF- β 1 by human dermal fibroblasts [87]. Glycolic acid (GA), commonly used in chemical peeling and skin rejuvenation, increases collagen synthesis by fibroblasts and modulates matrix degradation and collagen synthesis through keratinocyte-released IL-1 α . These results suggest that GA contributes to the recovery of photodamaged skin through various actions, depending on the skin cell type [88].

Besides the use of dermal fibroblasts in almost all skin substitutes, there are currently trials attempting at transplanting genetically modified fibroblasts carrying a stably integrated transgene as a vehicle for normal genes [89].

Dermal Endothelial Cell Culture

Dermal endothelial cells involved in tumor angiogenesis, wound healing, and inflammation are predominantly of micro vascular origin and are functionally distinct from large vessel-derived endothelial cells which have been largely used for in vitro vascular research. Initially, cultivation of dermal endothelial cells has been achieved using high concentrations of serum and conditioned media from tumors to achieve optimal growth [90]. Contamination of the cultures with other cell types, such as fibroblasts was evident, however, with some modifications of the culture procedure in which continuous Percoll gradient was applied; pure endothelial cells could be obtained. The culture medium consisted of endothelial cell basal medium supplemented with EGF and hydrocortisone [91]. The cultivation of endothelial cells in vitro was later facilitated by the incorporation of the potent mitogen vascular endothelial growth factor (VEGF) that also functions as a survival factor for endothelial cells by up-regulating Bcl-2 expression (Fig. 10) [92]. Several cellular components of the skin synthesize and release VEGF in vivo and in vitro (Table 1).

Clearly, the growth and turnover of endothelial cells in the skin is fundamental not only in normal development, but also in wound repair, hair follicle cycling, tumor cell metastasis, and in many different states of cutaneous pathology. Endothelial cells in vitro acquire a slightly elongated epithelioid (cobblestone) shape in the modified and now commercially available endothelial cell growth medium MV and are labeled with Ulex europaeus Agglutinin I and an antibody against Factor VIII-related antigen [93]. To further overcome the problems commonly involved in the culture of microvascular endothelial cells, including unreliable isolation techniques and low cell yields, a simplified protocol for the selective isolation and cultivation of human dermal microvascular endothelial cells from neonatal foreskins has been proposed. It utilizes immunomagnetic beads and it is based on the transient, endothelial cell-specific induction of E-selectin by TNF- α [94].

The effects of several cytokines have been examined on cultivated dermal endothelial cells in vitro. GM-CSF and IL-1 α and β induced endothelial cell proliferation, whereas IFN- γ and TNF α activated them by induction of HLA-DR and ICAM-1 expression [91]. Activated endothelial cells also help in initiating immune reactions as antigen presenting cells as they express adhesion molecules such as E selectin,

ICAM-1 and VCAM-1 that are essential in memory and effector T cell recruitment [95]. In wound healing, a dynamic interaction occurs among endothelial cells, angiogenic cytokines, such as FGF, VEGF, TGF- β , angiopoietin, and mast cell tryptase, and the extracellular matrix environment [96]. Fibrin fragment E liberated at wounds stimulates the proliferation, migration and differentiation of human dermal microvascular endothelial cells in vitro, both in the absence and presence of additional endothelial growth factors, such as VEGF and bFGF indicating a possible synergy between the signaling pathways used by these three angiogenic factors [97]. Increase of Bcl 2 on endothelial cells is associated with intratumoral angiogenesis enhancement and accelerated tumor growth [98]. On the other hand, several agents inhibited endothelial cell proliferation and angiogenesis. Angiotensin type II (AT2) receptor stimulation is antiangiogenic through inhibition of VEGF-induced endothelial cell migration and tube formation via activation of a PTX-sensitive G protein [99].

Kaposi's sarcoma-associated herpes virus (KSHV) is consistently found in all forms of Kaposi's sarcoma. Infection of dermal microvascular endothelial cells with KSHV induces spindle cell formation in vitro by a c-kit dependent mechanism. Endothelial cells are used as host cells for culturing and propagation of this virus [100]. Similarly, Bartonellosis, a disease caused by motile intracellular bacteria, produces characteristic dermal eruption that results from a pronounced endothelial cell proliferation (Verruga peruana). Also an extract of Bartonella bacilliformis organisms induced endothelial cell proliferation in vitro [101].

The effect of physical agents and drugs has also been studied on endothelial cells in vitro. Hypoxia resulted in synthesis and release of VEGF by endothelial cells and possibly by pericytes which contributes to their proliferation and subsequent angiogenesis, i.e., autocrine effect [102]. Ultraviolet radiation induces nitric oxide synthase 2 expression by endothelial cells which may provide an explanation of the erythema, edema and inflammation [103]. On the other hand, hypertension in end stage renal failure has been attributed to uremic plasma factors inhibiting nitric oxide synthase 2 with subsequent lack of vasodilatation and persistent vasoconstriction [104]. Tumors promote angiogenesis by secreting growth factors, such as VEGF and VEGF-related molecules either spontaneously or in response to hypoxia that stimulates endothelial migration, proliferation, proteolytic activity, and capillary morphogenesis. Newly formed blood vessels supply the tumor with nutrients and oxygen, dispose of its metabolic waste products, and generate paracrine stimuli, which further promote tumor cell proliferation and invasiveness [105]. Endostatin exerts its powerful antiangiogenic effect through inhibition of endothelial cell proliferation by E selectin dependent mechanism [106].

Griseofulvin has been used as an antifungal drug for many years, but it has recently been shown to be effective in several inflammatory skin diseases. Griseofulvin inhibited the expression of TNF α -induced VCAM-1 dose-dependently, and this inhibition was fully reversible. Similarly, griseofulvin inhibited the induction of VCAM-1 expression on both TNF α - and IL-1 α -stimulated human endothelial cells indicating potent immunomodulatory properties, which may be associated with its feature as a microtubule antagonist [107]. Retinoids inhibit whereas steroids induce proliferation of endothelial cells without effecting HLA-DR, ICAM-1 induction by IFN γ , TNF α and IL-1 α [108]. Nevertheless, retinoids inhibited VCAM-1 gene expression and VCAM-1 T cell binding to cytokine-treated endothelial cells thus explaining their anti-inflammatory effect [109].

Successful reconstruction of human skin model containing keratinocytes, fibroblasts and endothelial cells has been described. The therapeutic implications of this culture have yet not been verified [101].

Hair Follicle Cell Cultures

Different culture models are used to investigate hair biology. With organ cultures the whole hair follicle

is maintained in the culture in order to investigate the effect of different substances on hair growth. The development of medications to stimulate or to inhibit the production of hair keratine may have great impact on the treatment of baldness and hypertrichosis, respectively [111].

Regarding selective cell culture models, two different compartments of the hair follicle are the main objective of research, namely hair keratinocytes from the outer root sheath (ORS) and mesenchymal cells of dermal papilla (DPC).

ORS keratinocytes can be easily obtained from plucked hairs, using the trichogramm technique [112], thus avoiding any surgical procedure to isolate the ORS keratinocytes. Culture medium and mitogens are almost the same that are used in selective cultivation of epidermal keratinocytes. With this culture model the outgrowth of keratinocytes from the ORS is seen as early as two days following inoculation (Fig. 11); they produce multilayered cell colonies, in which desmosomes could be identified [112]. Although they do not produce the hair keratin, these cells can be used to study hair biology and indeed the inhibition of androgens on ORS keratinocytes proliferation has already been shown [113]. Vellus hair follicle cells were also successfully cultivated [114].

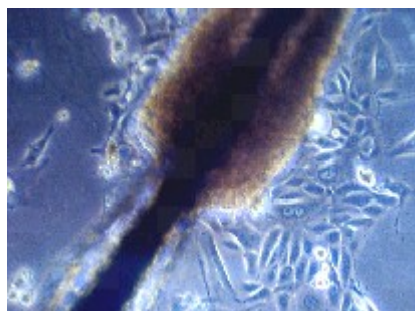


Figure 11: Outgrowth of keratinocytes from the outer root sheath.



Figure 12: Typical aggregative growth pattern of dermal papilla cells

As a source of keratinocytes and similar to epidermal cultures, ORS cells can also be used to treat recurrent leg ulcers [115]. Future models in this field may elucidate important aspects of hair graying and even may have therapeutic implications, since melanocytes of graying and white hair follicles can be induced to produce melanin in vitro [116].

DPC have a close contact to the keratin-producing hair matrix cells, being very important to study epithelial-mesenchymal interactions in the hair follicle. Complex culture-models have been developed [117]. They can be dissected from the hair follicle and grow in medium identical to that of dermal fibroblasts [118]. They have gained much interest due to their ability to induce hair growth after reimplantation in the skin [119,120].

When DPC were compared to dermal fibroblasts, many differences were found. They express muscle antigens [121], have a weaker capacity to contract collagen lattices and have an aggregative growth pattern [122] (Figure 12). Recently a difference in expression and activation of matrix metalloproteinases was also shown [123], reinforcing the concept that they are a specialized subpopulation of mesenchymal cells.

As a target of androgen effect in the hair follicle [113], [124], DPC were used to investigate androgenetic

alopecia [125]. Well established treatments for this condition were confirmed in vitro using DPC. Minoxidil has a proliferative and anti-apoptotic effect [126] and finasteride increases the expression of growth factors on DPC in vitro [127]

Other Cutaneous Cell Cultures

Other cutaneous cells of less importance have also been cultivated in vitro. Merkel cell is a highly specialized cell of neural crest origin that primarily acts as slowly adapting mechanoreceptors. Their in vitro cultivation and characterization has been hampered by their paucity, lack of information about specific mitogens and their state of differentiation. Nevertheless, they have recently been cultivated from animals and humans from both glabrous and hairy skin [128]. Merkel cells of sinus hair follicles of rats have been grown as a monolayer that exhibited flat round morphology with extended lamellae on their fringes in serum free medium, whereas they acquire a fibroblast-like morphology and survive longer in the presence of serum [129]. Establishment of a cell line from Merkel cell carcinoma in humans was possible. In general, cells grow as loosely arranged floating small aggregates on RPMI medium on irradiated fibroblast feeder layer. They also retain their in vivo properties, namely expression of cytokeratin 18, neuron-specific enolase, neurofilaments and synaptophysin. The expression of the distinct cytokeratin 20 has greatly facilitated their identification in vitro and in vivo and also improved detection of micro metastasis of Merkel cell carcinoma [130].

Epithelial cells were also cultivated from the secretory coil and collecting ducts of the sweat glands in a medium supplemented with fetal calf serum, insulin, transferrin, epidermal growth factor and hydrocortisone. Initially, cells of the secretory coil were elongated while of the collecting ducts were polyhedral [131]. Fibroblast overgrowth was markedly diminished by dispase separation and use of serum free medium similar to keratinocytes. Cells expressed carcinoembryonic antigen, K 8.37 and K 8.13 [132]. Ultrastructural studies revealed domes indicative of transepithelial active ion transport in cells of reabsorptive duct [131]. Sweat gland myoepithelial cells in culture expressed, in addition to unique cytokeratins, α smooth muscle actin (sma) [133]. Cells isolated from patients with cystic fibrosis were morphologically indistinguishable from normal except for high transepithelial resistance and increased amiloride sensitivity, i.e., functionally abnormal [134]. Skin equivalent studies revealed that keratinocytes of young donor could differentiate toward sweat ducts in vitro in the presence of serum, EGF and dermal fibroblasts [135].

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