Aus der Hautklinik
der Heinrich-Heine-Universität Düsseldorf

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Characterization of growth and differentiation of a spontaneously immortalized keratinocyte cell line (HaCaT) in a defined, serum-free culture system

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
Der Medizinischen Fakultät der Heinrich-Heine-Universität zu Düsseldorf

vorgelegt von

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2000

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

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Die in dieser Dissertation vorgestellten Experimente wurden im Rahmen einer Zusammenarbeit der Institute für Dermatologie der Heinrich-Heine-Universität Düsseldorf und der Mayo-Klinik in Rochester, Minnesota (USA), im Zeitraum von Oktober 1995 bis September 1996 in den USA durchgeführt.
Die Arbeit wurde durch Herrn Dr. Mark Pittelkow, Professor für Dermatologie an der Mayo-Klinik, mitbetreut. Aus diesem Grunde wurde die Dissertation in englischer Sprache verfaßt.
Finanziell wurde dieses Forschungsprojekt durch ein Stipendium der Heinrich-Hertz-Stiftung des Landesministeriums für Forschung und Wissenschaft Nordrhein-Westfalen zugunsten von Martin Rekus unterstützt.
An dieser Stelle bedanke ich mich für diese finanzielle Unterstützung. Mein besonderer Dank gilt meinem Betreuer in den USA Herrn Prof. Mark Pittelkow für die anregenden und kritischen Gespräche, meinem Doktorvater Herrn Prof. Thomas Ruzicka, welcher diese besondere Kooperation ermöglichte. Ebenfalls den medizinisch technischen Assistentinnen des dermatologischen Labors Frau Karen Squillace und Frau Nelle Swanson für ihre kontinuierliche Hilfsbereitschaft und die fröhliche Laboratmosphäre.

Für meine Mutter
Für meinen Vater, † 18.4.1999

1 INTRODUCTION

1.1 The skin

The smooth surface of the human skin suggests simplicity, but it embodies one of the most complex and metabolically active organs, which, as an external shield of the body, is designed to interact and cope with the environment. The skin is the largest organ system of the body which consists of an epithelial layer (epidermis), a connective tissue layer (dermis) and an adipose layer (hypodermis). The skin is an integrated tissue that performs its functions by means of a highly sophisticated communication network between the different compartments and cell types (e.g. epidermis, dermis, blood vessels, sweat and sebaceous glands, immunocompetent cells, invading leucocytes). As a physical barrier it prevents water loss and withstands mechanical, chemical and microbial assaults. It also functions to regulate temperature, produce hormones and vitamins, such as Vitamin D, and responds to environmental factors including ultraviolet radiation.

1.1.1 The keratinocyte and the epidermis

The outer layer, the epidermis, is a squamous epithelium that forms the protective covering of the skin. It is a perpetually renewing tissue with the main cell type, keratinocytes, superpositioned and organized into four histologically distinct cellular layers [1-4].

Keratinocytes arise from stem cells in the basal layer [1] and progressively mature through the spinous [2] and granular layers [3] until they reach the corneal layer [4] and are finally sloughed into the environment (desquamation). Thus, in the normal epidermis, there is a tightly controlled balance between proliferation and desquamation that results in a complete renewal or turnover approximately every 28 days. On its journey to the skin surface, the keratinocyte undergoes a complex program of terminal differentiation, also called keratinization. This cellular program is accompanied by highly regulated and marked changes in gene expression, cellular architecture and enzyme activity. Early steps in terminal differentiation of cells arising from the basal keratinocyte result in a permanent loss of growth potential, or clonogenicity known as commitment and the subsequent sequential expression of

differentiation markers. Among the major products of epidermal keratinization are the differentiated keratin intermediate filaments which are essential for maintaining structural integrity and resilience of the epidermis (reviewed in Fuchs, 1990, 1993; Fuchs and Byrne, 1994).

1.1.2 Epidermal growth and differentiation

The process of epidermal growth and differentiation can be subdivided into at least 4 functional compartments. As cells move through the distinct epidermal layers, they are converted from proliferative, undifferentiated keratinocytes into highly differentiated non-dividing or post-mitotic cells.

In large part, only the basal cell population of keratinocytes has the capacity to undergo DNA synthesis and mitosis. Basal cells are distinguished by an intracellular cytoskeleton composed of a relatively dispersed, but extensive, network of keratin filaments. These filaments are composed of a 1 : 1 ratio of two distinct keratin (K) proteins: K 5 and K 14. Similar to the suprabasal spinous cells, basal keratinocytes are connected by desmosomes, which are calcium-dependent adhesion proteins that form membrane junctions and interconnect the epithelial cells into a three-dimensional lattice.

The four to eight cellular layers of suprabasal spinous cells are postmitotic, but metabolically active. These cells primarily synthesize de novo two keratins, K 1 and K 10, that form a keratin pair cytoskeletal filaments and aggregate into thin tonofilament bundles. Additionally, spinous cells produce glutamine and lysine-rich envelope proteins, such as involucrin, which are deposited on the inner surface of the plasma membrane of each cell. During the differentiation process membrane coating granules (lamellar bodies or Odland bodies) are also formed within the cytoplasm, which subsequently fuse with the plasma membrane and release newly synthesized lipids into the intercellular spaces of granular and stratum corneum cells.

Upon reaching the granular layer of the epidermis, spinous cells stop generating keratin and envelope proteins, and induce expression of new proteins, including production of filaggrin, a histidine-rich, basic protein which appears to be involved in the bundling of tonofilaments into larger, macrofibrillar cables. The process of filament packing is thought to enable the keratin filaments to resist proteolysis and nuclear and cytoplasmic organelle destruction. Loricrin, a major component of the cornified envelope is also synthesized at this late stage. As each differentiating cell

becomes permeable, a calcium influx activates epidermal transglutaminase, which then catalyzes the formation of γ-glutamyl-ε-lysine isopeptide bonds, thereby biochemically cross-linking the envelope proteins into a cage-like network. As other lytic enzymes are released, all vestiges of metabolic activity terminate, and the resulting flattened squames become cellular skeletons, filled with macrofibrils of keratin filaments (reviewed in Eckert, 1989; Fuchs, 1990, 1993; Fuchs and Byrne, 1994).

The stratum corneum is composed of terminally differentiated keratinocytes sealed together by lipids as an impermeable, insoluble and highly protective fortress, which keeps microorganisms out and retains bodily fluids within. This 2 compartment system of cellular proteins held together by lipids is called the "bricks and mortar"– construct (reviewed in Nemes and Steinert, 1999).

Major research has focused on elucidating the cellular and molecular processes of proliferation and differentiation at both the extracellular and intracellular levels. The mechanisms by which these processes are regulated are largely unknown. However, several growth factors and cytokines, acting in an autocrine or paracrine manner have been implicated in the control of these critical biological processes and will be described herein.

1.2 *In vitro* propagation of keratinocytes

To further elucidate growth and differentiation characteristics of epidermis and epidermal keratinocytes *in vivo*, several *in vitro* culture systems have been developed and utilized. However, in many of these culture models, serum is routinely used. Serum is an undefined complex mixture of proteins, nutrients and solutes, and includes both growth promoting and inhibitory substances. As such, the presence of serum has limited the utility of many culture models to study various cytokine and growth factor effects because its complexity and variability in response has the potential to confound the interpretation of data.

A number of laboratories have contributed to methods aimed at optimizing terminal differentiation *in vitro*. From these studies, new clues to the extracellular control of keratinization have been identified. In these studies, an *in vitro* culture system has been developed to optimize growth and induction of terminal differentiation under defined conditions in which serum has been eliminated.

1.2.1 Serum-free cell culture of keratinocytes

The culture system used in the present studies allows the growth of keratinocytes to high density in a medium that does not require addition of undefined molecules and supplements. The cells can be maintained in an undifferentiated state from which they can be induced to differentiate by several techniques. These culture conditions allow the study of these cells in the absence of feeder layers, serum, exogenously added attachment factors and drugs which are used in other culture systems for the growth of these cells (Shipley and Pittelkow, 1987).

Serum not only contains a variety of proteins and other macromolecules, but also numerous low-molecular weight substances, including amino acids, vitamins, and trace elements. The development and use of MCDB 153 basal medium was based on experiments showing that specific essential substances are required for optimum keratinocyte growth *in vitro,* and the factors in this medium can be exactly quantified. MCDB 153 basal medium favors the selective multiplication of normal epidermal proliferative keratinocytes while suppressing the growth of fibroblasts and other cell types (Wille *et al.*, 1984). MCDB 153 basal medium supplemented with epidermal growth factor (EGF), insulin, hydrocortisone, phosphoethanolamine, ethanolamine and calcium chloride is known as defined medium. Clonal growth of keratinocytes in this medium can be obtained at initial plating densities of less than 200 cells/25 cm^2 (Boyce and Ham, 1983a, 1983b).

How these and other constituents of defined MCDB 153 medium propagate growth will now be described. Two hormones (insulin and hydrocortisone) and a polypeptide growth factor (EGF) regulate the growth of keratinocytes in the defined medium. Of these, EGF and insulin are required for clonal growth. Colony size, in the absence of hydrocortisone, is reduced but significant growth still occurs. Insulin must be added at supraphysiologic concentrations to elicit a growth response. The best conditions to support growth without differentiation have been achieved by using high concentrations of EGF (10 ng/ml) with low concentrations of calcium (0.1 mM) (Shipley and Pittelkow, 1987).

When bovine pituitary extract (BPE) is added to defined medium, it is called complete medium (CM). Although the presence of BPE seems to be beneficial for long term growth, it has been demonstrated that these cells will proliferate at clonal density (25 cells/cm^2) and at high density when the medium is supplemented with only defined

growth factors and hormones, including EGF, insulin and hydrocortisone, as used in defined medium (Pittelkow *et al.*, 1993).

Serum-free keratinocyte cell culture serves as a representative model to delineate the function of numerous biological and toxicologic agents. MCDB 153 basal medium offers several advantages over media requiring serum, feeder layers or toxic chemicals (e.g. cholera toxin) for epidermal cell growth. The constituents of the medium are completely defined and optimized for clonal growth of human keratinocytes.

Deletion or addition of certain chemicals can be performed easily and in a well-defined fashion. In turn, the response of keratinocytes can be readily monitored.

1.2.2 Proliferation and differentiation of normal human keratinocytes (NHK) in serum-free culture

Human keratinocyte proliferation, growth arrest, commitment, and terminal differentiation are highly coordinated and intricate biologic processes for which specific cellular control mechanisms have so far been elucidated in a serum-free culture system and now will be described.

Epidermal keratinocytes exhibit autoregulatory growth control mechanisms that coordinate proliferation and differentiation in serum-free and defined culture conditions (Shipley *et al.*, 1986; Cook *et al.*, 1991a). They require exogenous growth factors to initiate cell growth but once keratinocyte colonies are established, exogenous growth factors are no longer required, and cells exhibit autonomous proliferation (Pittelkow *et al.*, 1993). When keratinocytes become confluent, they growth-arrest, lose their clonogenic potential (become committed), and express the early differentiation transcripts, keratin 1 and 10, which are linked to terminal differentiation (Poumay and Pittelkow, 1995).

1.2.3 Biologic mechanisms for the regulation of normal human keratinocyte proliferation and differentiation: the role of growth factors, cell density and calcium medium concentration

The regulation of keratinocyte proliferation is mediated by peptide growth factors or growth regulators that are produced locally and exert their biological effects within the same tissue (Pittelkow, 1992). In this respect, several modes of growth factor/regulator activity have been identified, characterized and termed autocrine and paracrine activity among others. They attempt to explain biological mechanisms by which cells within tissues establish communication and control their proliferation and differentiation (Pittelkow *et al.*, 1991).

Several families of growth factors, including the epidermal growth factor (EGF) family and the fibroblast growth factor (FGF) family are potent mitogens for keratinocyte proliferation. Several of the EGF-related ligands, including transforming growth factor α (TGF-α), amphiregulin (AR) and heparin-binding EGF (HB-EGF) are expressed by cultured keratinocytes (Coffey *et al.*, 1987; Cook *et al.*, 1991b; Hashimoto *et al.*, 1994). These factors exert auto-stimulatory or auto-regulatory activities in keratinocytes implicating them as autocrine ligands for keratinocytes growth (Pittelkow *et al.*, 1993).

When keratinocytes grow autonomously in cell culture at high cell densities in the absence of exogenous growth factors, they express TGF-α mRNA and protein and TGF-α is autoinduced by TGF-α or EGF. The presence of TGF-α, among other growth factors, and a cognate receptor, the human EGF receptor = HER1, may explain in part why keratinocytes grow without exogenously added growth factors. This phenomenon has been designated “autonomous growth” (Pittelkow *et al.*, 1993). In contrast, when keratinocytes are growing at clonal densities, they require exogenous addition of EGF or EGF-related ligands to establish colonies. Once colonies form, however, they can grow autonomously, without exogenous growth factors.

Recently this laboratory reported that inhibition of EGF-receptor tyrosine kinase activity suppresses keratinocyte growth and induces terminal differentiation in exponentially proliferating cells. This fact supports the hypothesis that autocrine proliferation of keratinocytes is mediated in large part, if not solely, by endogenously produced EGF-related ligands that bind and activate the epidermal growth factor receptor (EGF-R) (Peus *et al.*, 1997). In summary, keratinocytes proliferate autonomously once clonal colonies are established, and control their proliferation

through autocrine and paracrine mechanisms involving endogenously expressed growth factors such as TGF-α and the EGF-R.

When keratinocytes in culture reach confluency they establish intimate cell-cell contacts, probably cell-matrix and other cellular interactions and initiate the program of terminal differentiation. Poumay and Pittelkow (1995) have shown that confluent cell density primarily controls keratinocyte commitment to terminal differentiation and differentiated keratin gene expression. Addition of EGF to confluent cultures was able to suppress keratin 1 and 10 expression, as early markers of terminal differentiation. However, cellular commitment to differentiation, as assessed by clonal growth assay, is not reversable. In summary, selected factors such as EGF are capable of modulating differentiated keratin gene expression but cell density is the primary signal responsible for keratinocyte commitment to differentiate.

Other than the influence of growth factors, which modulate keratinocyte growth and keratin gene expression, calcium plays an important role in these processes. In the epidermis, a calcium gradient is present within the stratified layers and is thought to promote differentiation, as cells move through the program of terminal differentiation, e.g., by inducing expression and activation of enzymes such as transglutaminase (Peterson *et al.*, 1983). However, in epidermis it is unlikely that calcium is the primary trigger for differentiation. Rather, it facilitates the molecular events in the differentiation process. In cell culture, however, calcium acts as a mitogen over a wide range of concentrations. Therefore, the role of calcium in regulating growth is permissive; i.e., if no other condition is limiting, calcium is mitogenic below 0.3 mM, but neither stimulates or inhibits growth above 0.3 mM. It is also known that the role of calcium in promoting differentiation is dependent on other factors that regulate growth such as EGF (Wille *et al.*, 1984).
Keratinocytes can be induced to express the early differentiation transcripts keratin 1 and 10 upon elevation of the calcium concentration to 1.5 mM at subconfluent densities but commitment to differentiation, as assessed by clonal growth capacities, cannot be observed. At cell confluence high medium calcium concentration does not significantly alter K 1 and K 10 expression. Therefore the calcium concentration does not primarily control commitment to terminal differentiation but is sufficient to modulate keratin 1 and 10 expression. In summary, rapidly growing human

keratinocytes maintain a high degree of clonogenic potential at subconfluent cell density regardless of high (1.5mM) or low (0.1mM) medium concentrations of calcium. Even at low (0.1mM) calcium concentration, cell confluence serves as the crucial sign to initiate the program of terminal differentiation, even though the calcium concentration seems to modulate differentiated keratin expression.

1.2.4 Correlation of cell culture growth and differentiation states to *in vivo* conditions and disease states of the epidermis

In vivo the stratified epidermis undergoes an orderly progression from proliferative, relatively undifferentiated cells in the basal layer to non-proliferative cells in the suprabasilar layers. The cells sequentially express a number of differentiation-specific proteins that ultimately result in formation of the stratum corneum. Using cell culture of human keratinocytes, several properties of growth and differentiation states *in vivo* can be mimicked. When cells are subconfluent, they exhibit a basal cell-like monolayer and therefore can be compared to basal cells in the epidermis which eventually become post-mitotic. When cell confluence is attained, cultured keratinocytes are similar to cells entering the suprabasal layer of the epidermis which become post-mitotic (irreversibly growth arrested) and committed to terminal differentiation. As a dynamic tissue, the epidermis renews itself approximately every 28 days. This transition state between the basal and suprabasal layers occurs irreversibly and regularly in the normal epidermis. The mechanisms involved in this transition can be studied in detail and applied to disease states of the epidermis.

For instance, when the epidermis is disrupted by injury, cellular events are activated to initiate reepithelization. This *in vivo* state can be mimicked by subconfluent culture. Under these similar conditions *in vitro* and *in vivo*, expression of K 1 and K 10 is suppressed. In addition, epidermal hyperproliferation, such as in psoriasis - with marked overexpression of EGF-related growth factors such as transforming growth factor-α, amphiregulin and heparin-binding EGF, and with delayed expression of K 1 and K 10 - may be represented by confluent cultures to which exogenous EGF has been added and in which K 1 and K 10 expression has been suppressed (Poumay and Pittelkow, 1995).

1.3 Cellular immortality

Normal human somatic cells are restricted to a limited life span *in vivo* and *in vitro*. After a limited number of cell divisions human somatic cells undergo an irreversible growth arrest through the process called cellular senescence, or replicative senescence. Cellular immortality is defined by the capacity of cells for unlimited proliferation under culture conditions when they escape the normally limited life span of adult somatic cells. Until now immortalization of human cells can only be reproducibly induced by certain DNA viruses such as simian virus 40, adenovirus types 5 and 12, and human papilloma virus types 16, 18, 33 and immortalization by chemical and physical agents is rare (reviewed in Rhim *et al.*, 1990), as is spontaneous immortalization. While it is a matter of discussion whether all carcinoma cells are necessarily immortal and whether immortality is an obligatory step for *in vivo* carcinogenesis, the loss of cellular senescence is regarded an essential, rate-limiting early clonal event in the transformation process *in vitro* (Fusenig and Boukamp, 1998).

1.3.1 Spontaneously immortalized keratinocytes

Spontaneous immortalization has been observed in both, rodent and human cells. In rodent cells it occurs routinely and has been described for mouse epidermal cells (Yuspa *et al.*, 1980), keratinocytes and other epithelial cells of the rat (Heimann and Rice, 1983; Phillips and Rice, 1983). In contrast to rodent cells, spontaneous immortalization of human epidermal cells in culture is a rare event, with only three cases so far reported (Baden *et al.*, 1987; Boukamp *et al.*, 1988; Rice *et al.*, 1993). In the original human tissue or upon cultivation of these rare human immortalized cell lines, only relatively low levels of DNA damage could be detected as compared to immortalized rodent cells. It has been concluded that human cells control cell proliferation and cell cycle events more rigorously than do rodent cells and thus posses greater karyotypic stability with less DNA mutational events.

Of the three human spontaneously immortalized keratinocyte cell lines reported, SIK immortalized keratinocytes exhibit persistently elevated cell cycle proteins cyclin A, cyclin B, and p34 cdc2 which may play a key role in immortalization of these cells. SIK cells displayed chromosomal aberrations involving chromosomes 6, 7 and 8 and with prolonged propagation developed additional cytogenetic damage. Regulation of major signal transduction pathways of SIK cells was reported not to be deregulated

since the effects of EGF, TGF-β and TPA on growth were indistinguishable from normal in serum-free culture (Rice *et al.*, 1993).

In NM-1 spontaneously immortalized keratinocytes, growth and differentiation characteristics were similar to normal keratinocytes. However, NM-1 cells showed trisomy of chromosome 8 (Baden *et al.*, 1987). In both NM-1 and SIK spontaneously immortalized keratinocytes, cytogenetic damage could be detected which indicates that certain senescence genes may be lost or mutated such that immortalization of cells results. Only hinted, growth and differentiation characteristics and major signal transduction pathway elucidation of these cells has been examined. In general these properties seem to be largely maintained similar to normal cells.

The spontaneously immortalized keratinocyte cell line HaCaT has been most widely used, since it has been available to many investigators.

1.4 HaCaT spontaneously immortalized keratinocytes

HaCaT is a spontaneously immortalized human keratinocyte cell line which developed through long-term culture of normal human adult skin keratinocytes at reduced calcium concentration and elevated temperature (Boukamp *et al.*, 1988). It was designated HaCaT (Ha = human adult, Ca = calcium, T = temperature) to indicate its origin and the initial culture conditions. HaCaT cells possess mutations in both alleles of the *p53* gene (Lehmann *et al.*, 1993), which are typical of UV-radiation induced mutations (Brash *et al.*, 1991; Ziegler *et al.*, 1993, 1994). *p53* is a tumor suppressor gene involved in DNA-repair which is considered to serve as “guardian of the genome” and is induced in response to DNA damage in human skin (Ziegler *et al.*, 1994). The current hypothesis for HaCaT immortalization involves a concerted action of *p53* mutations and elevated culture temperature as the major inducers of accumulated genetic alterations (reviewed in Fusenig and Boukamp, 1998). It is hypothesized that HaCaT cells have at least partly lost their protection mechanism against DNA-damage through mutation of the *p53* gene *in vivo,* and these cells were susceptible to accumulating cytogenetic changes in culture in response to elevated culture temperature. Another mechanism may involve increased activity of the enzyme telomerase that was found to be associated with immortalization of HaCaT cells (Harle-Bachor and Boukamp, 1996). Telomerase is a specialized cellular enzyme complex with reverse transcriptase activity that maintains stable telomere length. In normal cells, continuous shortening of the telomeres occurs as cells approach cellular

senescence. In contrast, HaCaT cells exhibit significantly increased telomerase activity resulting in largely maintained telomere length. These observations indicate that telomerase may play an important role in the immortalization process of these cells.
Distinct cytogenic changes of HaCaT cells generated by mechanisms described above, are thought to be essential for the early disturbances in growth control resulting in the prolonged and unlimited lifespan of HaCaT. Three distinct chromosomal translocations leading to the loss of one copy of chromosome arms 3p, 4p, and 9p as well as a gain of 9q due to formation of an isochromosome 9q could initially be detected (Boukamp *et al.*, 1988). Further studies indicate that the loss of the short arm of chromosome 3p in particular may account for the loss of senescence genes causing immortalization of HaCaT cells (unpublished data from Dr. Fusenig´s research group).

During continued culture of HaCaT cells additional chromosomal changes could be discovered, such a polyploidization (Boukamp *et al.*, 1988). However, although propagated for more than 300 population doublings and more than 6 years, HaCaT cells maintained a constant balance of genetic material and remained non-tumorigenic in contrast to virally transformed keratinocytes (Boukamp *et al.*, 1997).

1.4.1 HaCaT growth and differentiation under *in vivo* and *in vitro* conditions

In contrast to many virally transformed keratinocyte cell lines, HaCaT is capable of expressing differentiation-specific gene products including keratins 1 and 10 and differentiation markers such as Involucrin and Filaggrin (Boukamp *et al.*, 1988).
In contrast to normal human keratinocytes, subconfluent HaCaT grown in serum-containing medium express keratin 1 and 10 (Fusenig *et al.*, 1995). Moreover a much broader spectrum of keratins than that usually seen in primary keratinocyte cultures is expressed by HaCaT including keratins associated with simple epithelia, e.g. K 7, K 8, K 18, and K 19. When approaching very high cell densities the expression of the suprabasal keratins increased while the constitutively synthesized simple keratins, mainly K 7, K 8 and K 19 decreased. However in transplants of HaCaT cells K 1 appeared prematurely already in basal cells while K 10 was expressed fairly normally in the suprabasal position (Boukamp *et al.*, 1991).
Similar to normal keratinocytes, HaCaT can be induced to express keratin 1 and 10 by environmental stimuli such as depletion of retinoids while simple type keratins are

suppressed (Breitkreutz *et al.*, 1993). Although immortalized, HaCaT do not display tumorigenic or invasive properties *in vivo* (Boukamp *et al.*, 1985, 1988). Following subcutaneous injection HaCaT are non-tumorigenic and furthermore, are growth restricted, irrespective of the differentiation potential. They form epithelial cysts in which cell proliferation stops completely and irreversibly within a few weeks (Boukamp *et al.*, 1988). When transplanted onto nude mice HaCaT form an orderly structured and differentiated epidermal tissue (Breitkreutz *et al.*, 1998). In organotypic co-cultures on top of collagen gels containing human dermal fibroblasts, HaCaT cells form a remarkably well structured and differentiated squamous epithelium, although orthokeratotic keratinization was not achieved (Schoop *et al.*, 1999).

1.4.2 HaCaT growth and differentiation characteristics in serum-free culture

The mechanisms involved in control of growth and differentiation of human keratinocytes in serum-free medium have extensively been studied and are described under **1.4.1**. HaCaT cells have been widely used but never adapted to serum-free culture and further characterized. In this study HaCaT cells have been established in serum-free MCDB 153 basal medium with supplements. Based on this medium formulation, HaCaT cells can be directly compared with normal human keratinocytes (NHK).

Proliferative potential, cell cycle characteristics, colony-forming potential and keratin 1 and 10 expression as among the earliest representative markers of terminal differentiation that are expressed by basal and suprabasal keratinocytes as well as involucrin were examined. The results of these investigations demonstrate that several of NHK growth and differentiation properties are preserved by HaCaT. However, proliferation and differentiation of HaCaT cells are more stringently dependent on exogenous growth factors (GF), and GF depletion is associated with induction of apoptosis.

1.5 Skin multistep carcinogenesis

The development of cancer has been extensively studied. A large body of evidence suggests that the pathogenic process leading from a normal to a malignant cell can be characterized by step-wise events with accumulation of genetic and epigenetic changes. This multistep process is well defined for colorectal cancer where at least 7 genetic alterations render a cell malignant (Fearon and Vogelstein, 1990).

The pathogenesis of squamous cancer has been subsequently elucidated using mouse skin as an experimental model system which has helped to dissect the biological and genetic changes that contribute to specific stages of skin carcinogenesis: initiation, exogenous promotion, premalignant progression, and malignant conversion (Yuspa and Poirer, 1988; reviewed in Yuspa *et al.*, 1994, 1996; Yuspa, 1998). In this model system, initiation of mouse skin keratinocytes can be achieved by treatment with the polycyclic aromatic hydrocarbon dimethylbenzanthracene (DMBA) which results in specific gene mutation of the *Ha*-ras gene and is associated with differentiation defects of mouse keratinocytes. The resulting initiated cells are unrecognizable in the context of intact epidermis.

Exogenous tumor promotion e.g. with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) causes the selective clonal expansion of initiated mouse keratinocytes which resist the induction of differentiation by TPA. This imparts a growth advantage to initiated cells in contrast to normal mouse skin keratinocytes. The process of tumor promotion is reversible, epigenetic in nature and produces squamous cell papillomas.

Premalignant progression in this model system is mostly a spontaneous event and is associated with generalized genomic instability, namely chromosomal imbalance and aneuploidy (trisomy of chromosomes 6 and 7).

Malignant conversion is a final stage in carcinogenesis and has been shown to be associated with mutations in the *p53* gene and upregulation of the AP-1 family of transcription factors (reviewed in Yuspa *et al.*, 1996; Yuspa, 1998).

As demonstrated in this model system, both genetic and epigenetic changes can be attributed to specific stages of carcinogenesis. The genetic and biological pathways which determine each specific stage are currently being elucidated in the context of this mouse skin carcinogenesis system.

Because there are major discrepancies between sensitivity to carcinogenic agents and the incidence for development of tumors between rodent and human cells (compare

1.6.1 for the incidence of spontaneous immortalization) (Soballe *et al.*, 1996), it is a matter of debate to what extent data from rodent models can be extrapolated to the human situation. Therefore it is of major importance to establish carcinogenesis model systems using human cells.

1.5.1 HaCaT keratinocytes and *in vitro* multistep carcinogenesis

An experimental *in vitro* model system using spontaneously immortalized HaCaT keratinocytes for studying neoplastic transformation has provided insight into the mechanisms of human epidermal multistep carcinogenesis. (Boukamp *et al.*, 1988, 1990, 1994, 1995, 1997; reviewed in Fusenig *et al.*, 1995; Fusenig and Boukamp, 1998). Immortalization of a cell is considered an early and essential step in the transformation process *in vitro* (Fusenig and Boukamp, 1998). Immortalized cells have morphological alterations, chromosome abnormalities, unlimited growth potential, all properties not found in any cell of the human body except for cancer cells. Once immortalized, human cells can be easily transformed by oncogenic agents (Rhim *et al.*, 1990).

When HaCaT cells were transfected with the rasH oncogene two tumorigenic clones (HaCaT-ras) developed which represented two different phenotypes: (a) clones that formed slowly growing epidermal cysts (benign tumors) and (b) clones that gave rise to progressively growing and locally invasive squamous cell carcinomas (Boukamp *et al.*, 1990). Since the level of expressed mutant ras protein did not differ between the two tumorigenic cell clones it was suggested that the malignant phenotype resulted from additional genetic events. Further studies revealed a correlation between malignancy and loss of copies of chromosome no. 15 which was associated with a decrease in thrombospondin-1 (TSP-1) expression. At cellular level (phenotypically) both HaCaT-ras clones lost their dependence for exogenous growth factors. However, when transplanted onto nude mice both cell clones exhibited a differentiation potential similar to non-transfected HaCaT cells (Boukamp *et al.*, 1990). The only difference detectable at cellular level was that the malignant HaCaT-ras clones showed a decreased sensitivity to transforming growth factor β-mediated growth inhibition *in vitro* (Game *et al.*, 1992).

In addition, tumorigenic conversion of HaCaT cells was achieved by exposing HaCaT cells to culture stress such as growth over many passages in serum-free medium (Hill *et al.*, 1991) and propagation of HaCaT cells at elevated temperature (40 ^{0}C) (Fusenig

and Boukamp, 1998). Heat-induced malignant conversion was associated with gain of chromosomal material on 11q which could be correlated with overexpression of cyclin D (Boukamp *et al.*, in preparation).

Furthermore the microenvironment was shown to play a significant role in tumorigenic transformation of HaCaT cells. When platelet-derived growth factor B (PDGF-B) was overexpressed in HaCaT cells grafted to mice the cells became tumorigenic (Skobe and Fusenig, 1998). HaCaT cells do not express PDGF-receptor and it was concluded that PDGF expressed by HaCaT is sufficient to activate stromal cells to produce growth factors which in turn stimulate HaCaT proliferation.

Progression to more aggressive tumor phenotypes required the *in vivo* environment and was associated with loss of chromosome 2, 11, and 9q. At cellular level it was shown to be connected to increased growth potential *in vivo* mediated through autocrine regulation of growth and migration by expression of granulocyte-colony stimulating factor and granulocyte-macrophage colony-stimulating factor (G-/GM-CSF) (Fusenig and Boukamp, 1998).

With this *in vitro* carcinogenesis model system distinct steps associated with genetic and cellular alterations could be defined for human immortalized keratinocytes. In the present study effects of the tumor-promoting phorbolester, 12-O-tetradecanoylphorbol-13-acetate (TPA) on HaCaT growth and differentiation were studied to further elucidate cellular alterations associated with immortalized cells and tumor promotion in a defined, serum-free culture system.

1.6 The tumor-promoting phorbolester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and epidermal tumor promotion

Skin is a known target for phorbol ester-induced cellular alterations. Cell and tissue effects of TPA have been extensively examined in epidermis. TPA has been shown to markedly alter epidermal proliferation and differentiation (Argyris, 1981). Pronounced thickening of the epidermal cell layers occurs in skin following TPA treatment, and mitogenic activity of the geminative subpopulations of epidermis is dramatically increased in response to phorbol ester application. Epidermal thickening and hyperplasia are transient and the epidermis promptly returns to normal after discontinuation of phorbol ester administration. Despite increasing mitogenic activity of the germinative subpopulations of the epidermis TPA also is capable of initiating the program of terminal differentiation in some of these cells (Yuspa *et al.*, 1981).

TPA has long been known as a potent promoter of mouse skin carcinogenesis in the two-stage and multi-stage carcinogenesis systems (Boutwell, 1978; Yuspa *et al.*, 1981) which can elicit pleiotropic effects on a wide variety of cells *in vivo* and *in vitro* (Diamond *et al.*, 1980). TPA exerts its tumorigenic effect by preferentially expanding the population of skin epidermal cells which have undergone tumor initiation, thus providing growth advantage for these cells (Yuspa *et al.*, 1981, 1986; Yuspa and Poirer, 1988). The likelihood of subsequent malignant conversion and progression are consequently favored following the stepwise events of initiation and promotion. Exogenous promotion is a rate-limiting early event in carcinogenesis since initiated skin rarely develops tumors in the absence of exposure to exogenous promoters.

The mechanisms that regulate tumor promotion have been studied in mouse skin and show that exogenous skin tumor promoters such as TPA activate protein kinase C (PKC). This enzyme activation accelerates epidermal terminal differentiation. Initiated cells resist the induction of terminal differentiation by activators of PKC and the differential response to phorbol esters favors the growth of the initiated subpopulation. (Yuspa *et al.*, 1995)

1.6.1 Effects of TPA on normal human keratinocyte growth and differentiation in serum-free culture

Addition of TPA to rapidly growing normal human keratinocytes causes rapid cessation of growth and modulates the differentiation responses. The growth arrest induced by TPA is irreversible in that treated cells lose their colony-forming potential (Wille *et al.*, 1985). Phorbol ester-mediated loss of clonogenicity is not sufficient for suprabasal keratin expression (Poumay and Pittelkow, 1995). However, at cell confluence TPA suppresses directly the expression of K 1 and K 10 mRNA induced by cell confluence (data from the present study).

Further studies demonstrated that TPA markedly enhances accumulation of transforming growth factor α (TGF-α) mRNA and secretion of TGF-α protein through activation of protein kinase C (Pittelkow *et al.*, 1989). However TPA suppresses the expression of c-myc (Younus and Gilchrest, 1992) which is an important growth controlling transcription factor for human keratinocytes and was reported to down-regulate expression of the epidermal growth factor receptor (Yaar *et al.*, 1993).

1.6.2 Tumor promotion of initiated cells: TPA and growth and differentiation properties of HaCaT cells in serum-free culture

Little is known about the effect of TPA on HaCaT *in vitro* growth and differentiation. However, immortalized HaCaT cells are considered initiated and thus represent an early stage in the *in vitro* carcinogenesis context. Investigation of the effects of a tumor-promoting agent such as TPA on HaCaT growth and differentiation is therefore of major interest. In this study TPA-mediated effects on HaCaT growth and differentiation were determined and could be directly compared to these on normal human keratinocytes (NHK) which had previously been described in the same serum-free culture system (Wille *et al.*, 1985).

Proliferative potential, cell cycle characteristics, clonal capacities and keratin 1, 10 and involucrin mRNA expression were examined. The results of these studies demonstrate that HaCaT differ from NHK in that they lack the growth-inhibitory and commitogenic effect of TPA. In addition these studies show that TPA is capable of increasing clonogenic potential of HaCaT under culture conditions favoring loss of clonogenicity and such irreversible commitment to terminal differentiation.

1.7 c-jun/AP-1 family of transcription factors

In keratinocytes, as in other cell types, the expression of most genes is regulated at the transcriptional level by a class of proteins called transcription factors. Transcription factors are nuclear proteins that regulate transcription by mediating the final steps in the relay of information from the cell surface to the nucleus and to gene expression. In its simplest form, this is accomplished by binding of the transcription factor to a specific DNA sequence element located in the target gene, which has been designated as either silencer or enhancer, based on its potential to suppress or to facilitate gene activation, respectively.

Activator protein 1 (AP-1) is a dimeric transcription factor composed of proteins which are members of two different families of proto-oncogene products: jun (c-jun, junB, junD) and fos (Fra-1, Fra-2, c-fos, fosB) (Ransone and Verma, 1990), which bind to the DNA sequence 5`-GTGAGTCAG-3. These family members associate to form a variety of homo- and heterodimers all of which recognize the TPA-responsive element (TRE) (reviewed in Angel and Karin, 1991). AP-1 transcription factor mediates gene expression following a variety of extracellular stimuli, including the

action of growth factors, lymphokines, tumor necrosis factor α, phorbol-esters and stress-induced by u.v. irradiation. Jun and fos family members are also known to modulate events involved in proliferation, differentiation and transformation of keratinocytes (reviewed in Angel and Karin, 1991; Welter and Eckert, 1995).

1.7.1 Dominant-negative mutants/c-jun dominant negative mutant (TAM 67)

Several studies using knock-out mice, cell lines deficient in specific AP-1 components and cell lines in which non-functional transcription proteins are expressed have helped to understand the physiological function of AP-1 factors (reviewed in Karin *et al.*, 1997; Eckert *et al.*, 1997). Among others, a dominant-negative mutant of c-jun (TAM-67) has been developed, and has been shown to disrupt the normal activity of AP-1 when expressed in various cells (Brown *et al.*, 1993).

Transcription factors usually contain several functional domains, such as an DNA-binding domain, a transcription-regulating domain (transactivation domain) and also often a domain which controls their activity (i.e. via a phosphorylation site or a ligand binding site). The c-jun and c-fos proteins contain basic DNA-binding domains, leucine zipper motifs involved in dimerization, and transactivation domains. Generally those domains are functionally and physically separable and can be mutated independently. In the case of TAM-67, a dominant-negative mutant of c-jun has been developed that retains an intact, functional subset of the domains of the parent, wild-type c-jun protein but that lacks amino acids 3-122 of c-jun, the major transactivation domain, so as to be non-functional (Brown *et al.*, 1993). This mutant c-jun protein can dimerize and bind DNA, but it fails to activate the transcription of an AP-1 responsive gene. In addition, the mutant c-jun protein can suppress the functions of wild-type c-jun and c-fos proteins by inhibiting c-jun and c-fos-mediated DNA binding and transcriptional activation and therefore functions as a dominant-negative mutant of c-jun as defined by Herskowitz (1987).

1.8 The role of AP-1 family members for growth, differentiation and tumor progression of keratinocytes

AP-1 binding sites are involved in response to various signaling events that regulate cell proliferation and differentiation in epidermis (Briata *et al.*, 1993; Welter and Eckert, 1995; reviewed in Angel and Karin, 1991; Eckert *et al.*, 1997). AP-1 also regulates genes expressed during tumor promotion, and is known to play an important role in tumor progression and invasion (Dong *et al.*, 1994, 1995; Li *et al.*, 1996; Saez *et al.*, 1994).

The AP-1 family of transcription factors seems to play a central role in the regulation of gene expression in epidermis, because AP-1 binding sites are present in many keratinocyte genes. Several of these binding sites have been shown to mediate calcium- and phorbol-ester-dependent responses for several epidermal genes such as keratin 6, transglutaminase 1 and involucrin. Moreover AP-1 transcription factors are expressed in a differentiation-dependent manner in cultured keratinocytes and in *in vivo* epidermis (reviewed in Eckert *et al.*, 1997).

Regarding epidermal tumorigenesis, several factors of the AP-1 family including c-jun have been described to play crucial roles in tumor progression and invasion. Fos-/- mice fail to develop malignant progressing tumors of skin (Saez *et al.*, 1995). In malignant mouse keratinocytes constitutive high AP-1 activity was observed and stable expression of a dominant negative mutant of c-jun efficiently blocked tumor formation *in vivo* (Domann *et al.*, 1994). *In vitro*, a dominant negative mutant of c-jun was capable to inhibit AP-1 transactivation of malignant squamous carcinoma cells and further suppressed the tumorigenic phenotype of theses malignant mouse epidermal cells, when they were injected into athymic nude mice (Bowden *et al.*, 1994).

Other studies implicate a role of c-jun in tumor invasion. EGF-induced invasion of squamous cell carcinoma is inhibited by a dominant negative mutant of c-jun (Malliri *et al.*, 1998). In addition to blocking TPA-induced AP-1 transactivation and neoplastic transformation of mouse epidermal JB 6 cells (Dong *et al.*, 1994) TPA-induced Matrigel invasion is also blocked by a dominant negative mutant of c-jun (Dong *et al.*, 1997). Transformation of rat embryo cells induced by an activated ras gene in presence of the tumor promoter TPA was also demonstrated to be inhibited by this dominant-negative c-jun mutant, designated TAM 67 (Brown *et al.*, 1993).

1.8.1 Growth and differentiation characteristics of HaCaT cells transfected with dominant negative mutant of c-jun (TAM 67) in serum-free medium

The c-jun/AP-1 family of transcription factors is known to modulate events involved in proliferation, differentiation and transformation of keratinocytes. In this study, HaCaT cells were stably transfected with the dominant-negative c-jun cDNA, pmm 67, to examine effects of jun and AP-1 on HaCaT cell growth and differentiation. TAM 67 expression was confirmed by metabolic labeling. Proliferative potential and keratin 1 and 10 mRNA expression were examined by cell enumeration and Northern Blot analysis in pmm 67 transfectants and neo HaCaT control.

The results of these studies demonstrate that pmm 67 transfectants preserve most proliferation characteristics and differentiated keratin 1 and 10 expression patterns of HaCaT cells transfected with neo. However, TAM 67 protein is sufficient to inhibit TPA-mediated suppression of K 1 and K 10 gene expression at subconfluency.

1.9 Objective of these studies

The objective of these studies was to establish and propagate a widely used spontaneously immortalized keratinocyte cell line (HaCaT) in a defined, serum-free culture system. Characterization of its growth and differentiation properties and comparison of HaCaT responses in this culture system with these of normal human keratinocytes (NHK) that are also routinely cultured in this culture medium, was performed to yield new insights into altered biological mechanisms of immortalized cells. Proliferative potential, cell cycle characteristics, clonal capacities and keratin 1 and 10 expression as among the earliest representative markers of terminal differentiation that are expressed by basal and suprabasal keratinocytes, as well as involucrin, were examined.

HaCaT are immortalized and thus considered initiated in the *in vitro* carcinogenesis context. It was anticipated to use HaCaT cells in subsequent studies as an *in vitro* model to further delineate epidermal carcinogenesis in a serum-free culture system. In particular, the role of the tumor-promoting phorbol-ester TPA on HaCaT cell growth and differentiation was investigated to further elucidate cellular alterations associated with immortalized cells and tumor promotion. The methods mentioned above were used to determine TPA-responses in HaCaT cells.

AP-1/c-jun transcription factor family is known to modulate events involved in proliferation, differentiation and transformation of keratinocytes. In several studies a dominant negative mutant of c-jun was demonstrated to inhibit the transformed phenotype of keratinocytes. In this study stable transfection of immortalized HaCaT cells with a dominant-negative c-jun c-DNA, pmm 67, was performed to more specifically examine effects of jun and AP-1 on HaCaT cell growth and differentiated keratin 1 and 10 expression.

2 MATERIALS AND METHODS

2.1 Methods

2.1.1 Cell culture and kinetic studies

The keratinocyte cell line HaCaT was kindly provided by Dr. Fusenig, German Cancer Research Center (DKFZ), Heidelberg. Cell culture was performed in a 5% humidified CO_2 incubator (Forma Scientific) at 37°C. HaCaT cells from passages 60-80 were maintained in a replicative state with serum-free MCDB 153 medium supplemented with 25 µg/ml protein bovine pituitary extract (BPE), EGF (10 ng/ml), insulin (5 µg/ml), hydrocortisone ($5x10^{-7}$M), ethanolamine ($1x10^{-4}$M), and phosphoethanolamine ($1x10^{-4}$M) (Wille *et al.*, 1984). This medium is designated "complete medium" (CM). "Standard medium" (SM) is complete medium without growth factors and hormones, specifically EGF, insulin, and bovine pituitary extract, and is supplemented with the antibiotics penicillin at 50 units/ml and 50 µg/ml streptomycin. The standard medium calcium concentration was 0.1 mM and was designated "low" calcium medium. "High" calcium concentration was prepared by addition of $CaCl_2$ to a final concentration of 1.5 mM. In all studies, the cell culture medium was changed every 2 days. Cultures were washed repeatedly with Solution A when switched from complete to standard medium.

Serial passaging of HaCaT cells was performed by trypsinization (0.025% trypsin with 0.01% EDTA) of cultures for about 5 minutes followed by inactivation of this process with 2% dialyzed fetal bovine serum (dFBS) in Solution A. After centrifugation of HaCaT cells at 1000 rpm for 10 minutes at 4°C (Beckman J6M centrifuge, CA, USA) and resuspension in complete medium, HaCaT cells were replated into culture flasks.

For cell kinetic studies, HaCaT cultures were initiated at a cell density of $3x10^3/cm^2$ in complete medium. After 3 days of culture, cells were switched to standard medium and designated culture factors (EGF 10 ng/ml, insulin 5 µg/ml, or EGF + insulin) or TPA (10 ng/ml) were added as indicated in the figures. Cultures of all growth conditions were refed every 2 days. Cell enumeration was performed with a standard cell counting chamber and at least two flasks were counted per time point and growth

condition. The cell density was calculated by dividing the average total cell number per culture by the total surface area per culture flask.

2.1.2 Clonal assays

Clonal assays were performed as described by Wille *et al.* (1984) for normal human keratinocytes. After specific treatment in cell culture, HaCaT cells were trypsinized, washed and about 500 cells were inoculated into 60 mm dishes containing MCDB 153 medium with designated additives (10 ng/ml EGF, 5 µg/ml Insulin and Penicillin at 50 U/ml). After a growth period of 10 days in a 5% humidified CO_2 incubator at 37°C, the cells were fixed with formaldehyde, stained with crystal violet and representative plates were photographed.

2.1.3 Cell cycle analysis

Following trypsinization and washes in phosphate buffered saline (PBS), HaCaT cells were collected from different culture conditions and fixed in 2.5 x vol. 95 % Ethanol. At a later time point, fixed cells were washed and treated with DNAse-free RNAse A, 180 µg/ml for 30 min. After washing, Propidium Iodide (PI) was added at a final concentration of 20 µg/ml and the cells were incubated on ice for 20 min before fluorescence-activated cell sorter analysis (FACS), using a Becton-Dickinson FACScan flow cytometer (California, USA). The PI signal was detected through a 630 nm long-pass filter. The data was further analyzed by a Modfit cell cycle analysis program (DNA Modeling System, Maine, USA) to determine the distribution of the cell cycle phases.

2.1.4 DNA-Labeling with BrdU, Nuclear Extraction and Staining Procedure

HaCaT cells were either pulse (2 hour) or continuously (26 hour) labeled with bromodeoxyuridine (BrdU) at a concentration of 200 µmol/l in complete medium or standard medium or in complete medium containing TPA (10 ng/ml). Nuclei were prepared by trypsinizing, washing and resuspending cells in 100 µl PBS/1% BSA, following by lysis in 400 µl PBS containing 0.5 M EDTA (pH 7.2), RNAse 0.2 mg/ml and 0.5% NP-40 on ice for 15 minutes. Hydrolysis was initiated by addition of 100 µl 1 M HCl solution at room temperature for 30 minutes and was terminated by adding

300 µl 1 M Trisbase on ice for 5 minutes. BU-1 monoclonal Antibody was added at 1:50 dilution for 20 minutes followed by fluorescein-conjugated rabbit, anti-mouse Antibody at 1:1000 dilution. Antibody marked nuclear extracts were washed and resuspended in 500 µl 1% BSA. The fluorescent signal was detected using a Becton Dickinson FACScan and the data was further analyzed by PC-Lysys sofware (Becton Dickinson, CA, USA) to determine the percentage of BrdU labeled nuclei.

2.1.5 Northern blot procedure

2.1.5.1 Oligo-dt cellulose preparation

Oligo-dt was weighed out in specific amounts i.e. 0.025g per 75 cm^2 flask or 0.06g per 175 cm^2 flask, washed subsequently with 0.1 NaOH, distilled water and finally treated with water containing 0.1% DEPC by rocking for several hours. Following several washes with DEPC treated autoclaved water, the oligo-dt cellulose was resuspended in 2 ml High Salt buffer. In between all washes, the samples were centrifuged at 1200 rpm (500 x g) for 1 minute.

2.1.5.2 Poly(A) RNA isolation

Poly(A) RNA was isolated from HaCaT cultures by a modified method of Schwab *et al.* (1983), as previously described (Pittelkow *et al.*, 1989). HaCaT cells were lysed with lysis buffer containing 25 µg/ml Proteinase K, i.e.6 ml per 75 cm^2 flask or 12 ml per 175 cm^2 flask. The DNA of the samples was sheared by repeated passage through a 21 gauge needle. Following 30 minutes incubation at 37°C with 75 µg/ml Proteinase K, 2 ml of prewashed Oligo-dt solution were added to each sample and concentration of NaCl was adjusted to 0.5 M. The mixture was placed on a rocker for at least two hours before samples were centrifuged at 1000 rpm for 1 minute, washed and resuspended in High salt buffer. The RNA was then separated from the cellulose by passage through a DEPC treated Bio-Rad econo column (Bio-Rad, CA, USA). Low salt buffer containing 1 µl proteinase K per 10 ml was poured through the column before the RNA could be eluted by 55 °C warm No Salt buffer. The salt concentration was adjusted to 0.5 M NaCl and two times the volume of the sample of 95% Ethanol was added. The RNA was precipitated overnight at -20°C or for at least 30 minutes at

-70°C . The following day, samples were repeatedly spun down at 4°C in a microfuge for 15 minutes and the pellet was resuspended in TE buffer containing 1 µl proteinase K per 10 ml. Optical density of the samples at 260 and 280 nm wavelength was determined with a spectrophotometer DU-64 (Beckman, CA, USA) and the concentration of the RNA was calculated.

2.1.5.3 Gel electrophoresis and Northern blot analysis

The RNA samples were dried in a speed vacuum (Savant integrated Speedvac System ISS 100), resuspended in 20 µl sample buffer and 2 µl tracking dye was added. Samples and an RNA standard ladder (Gibco BRL, Life technologies, Inc., Maryland, USA) were separated on 1.2% agarose-formaldehyde gels at 100 Volts for 3-4 hours. The gel was photographed under high intensity UV light and samples were transferred to a Nytran nylon membrane overnight, utilizing a Turboblotting system (Schleicher & Schuell, New Hampshire, USA). Crosslinking of the RNA to the membrane was performed in a UV stratalinker 1800 (Stratagene, CA, USA) and after subsequent rinses with 2 x SSC the membrane was hybridized at 43°C with randomly primed DNA probes labeled with [α-^{32}P]dCTP (Coffey *et al.*, 1987). (Probes were prepared using a Gibco BRL random priming label kit (Life Technologies, MD, USA) according to directions. 50–100 ng of Qiaex extracted probe DNA were used). The probes included cDNAs specific for the human keratin 1 or 10 or involucrin (Roop *et al.*, 1988). 4 high stringency washes (0.1x SSC/0.1% SDS at 65°C) of 15 minutes duration were applied to the hybridized membranes before autoradiographic exposure. Each membrane was also hybridized with an identically labeled glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA probe to quantitate a constitutively expressed gene and assure equivalent loading and transfer of RNA. Between each subsequent hybridization, probes were stripped from the membranes by two washes with 0.1xSSC/0.5% SDS at 95°C for 10 min.

2.1.6 Nuclear fragmentation (apoptosis) and cell necrosis

HaCaT cells, grown on glass coverslips, were incubated with both PI, 2 µg/ml and 4,6 - Diamidin -2- phenylindoldihydrochloride (DAPI), 15 µg/ml at 37 °C for 20 minutes. The coverslips were transferred to a glass slide and nuclear morphology was assessed under an inverted Carl Zeiss Photofluoreszenz microscope 3 (Jena, Germany). DAPI-

stained nuclei were counted as apoptotic if at least 3 separate fragments of condensed chromatin were identified in cell nucleus (Oberhammer *et al.*, 1992). At least 200 cells were randomly counted, and nuclear fragmentation (apoptosis) was expressed as a percentage of total living cells that did not stain by PI (Kwo *et al.*, 1995).

2.1.7 Stable transfection with dominant-negative c-jun mutant and G 418 selection

The dominant negative c-jun mutant TAM 67 (Brown *et al.*, 1993) has been cloned into the pMEX MTX vector and is designated pmm 67 (Dong *et al.*, 1994). Stable transfection of HaCaT cells was performed with pmm 67 (pMexMTX-neoTAM 67) or neo plasmid (pMexMTX-neo) generously provided by Dr. Dong (Hormel Institute, Austin, MN, USA). pMexMTX-neo is a mammalian expression vector in which the gene of interest is under the transcriptional control of the mouse metallothionein promoter. 15 μg of plasmid DNA was transfected using 1.5 ml standard medium and 50 μl Lipofectin with HaCaT cells at 50% confluence in 60 mm dishes and incubated for 6 hrs at 37°C. Cells were selected in complete medium containing Geneticin (G418) at 100 μg/ml. Individual clones were isolated and expanded in the presence of G418 and analyzed for TAM 67 expression through metabolic labeling followed by immunoprecipitation.

2.1.8 Metabolic Labeling and Immunoprecipitation

pmm 67 transfectants and neo HaCaT control cultures (vector only transfectants) were labeled overnight (16 hours) with (^{35}S)methionine (150 μCi/ml) in DMEM medium supplemented with BPE, EGF (10 ng/ml), insulin (5 μg/ml), hydrocortisone (5x10 $^{-7}$ M), ethanolamine (1x10 $^{-4}$ M), and phosphoethanolamine (1x10 $^{-4}$ M). Cell lysates were obtained by scraping cultures into 1 ml of cold Frackelton buffer, followed by centrifugation for 15 minutes at 4°C. The proteins in the supernatant were equilibrated using a Bio Rad protein reagent (Bio Rad, CA, USA). 10 μl c-jun/AP-1 (Ab-1) Antibody coupled to 15μl of Protein G-Plus Agarose beads were added and samples were rocked for several hours at 4°C and repeatedly washed with Frackelton buffer. The beads were released after adding 20 μl of sample buffer by incubation at 100°C for 5 minutes, and separated by centrifugation at 2000 rpm for 5 minutes.

Immunoprecipitates and a low molecular weight protein marker (Biorad, CA, USA) were separated by 12,5 % SDS-PAGE and the gel was fixed in 50 % methanol + 10 % glacial acetic acid for 30 minutes, rinsed with water and exposed by autoradiography for 30 minutes.

2.2 Materials

2.2.1 chemicals and radiochemicals

Acetic Acid, Glacial	J.T. Baker Chemical Company, New Jersey, USA
Acrylamide/Bisacrylamide 29:1	Bio Rad Laboratories Life Science Group, CA, USA
Agarose	FMC BioProducts, ME, USA
Ammoniumpersulfate	Serva Biochemicals, New York, USA
Boric Acid	Sigma Chemical Company, Missouri, USA
Bovine serum albumin (BSA)	Sigma Chemical Company, Missouri, USA
5-Bromo-2´-Deoxyuridine (BrdU)	Sigma Chemical Company, Missouri, USA
Butanol	Fisher Scientific, New Jersey, USA
Calcium Chloride	Fischer Scientific, PA, USA,
Crystal Violet	Allied Chemical, New York, USA
4`,6-Diamidine-2`-Phenylindole Dihydrochloride (DAPI)	Boeringer Mannheim, Germany
Dialyzed fetal bovine serum (dFBS) (dialyzed in the laboratory)	Sigma Chemical Company, Missouri, USA
Diethyl Pyrocarbonate (DEPC)	Sigma Chemical Company, Missouri, USA
DMEM (w/o methionine and cystine, + L-glutamine)	Sigma Chemical Company, Missouri, USA
EDTA	Sigma Chemical Company, Missouri, USA
Ethanol	Fisher Scientific, New Jersey, USA
Formaldehyde solution (37%)	Sigma Chemical Company, Missouri, USA
Formamide (ultrapure)	Boehringer Mannheim, Germany, Cat.No: 100731

Geneticin	Sigma Chemical Company, Missouri, USA
L-Glutamine	Sigma Chemical Company, Missouri, USA
Glycerol	American Scientific Products, IL, USA
HCl	Sigma Chemical Company, Missouri, USA
HEPES	US Biochemical Corp., OH, USA
KCl	Sigma Chemical Company, Missouri, USA
Lipofectin	Gibco BRL, MD, USA
Methanol	Fisher Scientific, New Jersey, USA
MOPS	Sigma Chemical Company, Missouri, USA
NaCl	Sigma Chemical Company, Missouri, USA
NaOH	Sigma Chemical Company, Missouri, USA
Nonidet P-40 (NP-40)	Sigma Chemical Company, Missouri, USA N-3516
Oligo dt cellulose	Collaborative Research Labs, MA, USA
Phorbol 12-Myristate 13 Acetate (PMA) =TPA	Sigma Chemical Company, Missouri, USA, St. Louis P-8139
Phosphate buffered saline (PBS)	10x concentrated, Dulbecco't PBS
Propidium Iodide (PI)	Sigma Chemical Company, Missouri, USA P-4170
Protein G Plus Agarose beads	Oncogene Research Products, MA, USA
Sodium acetate	Sigma Chemical Company, Missouri, USA
Sodium dodecyl sulfate (SDS)	Sigma Chemical Company, Missouri, USA
N,N,N',N' Tetramethylethylenediamine (TEMED)	Bio Rad Laboratories Life Science Group, CA, USA
12-O-tetradecanoylphorbol-13-acetate(TPA)	Refer to Phorbol 12-Myristate 13 Acetate (PMA)
Trizma-Base [Tris(hydroxymethyl)aminomethane]	Sigma Chemical Company, Missouri, USA T-1503
Trizma hydrochloride (Tris-HCl)	Sigma Chemical Company, Missouri, USA
Trypsin	Sigma Chemical Company, Missouri, USA T-8253

Radiochemicals

[α-^{32}P] dCTP	Amersham life Science, Cat.No: AA 0005, Arlington Heights, Illinois, USA
35S-protein labeling mix	NEN/Dupont, MA, USA

2.2.2 culture media and additives

Basal media, MCDB 153*:* Dr. Pittelkow´s laboratory prepared the basal media MCDB 153 instead of purchasing it. Therefore, different stock solutions were made.

Stock 1 (100x)	Arginine•HCl ($1x10^{-3}$ moles/l) Histidine•HCl•H_2O ($8x10^{-3}$ moles/l) Isoleucine allofree ($1.5x10^{-3}$ moles/l) Leucine ($5x10^{-2}$ moles/l) Lysine•HCl ($1x10^{-2}$ moles/l) Methionine ($3x10^{-3}$ moles/l) Phenylalanine ($3x10^{-3}$ moles/l) Threonine ($1x10^{-2}$moles/l) Tryptophan ($1.5x10^{-3}$ moles/l) Tyrosine ($1.5x10^{-3}$ moles/l) Valine ($3x10^{-2}$ moles/l) Choline chloride ($1x10^{-2}$ moles/l) Serine ($6x10^{-2}$ moles/l)
Stock 2 (100x)	Biotin ($6x10^{-6}$ moles/l) Ca pantothenate ($1x10^{-4}$ moles/l) Niacinamide ($3x10^{-5}$ moles/l) Pyridoxine HCl ($3x10^{-5}$ moles/l) Thiamine Hcl ($1x10^{-4}$ moles/l) Kcl ($1.5x10^{-1}$ moles/l)
Stock 3 (50x)	Folic acid ($9x10^{-5}$ moles/l) Na_2HPO_4•$7H_2O$($1x10^{-1}$ moles/l)
Stock 4a / 4b / K2	$CaCl_2$•$2H_2O$ ($2x10^{-1}$ moles/l) / $MgCl_2$•$6H_2O$ ($6x10^{-1}$ moles/l) / $FeSO_4$•$7H_2O$ ($5x10^{-3}$ moles/l)
Stock 5 (1000x)	Phenol Red ($3.3x10^{-3}$ moles/l)
Stock 6c	Riboflavin ($1x10^{-4}$ moles/l)
Stock 8 (100x)	Asparagine ($1x10^{-2}$ moles/l) Proline ($3x10^{-2}$ moles/l) Putrescine ($1x10^{-4}$ moles/l) Vit B_{12} ($3x10^{-5}$ moles/l)

Stock 9 (100x)	Alanin ($1x10^{-2}$ moles/l) Aspartic Acid ($3x10^{-3}$ moles/l) Glutamic Acid ($1x10^{-2}$ moles/l) Glycine ($1x10^{-2}$ moles/l) Phenol Red (0.75 ml)
Stock 10 (100x)	Adenine ($1.8x10^{-2}$ moles/l), myo-Inositol ($1x10^{-2}$ moles/l), Lipoic Acid ($1x10^{-4}$ moles/l), Thymidine ($3x10^{-4}$ moles/l), $CuSO_4 \cdot 5H_2O$ ($1x10^{-6}$ moles/l)
Stock L (Trace Elements)	$CuSO_4 \cdot 5H_2O$ ($1x10^{-7}$ moles/l)H_2SeO_3 ($3x10^{-6}$ moles/l) $MnSO_4 \cdot 5H_2O$ ($1x10^{-7}$ moles/l), $Na_2SiO_3 \cdot 9H_2O$ ($5x10^{-5}$ moles/l), $(NH_4)_6MO_7O_{24} \cdot 4H_2O$ ($1x10^{-7}$ mol/l), NH_4VO_3 ($5x10^{-7}$ moles/l), $NiCl_2 \cdot 6H_2O$ ($5x10^{-8}$ moles/l) $SnCl_2 \cdot 2H_2O$ ($5x10^{-8}$ moles/l) $ZnSO_4 \cdot 7H_2O$ ($5x10^{-5}$ moles/l)

To obtain **4 liters** of MCDB 153 media 40 ml of Stock 1, 40 ml of Stock 2, 80 ml of Stock 3, 2 ml of Stock 4a, 4 ml of Stock 4b, 4 ml of Stock K2, 4 ml of 6c, 4 ml of Phenol Red, 40 ml of Stock 8, 40 ml of Stock 9, 40 ml of Stock 10 and 40 ml of Stock L were added to 3 liters of Millipore filtered H_2O.

Also added were: 4.324 g Glucose, 30.398 g NaCl, 26.4 g Hepes (US Biochemical Corp., OH, USA), 2.00 g Na Acetate$\cdot 3H_2O$, 3.5 g L-Glutamine, 0.22 g Na Pyruvate, 0.168g Cysteine.

The pH of the media was then adjusted to 7.4 using NaOH before addition of 4.704 g $NaHCO_3$. Millipore filtered H_2O was added up to a final volume of 4 liters and the media was filtersterilized with 0.2µ filters into 500 ml bottles.

The Ca^{2+} concentration of MCDB 153 media was 100 µM. When standard- or complete medium was prepared by addition of designated exogenous growth factors, $CaCl_2$ and antibiotics, as described under **2.1,** a 1:33 dilution of the High amino acid stock solution was also added

Exogenous growth factors, antibiotics and high amino acids:

Bovine pituitary extract (BPE)	BPE was prepared by homogenizing 150 grams of mixed sex bovine pituitaries in 250 ml cold 0.15 M NaCl for 10 minutes. The homogenate was transferred to a cold beaker and stirred for 90 minutes at 4 °C. Next it was centrifuged for 10 minutes at 9800 g. The supernatant was saved, divided into aliquots and stored frozen at -70°C. The aliquots were thawed as needed and centrifuged at 9800 g for 15 minutes in the cold room and filtersterilized.
Epidermal growth factor (EGF) (human recombinant)	Upstate Biotechnology, New York, USA, Catalog.#: 01-107
Ethanolamine	Sigma Chemical Company, Missouri, USA
Stock for 6 high amino acids	L-Histidine•HCl•H_2O ($8x10^{-3}$ moles/l) L-Isoleucine ($2.5x10^{-2}$ moles/l) L-Methionine ($3x10^{-3}$ moles/l) L-Phenylalinine ($3x10^{-3}$ moles/l) L-Tryptophan ($1.5x10^{-3}$ moles/l) L-Tyrosine ($2.5x10^{-3}$ moles/l)
Hydrocortisone	Sigma Chemical Company, Missouri, USA
Insulin	Sigma Chemical Company, Missouri, USA, Catalog.#: I-5500
Penicillin	Sigma Chemical Company, Missouri, USA
Phosphorylethanolamine	Sigma Chemical Company, Missouri, USA
Streptomycin	Sigma Chemical Company, Missouri, USA

Washing buffer (Solution A): With reference to Shipley and Ham, 1981, Solution A consists of glucose (10.0 mM), NaCl (130 mM), KCl (3 mM), Sodiumphosphate (1.0 mM), phenolred (0.0033 mM) and Hepes (30 mM). It is at a pH of 7.4.

If not otherwise indicated, all basal media ingredients were obtained at Sigma Chemical Company, Missouri, USA

2.2.3 enzymes

Ribonuclease 1 "A" (RNAse A) Bovine Pancrease	Pharmacia, NJ, USA, Cat. No. 27-0323-01
Proteinase K	Life Technologies, Gaithersburg, MD, USA

2.2.4 antibodies

BU1-BrdU Ab	Mayo Clinic, Division of Laboratory Medicine, provided by Dr. Katzmann , MN, USA Ref: Gonchoroff *et al.*: A monoclonal antibody reactive with 5-Bromo-2-Deoxyuridine that does not require DANN denaturation, *Cytometry,* 6:506-512, 1985
FITC rabbit anti-mouse (Rabbit F(ab`)2 Anti-Mouse IgG)	Southern Biotechnology Associates, Alabama, USA, Cat. No. 6120-02
c-jun/AP-1 (Ab-1)	Oncogene Research Products, MA, USA, Cat. No. PCO6L, polyclonal rabbit IgG

2.2.5 RNA solutions

Lysis buffer	For 500 ml, use 5.0 ml 1.0 M Tris-HCl, 10.0 m 5.0 M NaCl, 5.0 ml 0.2 M EDTA, 429.5 ml H_2O, 0.5 ml DEPC, 50.0 ml 10 % SDS
High salt buffer	For 500 ml, use 5.0 ml 1.0 M Tris-HCl, 50.0 ml 5.0 M NaCl,2.5 ml 0.2 M EDTA 432.0 ml H_2O, 0.5 ml DEPC, 10.0 ml 10 % SDS
Low salt buffer	For 500 ml, use 5.0 ml 1.0 M Tris-HCl, 10.0 ml 5.0 M NaCl, 2.5 ml 0.2 M EDTA, 472.0 ml H_2O, 0.5 ml DEPC, 10.0 ml 10 % SDS
No Salt buffer	For 100 ml, use 0.5 ml 1.0 M Tris-HCl, 0.5 ml 0.2 M EDTA, 97 ml H_2O, 0.1 ml DEPC, 2.0 ml 10 % SDS
TE buffer	1x (Tris EDTA) for 500 ml use 0.6055 g Tris Base, 0.186g EDTA $2H_2O$, concentrated HCl to pH 8, H_2O to 500 ml total

SCC	NaCl, Na Citrate, pH 7.4
Hybridization buffer for northern blot analysis	Amersham Life Science, IL, USA (Hybridization buffer tablets)
10 x MOPS buffer	0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0 adjusted with NaOH
Tracking dye	50 % glycerol, 1 mM EDTA, 0.4 % Bromophenol blue, 0.4 % Xylene cyanol
Sample buffer	500 µl ultrapure formamide, 100 µl 10x MOPS, 178 µl 37 % formaldehyde, 222 µl DEPC H_2O
Gel buffer	10 ml 10 x MOPS, 22.2 ml DEPC treated H_2O, 17,8 ml 37 % formaldehyde (The gel was made up of 1,2 g Agarose, 50 ml DEPC treated H_2O and 50 ml gel buffer)
Running buffer	100 ml 10 x MOPS, 89.3 ml 37% formaldehyde, q.s. to 1 liter with H_2O

2.2.6 Immunoprecipitation buffers

Frackelton buffer	Tris Base pH7.4, 20mM, $Na_4P_2O_7$ 30 mM, NaCl 40mM, NaF 50mM, EDTA 5mM, Trition-X 100 0.5%, Glycerol 5%, Day of use add the following: 0.1mM Aprotinin 0.12 TIU/ml, Leupeptin 10µg/ml, PMSF EtOH 1.0mM, Pepstatin EtOH 5µg/ml (all these chemicals despite glycerol were obtained at Sigma Chemical Company, Missouri, USA)
Sample buffer	62.5 mM Tris Hcl, 10 % glycerol, 1.5 % SDS, 2.5 % β-mercaptoethanol (all these chemicals despite glycerol were obtained at Sigma Chemical Company, Missouri, USA)

2.2.7 SDS-PAGE solutions

Lower Gel buffer pH 8.8	Tris Base 1.5 M, SDS 0.4% in Millipore water, pH was adjusted to 8.8 with HCl. The gel for 12.5% SDS PAGE was made up of 8 ml Lower Gel Buffer, 13.35 ml 30% acrylamide, 50 µl 10% Ammoniumpersulfate, 25 µl TEMED and 10.65 ml Millipore H_20
Upper Gel buffer pH 6.8	Tris Base 1.5 M, SDS 0.4% in Millipore water, pH was adjusted to 6.8 by HCl. The stacking gel was made up of 2.5 ml Upper Gel buffer, 1.6 ml 30% acrylamide, 30 µl 10% Ammoniumpersulfate, 10 µl TEMED and 5.9 ml Millipore H_20
SDS running buffer (10x stock)	Tris Base 60.6 g, glycine 288 g, SDS 20 g and Millipore water up to 2 liters. Dilute 1:10 before using
30 % acrylamide stock	Acrylamide/Bis 29:1 30 g + Millipore filtered H_2O up to 73 ml

3 RESULTS

3.1 Growth, differentiation and viability of HaCaT cells

3.1.1 Cell growth rates kinetics and growth factor dependency

HaCaT cells grown in complete MCDB 153 medium exhibit a cell generation time (G_T) of approximately 26 hours. Plateau phase of growth was observed when cultures reached confluence at a density of 1-2 x $10^5/cm^2$ similar to normal human keratinocytes (Wille *et al.*, 1984). Removal of exogenous growth factors resulted in an initial increase in cell G_T to approximately 40 hours. After 5-6 days, the rate of cell growth diminished to > 96 hours **(Figure 1A)**.

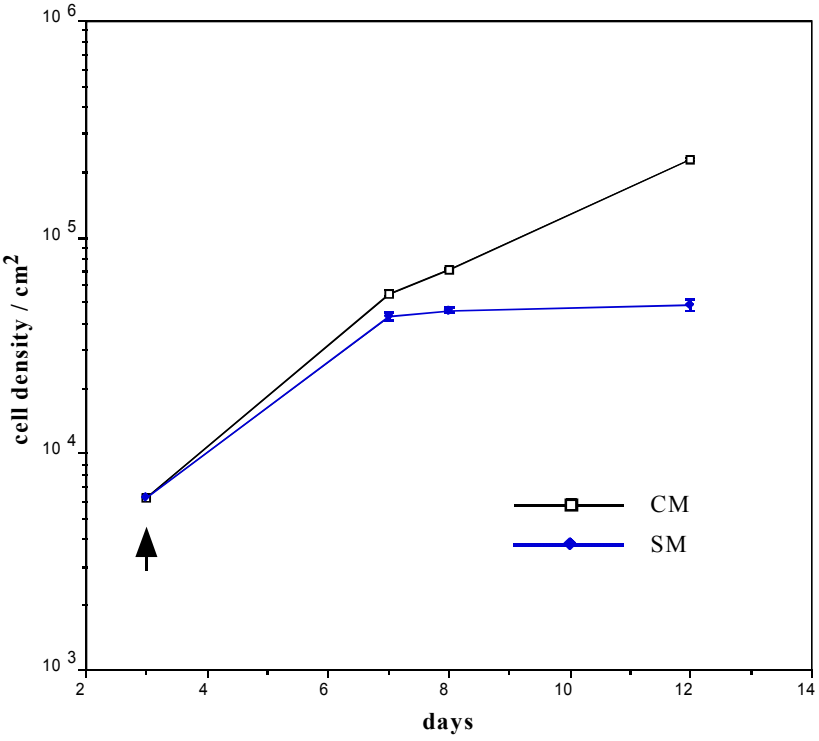


Figure 1A. Growth response of HaCaT cells. HaCaT were cultured in complete medium (CM) (—□—) for 3 days prior to switching (↑) cultures to standard medium (SM)(—◆—).

Elevation of medium calcium concentration to a more physiological level of 1.5 mM failed to promote replication significantly although growth rate was slightly enhanced in both standard medium $G_T \approx 38$ hours and complete medium $G_T \approx 24$ hours (data not shown). These findings indicate that HaCaT, unlike normal keratinocytes, require

exogenous growth factors to maintain logarithmic growth phase. Furthermore calcium concentration (1.5 mM) does not markedly stimulate growth in the presence or absence of growth factors and low calcium medium concentration is not a limiting factor for proliferation similar to normal keratinocytes (Wille *et al*., 1984).

The effects of EGF and/or insulin on proliferation kinetics were examined. Either EGF or insulin stimulated proliferation similar to complete medium **(Figure 1B)**.

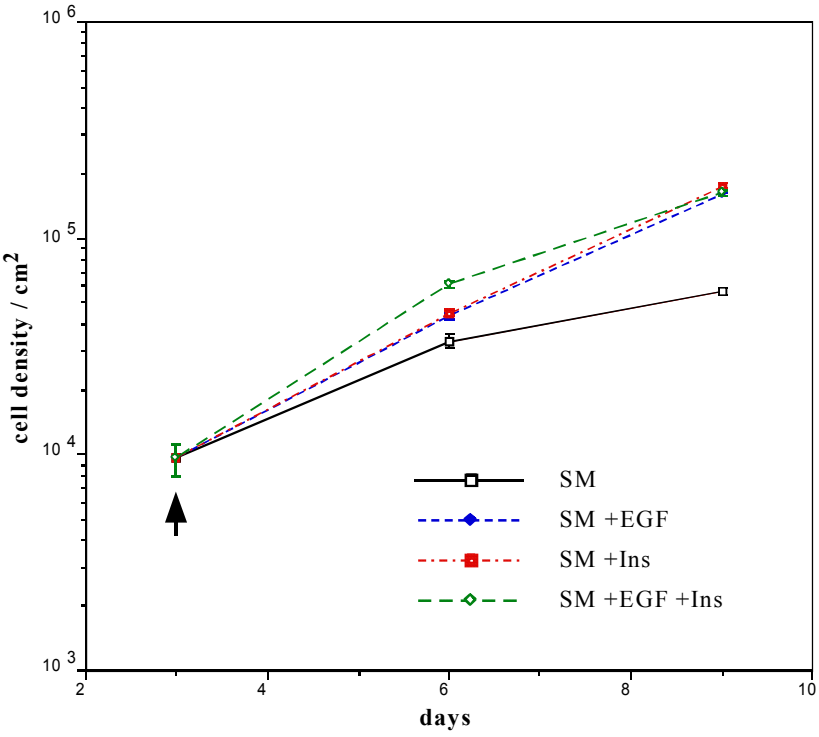
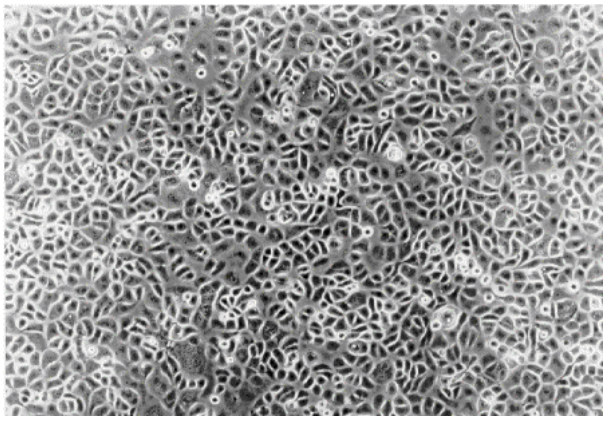


Figure 1B. Growth response of HaCaT cells. HaCaT were cultured in complete medium (CM) for 3 days prior to switching (↑) cultures to standard medium (SM)( ) or SM containing EGF (10 ng/ml,), insulin (5 ng/ml,), or EGF+insulin ( ).

Initially following medium switch, HaCaT cells cultured in standard medium exhibited similar morphologic appearance to cells grown in complete medium **(Figure 2A)**. However, several days after growth factor withdrawal the number of mitotic cells decreased markedly, and several days later pyknotic appearing cells could be observed. These cultures lost the ability to grow to confluent density **(Figure 2B)**.

A.



B.

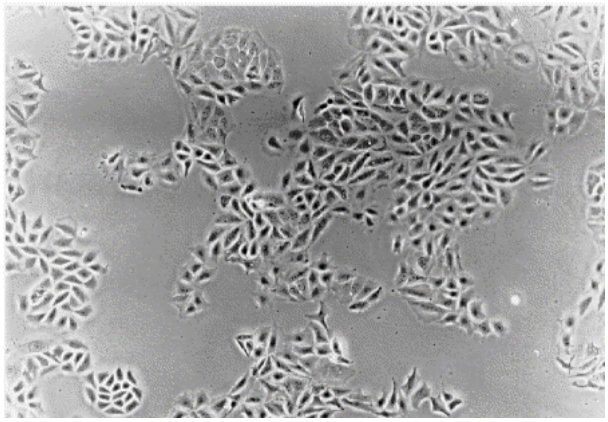


Figure 2. phase-contrast photomicrographs of HaCaT cells. HaCaT were cultured in complete medium (CM) for 7 days **(A)** or CM was switched to standard medium (SM) after 3 days of growth, and cells were maintained in SM for 4 days **(B)**.

3.1.2 DNA synthesis, cell cycle kinetics and effects of EGF, insulin or calcium

BrdU incorporation into DNA demonstrated that cultures of exponentially growing HaCaT cells undergo S-phase DNA synthesis at the same rate as the overall population growth rate. 26 hour BrdU label exposure showed that greater than 90 % of the cells incorporated BrdU into DNA approximating the population doubling time in complete medium **(Table 1)**. The DNA synthetic rate was equivalent in cells reared in

either low (0.1 mM) or high (1.5 mM) Calcium containing medium (data not shown). DNA incorporation of BrdU using a 2 hour exposure demonstrated 49 % of HaCaT cells labeled in complete medium, but only 21 % of cells labeled in standard medium. These results confirm that the decreased growth rate after growth factor depletion is correlated with a proportionate decrease in DNA synthesis. The rate of DNA synthesis was not significantly different for high calcium (1.5 mM) medium, paralleling the findings in keratinocytes (Wille *et al.*, 1984). Interestingly with prolonged (26 hour) BrdU exposure, only a slightly greater percentage of HaCaT growing in standard medium, 29 %, labeled **(Table 1)**.

	2 hours	**26 hours**
CM	49	91
SM	21	29

Table 1. Percent BrdU-positive cells. HaCaT were exposed to BrdU in complete (CM) or standard (SM) medium for 2 hours or 26 hours. Cultures in CM were switched 4 days earlier to SM. Percent BrdU-incorporated nuclei determined by flow cytometry as described in Materials and Methods.

Cell cycle analysis of HaCaT grown in complete or standard medium was performed. Rapidly growing HaCaT have the following cell cycle distribution 34 % G1, 51 % S and 13 % G2/M. 3 days of growth factor depletion shows an increase in G1 phase to 75 % and a decrease in S and G2/M phase to 18 % and 7 %, respectively **(Table 2)**. By 6 days of culture without growth factors, S phase percent decreased to 5 %. These results demonstrate that depletion of growth factors progressively decreased S and G2/M phase percentages with a coordinate increase in G1-arrested cells.

The effects of EGF, insulin, or EGF + insulin on growth and cell cycle distributions of HaCaT were next examined **(Table 2)**. EGF had stimulated DNA synthesis with 38-41 % of cells in S-phase at day 3 or 6, respectively. Addition of insulin to standard medium resulted in 38 % or 33 % S-phase cells at 3 or 6 days respectively. EGF + insulin were no more potent in stimulating DNA-synthesis than EGF or insulin alone. These results of cell cycle analysis correlated with the growth curves of EGF, insulin or complete medium suggest, that EGF or insulin are sufficient to stimulate DNA-synthesis in HaCaT cells similar to complete medium.

	Day 3			day 6		
	G1	**S**	**G2/M**	**G1**	**S**	**G2/M**
SM	75	18	7	88	5	7
SM +EGF	52	41	7	58	38	4
SM +Ins	61	38	1	56	33	11
SM +EGF +Ins	52	41	7	44	52	4

Table 2. **Cell cycle distribution in %.** Effect of selected culture factors on HaCaT cell cycle distribution. Cells were switched to standard medium (SM) and designated culture factors (EGF, insulin or EGF + insulin) were added as indicated. Cell cycle analysis was performed 3 and 6 days after switch to specific culture medium.

3.1.3 Loss of clonogenicity in standard medium

Clonogenic growth was examined over 7 days of growth factor deprivation. Progressive loss of clonogenic potential was observed for HaCaT cells that had been cultured in growth factor deficient, standard medium compared to complete medium prior to clonal assay **(Figure 3)**.

Within 2 days following switch to growth factor depleted medium, marked decrease in size and number of clone-forming HaCaT cells were observed. By 4 days, further decrease was observed, similar that seen at 7 days growth in standard medium.

These findings indicate that the majority of HaCaT cells cultured in depleted medium of growth factors loses replicative potential in a time dependent-manner and becomes irreversibly committed at subconfluent cell densities. This finding differs from normal keratinocytes that retain clonogenic potential in growth factor deficient medium at subconfluence (Poumay and Pittelkow, 1995). By contrast, at culture confluence, decreased clonogenicity was observed for HaCaT cells cultured in standard or complete medium (data not shown) similar to results for normal keratinocytes (Poumay and Pittelkow, 1995).

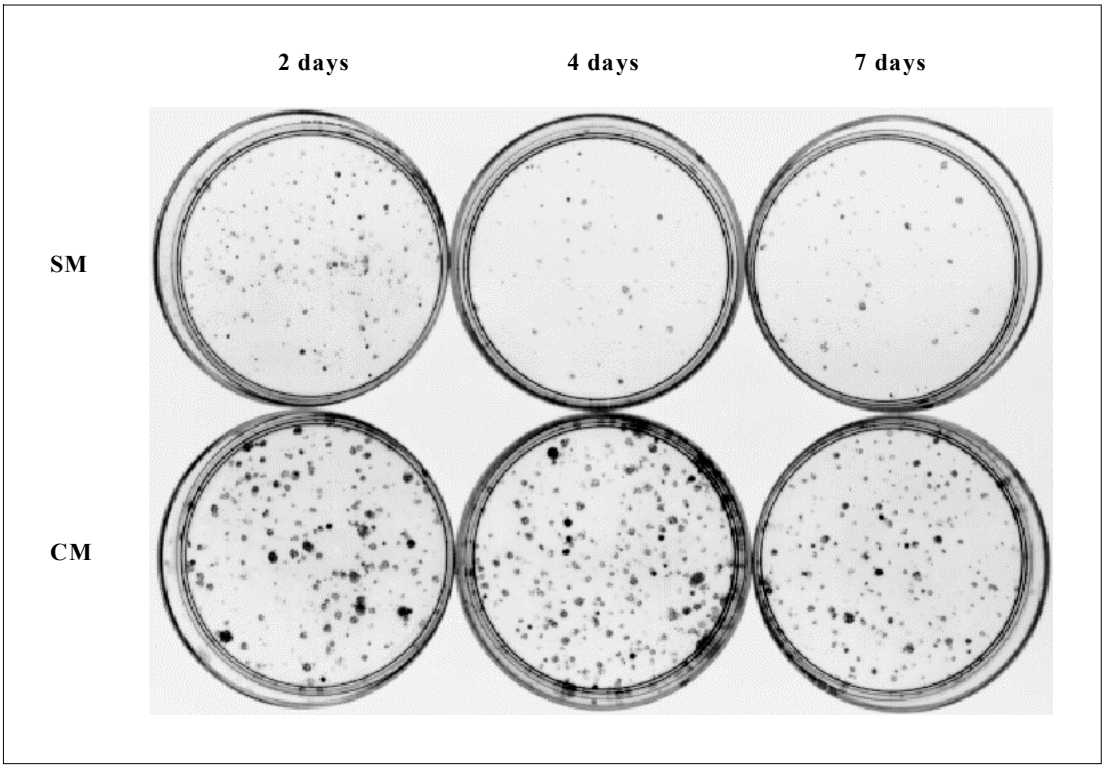


Figure 3. Growth factor depletion induces loss of clonogenicity. HaCaT were cultured in complete medium (CM) or in standard medium (SM), containing 0.1 mM calcium concentration, at subconfluent cell densities. Cells grown in SM had been switched from CM and cultured for 2, 4 or 7 days before re-plating in clonal assay.

3.1.4 Subconfluent HaCaT cells express keratins 1 and 10 and involucrin after growth factor removal

Expression of suprabasal differentiation markers K 1, K 10, and involucrin which are closely linked to terminal differentiation, was examined in various culture conditions. mRNA was harvested from subconfluent (70-90 % cell confluence, as assessed by phase contrast microscopy) or from fully confluent cultures. Cultures were also switched to standard medium and cultured for 48 hours before RNA isolation. Northern analysis demonstrates induction of mRNA expression for K 1 and K 10 in subconfluent cultures of HaCaT after removal of growth factors **(Figure 4)**.

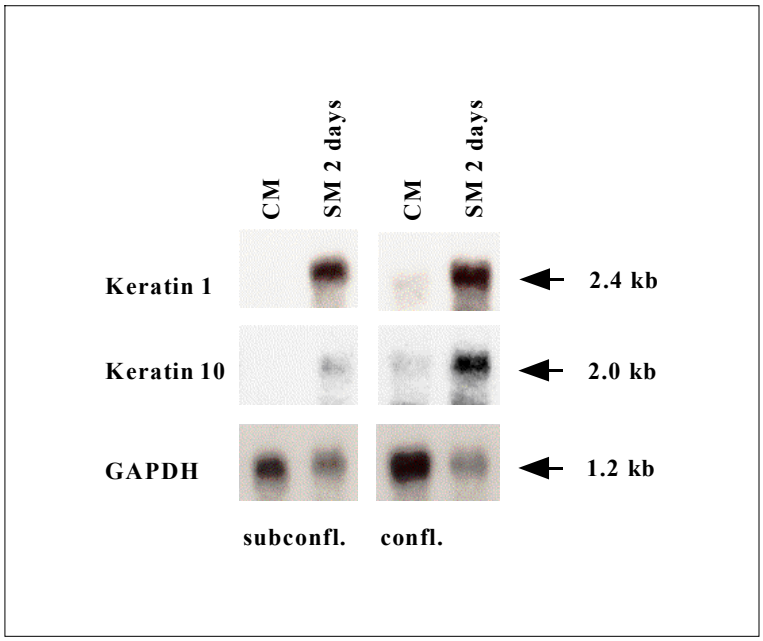


Figure 4. Northern analysis of HaCaT expressing terminal differentiation-specific K 1 and 10 mRNA at subconfluent or confluent cell densities. HaCaT plated at $3x10^3$ cells/cm^2 (subconfl.) or $3x10^4$ cells/cm^2 (confl.) and grown in complete medium (CM) for approximately 7days and switched to standard medium (SM) for two days. Poly(A)RNA was harvested, and 2.5 µg samples loaded and analyzed by Northern blot hybridization with specific probes to K 1, K 10 or GAPDH constitutive probe.

In rapidly growing, complete medium, HaCaT do not express K 1 or K 10, similar to normal keratinocytes (Poumay and Pittelkow, 1995). These results demonstrate that rapidly growing HaCaT cells initiate terminal differentiation after growth factor withdrawal, concomitant with loss of clonogenic potential. At culture confluence in complete medium limited expression of K 1 and K 10 was observed that was markedly enhanced by removal of growth factors for 2 days. Clonal growth analysis indicated that greater loss of proliferative potential was induced by growth factor depletion compared to subconfluent culture (data not shown).

The kinetics of K 1 and K 10 and involucrin mRNA expression induced in HaCaT cells cultured in standard medium were next investigated. **Figure 5** shows the time-dependent induction of K 1,10 and involucrin mRNA expression over 96 hours of culture in growth factor deficient medium at subconfluent densities. K 1 mRNA expression is weakly induced in subconfluent cultures within 24 hours of removal of growth factors, and is more strongly induced at confluence following 24 hours of culture in standard medium (data not shown). Involucrin mRNA expression is induced more strongly at later times (by 96 hours) after culture in standard medium. These findings suggest that both growth factors and cell density control the irreversible growth arrest, loss of clonogenic potential and commitment to terminal differentiation in HaCaT.

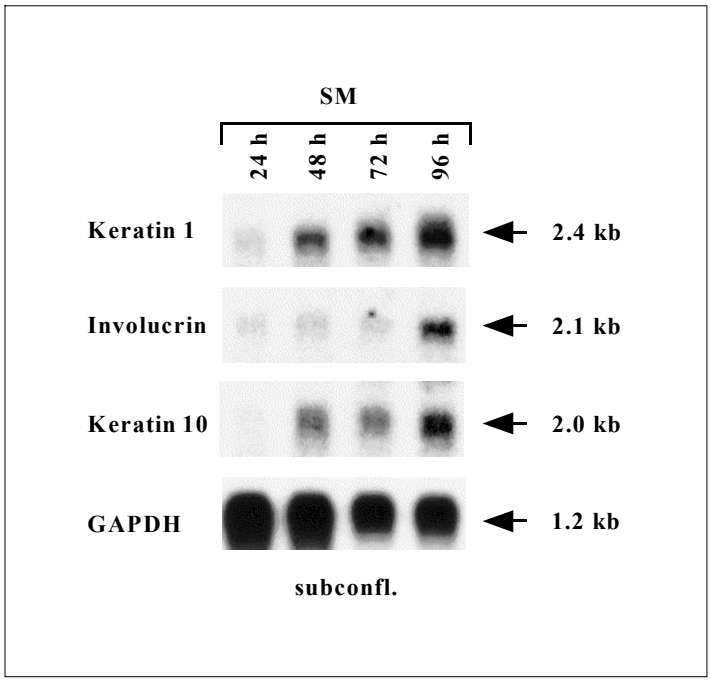


Figure 5. Northern analysis of time-dependent K 1, K 10 and involucrin mRNA expression in standard medium. HaCaT were switched to standard medium (SM) at subconfluence and cultured for 24, 48, 72 or 96 hours. Poly(A)RNA was harvested and samples were analyzed by northern blot hybridization with the specific probes to K 1, K 10, involucrin or a GAPDH probe.

The effect of EGF-addition to cultures of HaCaT expressing K 1, K 10 and involucrin was examined. EGF (10 ng/ml) exposure of HaCaT in standard medium suppressed mRNA levels of these differentiation markers **(Figure 6)**.

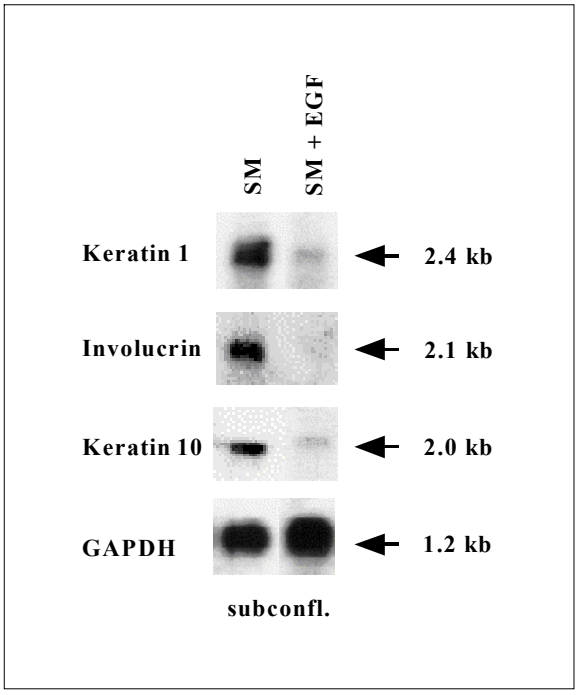


Figure 6. Keratin 1,10 and involucrin mRNA expression suppressed by EGF. HaCaT cells cultured in standard medium (SM) for 3 days at subconfluence and treated with EGF (10 ng/ml) for 24 hours. mRNA samples analyzed by northern hybridization with designated probes.

Calcium concentration has been reported to modulate expression of differentiation markers in normal keratinocytes under selected culture conditions (Poumay and Pittelkow, 1995). HaCaT are normally cultivated in medium containing > 1 mM calcium. Therefore the effects of calcium on expression of K 1 mRNA were examined in complete or standard medium at subconfluence or confluence. Compared to subconfluent, HaCaT cells grown in complete medium, both growth factor depletion and confluence enhanced K 1 mRNA expression in high calcium medium **(Figure 7)**. K 1 steady state levels of mRNA increased 4-5 times when growth factors were removed, regardless of whether HaCaT were at subconfluent or confluent cell densities. As HaCaT attained culture confluence, 1.5 mM calcium stimulated K 1 mRNA expression. These findings are similar to those observed for normal keratinocytes grown in standard medium at high (1.5 mM) calcium concentration (Poumay and Pittelkow, 1995).

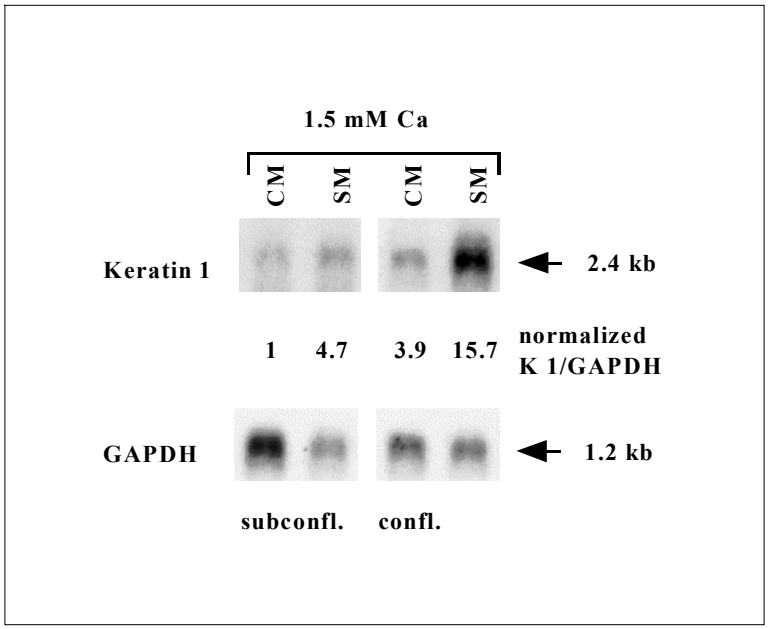


Figure 7. Effect of medium calcium concentration and growth factors on K 1 mRNA expression. HaCaT cells were plated at $3x10^3$ cells/cm^2 or $3x10^4$ cells/cm^2 and grown in complete medium (CM) to subconfluence (80 %) or confluence, then switched to CM or standard medium (SM) containing 1.5 mM Ca^{2+} for 24 hours. RNA samples analyzed by northern blot hybridization with the specific probes to K 1 or GAPDH.

3.1.5 Growth factor removal stimulates HaCaT cell apoptosis

The proliferative potential and altered cell morphology, including cell elongation and appearance of crenated HaCaT cells in growth factor depleted medium, suggested cells were undergoing apoptosis. To test this possibility HaCaT cells were switched to standard medium at low cell density (40-60% confluence), cultured for specific times and stained with propidium iodide and DAPI. Over 8 days of culture in growth factor depleted medium, the apoptosis rate increased significantly from 2-5 % early in culture to 11 % by 8 days **(Figure 8)**. A lower, more constant rate of apoptosis was observed for HaCaT grown in complete medium that ranged from 1-4 % over 8 days of culture. These results show that the increasing rate of apoptosis in growth factor depleted cultures of HaCaT could account for the lack of further increase in the number of cells after switch to standard medium **(Figure 1)**. HaCaT cells which replicate do not increase significantly in percent as measured by BrdU incorporation **(Table 1)**. Preferential apoptosis of HaCaT cells undergoing DNA synthesis may account for the decrease in growth rate observed in standard medium.

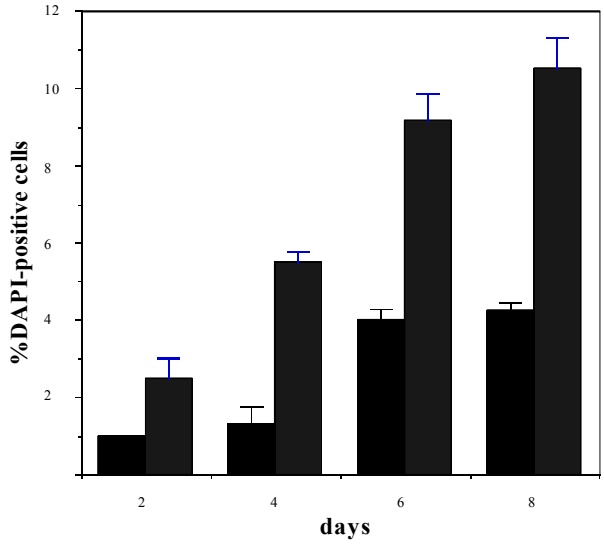


Figure 8. Growth factor depletion stimulates apoptosis of HaCaT. Cells were plated at 2-3x10^3/cm^2 onto glasscoverslips. After 3 days, cultures were switched to standard medium (SM) (grey), or cultured in complete medium (CM) (black) for 2, 4, 6 or 8 days. Cells were stained with PI and DAPI and cell counting was performed as described in Materials and Methods.

3.2 12-O-tetradecanoylphorbol-13-acetate (TPA) and HaCaT growth and differentiation characteristics

3.2.1 Cell growth rate - and cell cycle - kinetics of HaCaT cells treated with TPA

HaCaT cells grown in complete MCDB 153 medium exhibit a cell generation time (G_T) of approximately 26 hours **(Figure 1A)**. When TPA (10 ng/ml) is added to complete medium HaCaT continue exponential growth and exhibit a slightly increased G_T of approximately 28 hours. After 11 days of growth HaCaT attain culture confluence, similar to HaCaT cultures initiated at 3 x 10^3/cm^2 and grown in complete medium without TPA **(Figure 9A)**.

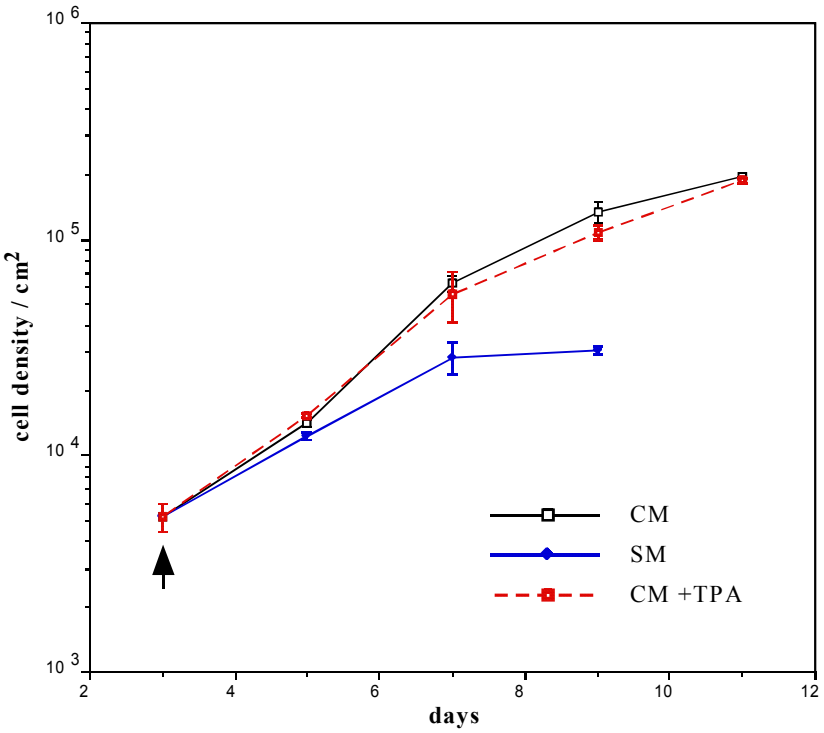


Figure 9A. Growth response of HaCaT cells. HaCaT were plated at 3x10^3/cm^2 and grown in complete medium (CM) for 3 days, switched to standard medium (SM), or CM containing TPA (10 ng/ml).

Addition of TPA to HaCaT cells propagated in standard medium without exogenous growth factors fails to promote growth. However, the cell generation time is slightly decreased ($G_T \sim 38$ hours) in contrast to HaCaT cells grown in standard medium ($G_T \sim 40$ hours). When TPA and insulin are added to standard medium, HaCaT cells proliferate at a similar overall rate as HaCaT cultured in standard medium supplemented with insulin **(Figure 9B)**.

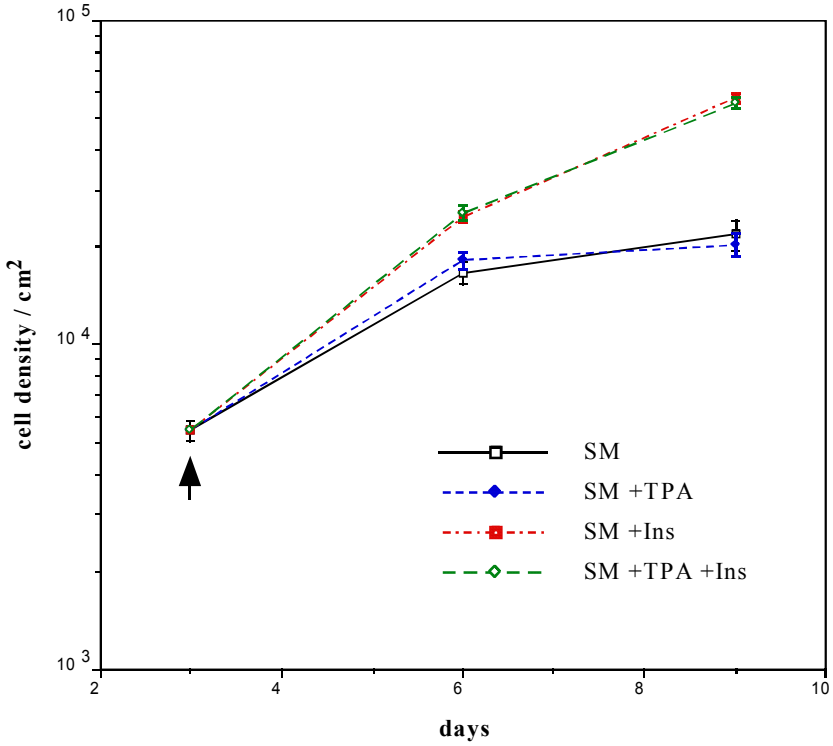


Figure 9B. Growth response of HaCaT cells. HaCaT were cultured in complete medium (CM) for 3 days prior to switching cultures to standard medium (SM), or SM containing TPA (10 ng/ml), Insulin (5 ng/ml), or TPA + Insulin.

Contrasting to normal human keratinocytes (NHK) which are growth-arrested by TPA (Wille *et al.*, 1985), these findings suggest that TPA neither inhibits nor promotes proliferation of HaCaT cells cultured in both, standard and complete medium, and in standard medium supplemented with insulin.

The effect of TPA on cell cycle distribution of HaCaT was next examined and compared to NHK. When TPA was added to HaCaT cells reared in complete medium the cell cycle distribution was not significantly altered with an average decrease of cells in S-phase from 46 % to 38 % over a time period of 6 days of TPA exposure in association with an increase in the G1 phase of the cell cycle **(Table 3)**. In contrast, NHK treated with TPA exhibited a decrease of cells entering the S-phase from 22 % to 9 % over a time period of 2 days with further accumulation of cells in both the G1 and G2/M phases of the cell cycle **(Table 3B)**. These results suggest that in contrast to NHK, HaCaT cell cycle progression is not significantly inhibited by TPA in complete medium.

A

	G1	**S**	**G2/M**
CM	44 ±10	46 ± 8	10 ± 3
CM +TPA	50 ± 9	38 ± 6	12 ± 5

B

	G1	**S**	**G2/M**
CM	58 ± 1	22 ± 3	21 ± 2
CM +TPA	56 ± 2	9 ± 4	36 ± 2

Table 3. Cell cycle distribution in %. Effect of TPA on HaCaT **(A)** and NHK (Wille *et al.*, 1985) **(B)** cell cycle distribution. Exponentially growing cells were exposed to TPA at 10 ng/ml **(A)** or 100 ng/ml **(B)** in complete medium (CM). Cell cycle analysis was performed every 2 days **(A)** or every other day **(B)** after addition of TPA. The numbers presented in the table are averaged based on a time period of 6 days (**A)** or 2 days **(B)** of TPA exposure.

Further experiments focused on the effect of TPA on cell cycle distribution of HaCaT cells grown in standard medium or in standard medium supplemented with insulin. Similar to HaCaT grown in complete medium and TPA **(Table 3A)**, cell cycle progression of HaCaT grown in standard medium supplemented with insulin and exposed to TPA was only slightly inhibited **(Table 4)**. A decrease from 25 % to 16 %

(33 % to 25 %) of cells in S-phase of the cell cycle could be detected after addition of TPA to standard medium supplemented with insulin after 3 (6) days of growth. When TPA was added to HaCaT cells reared in standard medium cells entering the S-phase decreased similarly only slightly from 15 % to 10 % after 3 days and from 12 % to 9 % after 6 days of growth. These results in correlation with the results presented in **Table 3A** indicate that HaCaT cell cycle progression is similarly controlled in both, complete medium and standard medium supplemented with insulin, after TPA treatment. TPA alone is not sufficient to induce cell cycle progression of HaCaT reared in growth factor-depleted, standard medium.

	Day 3			**Day 6**		
	G1	**S**	**G2/M**	**G1**	**S**	**G2/M**
SM	71	15	4	77	12	11
SM +Ins	63	25	12	56	33	11
SM +TPA	74	10	16	78	9	13
SM +Ins +TPA	76	16	8	64	25	11

Table 4. Cell cycle distribution in %. Effect of selected culture factors on HaCaT cell cycle distribution. After 3 days of growth in complete medium (CM), cells were switched to standard medium (SM) and designated culture factors (insulin) and/or TPA were added as indicated. Cell cycle analysis was performed 3 and 6 days after switch to specific culture conditions.

In summary, these results present evidence that HaCaT cell cycle progression is differentially affected compared to NHK in that it is much less pronounced inhibited and cells do not accumulate in the G2/M phase of the cell cycle. These results of cell cycle analysis correlated with the growth curves of HaCaT cells grown in the presence of TPA suggest, that TPA only slightly inhibits cell cycle progression of HaCaT under various culture conditions and growth further occurs at a similar overall rate after TPA treatment as in the absence of TPA. Other results showing that HaCaT labeling index is not inhibited by TPA in complete medium (data not shown) further substantiate the differential effect of TPA on HaCaT versus NHK proliferation and cell cycle progression.

3.2.2 TPA increases clonogenic potential of HaCaT cells

The effect of TPA on clonogenic potential of HaCaT cells was examined at subconfluent and confluent cell densities. HaCaT cells cultured in standard or in complete medium were treated with TPA for 24 hours prior to assay set-up. Clonogenic potential of exponentially growing HaCaT in complete medium was increased after TPA treatment **(Figure 10)**. In addition an increase in clonogenicity was observed in HaCaT cells exposed to TPA in standard medium without exogenous growth factors at subconfluence and in HaCaT cells exposed to TPA at culture confluence in both standard and complete medium. This enhancement of clonogenic potential was most pronounced at culture confluence in standard medium.

Commitment to terminal differentiation is defined as irreversible loss of clonal proliferative potential (Wille *et al.*, 1985). NHK lose clonal proliferative potential in response to TPA and become irreversibly committed (Wille *et al.*, 1985). Contrasting to NHK, these findings suggest that HaCaT resist the commitogenic response to TPA. In addition, these results indicate that TPA is capable to protect some HaCaT cells from commitment to terminal differentiation induced through withdrawal of exogenous growth factors at subconfluent cell densities, or induced through cell confluence in both complete and standard medium. HaCaT cells resist commitogenic signals after TPA treatment and such retain proliferative potential.

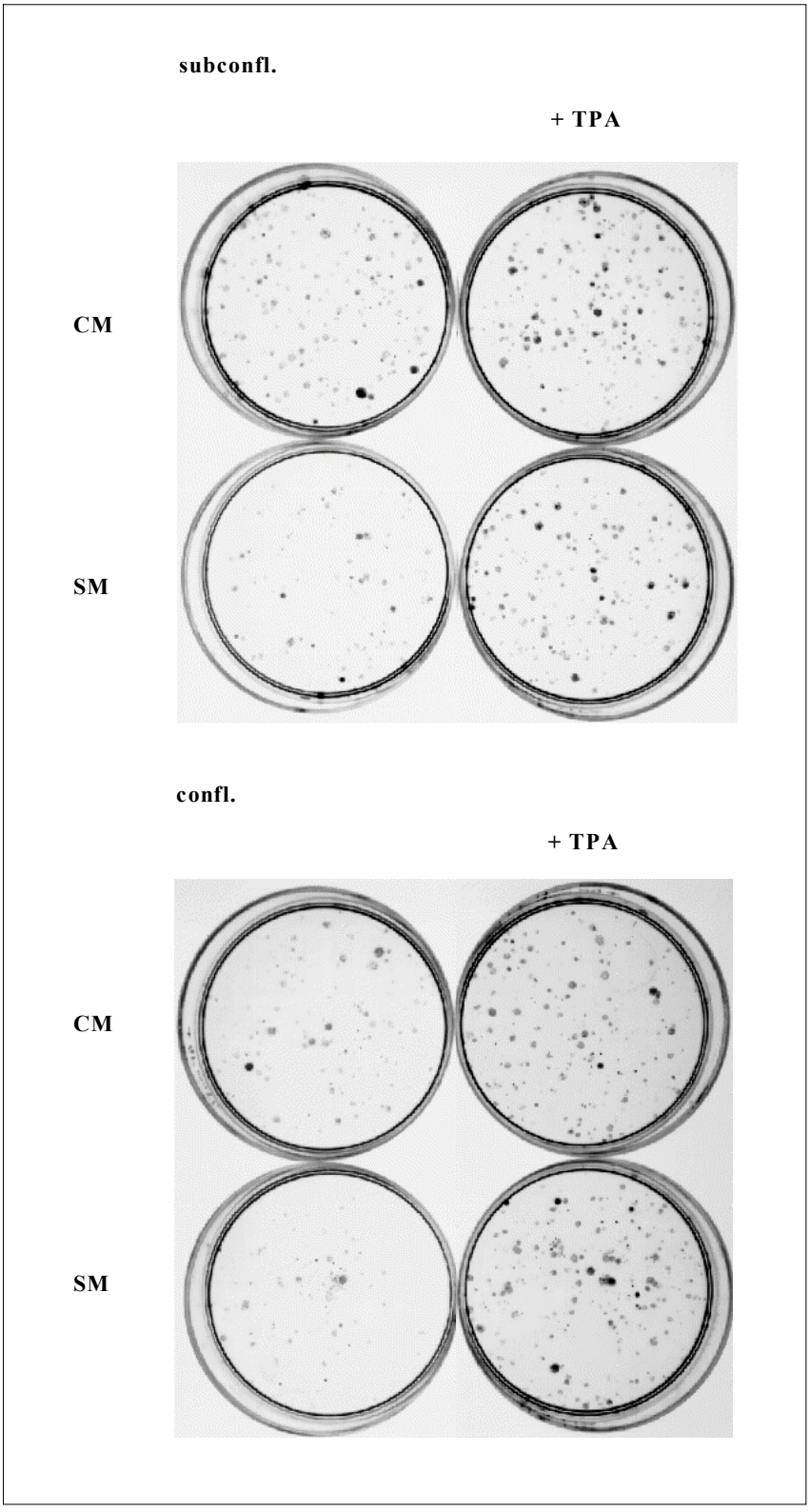


Figure 10. TPA increases clonogenic potential of HaCaT cells. Clonal growth assay of the effect of TPA on HaCaT cells previously cultured in complete medium (CM) or in standard medium (SM) for 24 hours, prior to addition of TPA to SM or CM containing cultures for another 24 hours. HaCaT cells were exponentially proliferating in subconfluent culture (A) or at stationary phase of growth in confluent culture (B).

3.2.3 TPA suppresses expression of keratin 1, 10 and involucrin mRNA in HaCaT cells induced to differentiate through growth factor withdrawal

The effect of TPA- and TPA+EGF-addition to subconfluent cultures of HaCaT cells expressing K 1, K 10 and involucrin mRNA was examined by Northern Blot analysis. Both, TPA- and TPA+EGF-exposure of HaCaT in standard medium suppressed mRNA levels of these differentiation markers **(Figure 11)**.

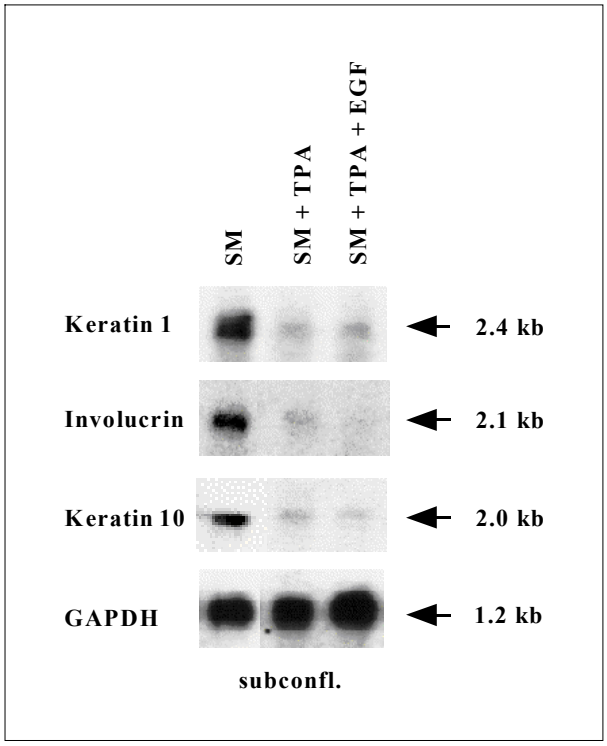


Figure 11. Keratin 1, 10 and involucrin mRNA expression suppressed by EGF and TPA. Keratin 1, 10 or involucrin mRNA transcripts and GAPDH transcripts probed in poly(A)RNA preparations isolated from cultures initiated simultaneously, plated at $3x10^2$ cells/cm^2 and cultivated in complete medium (CM) until cultures reached subconfluence (80%). Cells were switched to standard medium for two days prior to addition of EGF (10 ng/ml), TPA (10 ng/ml) or EGF and TPA for 24 hours before Poly(A)RNA extraction. Samples (4.4 µg) were subjected to northern analysis by hybridization with the specific probes.

These results suggest that TPA is a potent suppressor of K 1, K 10 and involucrin mRNA expression in HaCaT cells at subconfluent cell densities similar to EGF **(Figure 6)**. Concomitant with the suppressive effect of TPA on differentiation marker expression, HaCaT clonogenic potential is increased **(Figure 10)** and thus HaCaT cells maintain proliferative potential despite commitogenic signals.

3.2.4 TPA suppresses expression of keratin 1 in both normal human keratinocytes and HaCaT cells at culture confluence

Culture confluence is known to induce keratin 1 mRNA expression of normal human keratinocytes (NHK) proliferating autonomously in standard medium without growth factors or in the presence of growth factors in complete medium (Poumay and Pittelkow, 1995). Confluent NHK cultures expressing K 1 were exposed to TPA and compared to HaCaT cells. Northern blot analysis shows that in both, HaCaT cells and NHK, TPA actively suppresses K 1 mRNA expression induced by culture confluence in complete- or standard medium **(Figure 12)**.

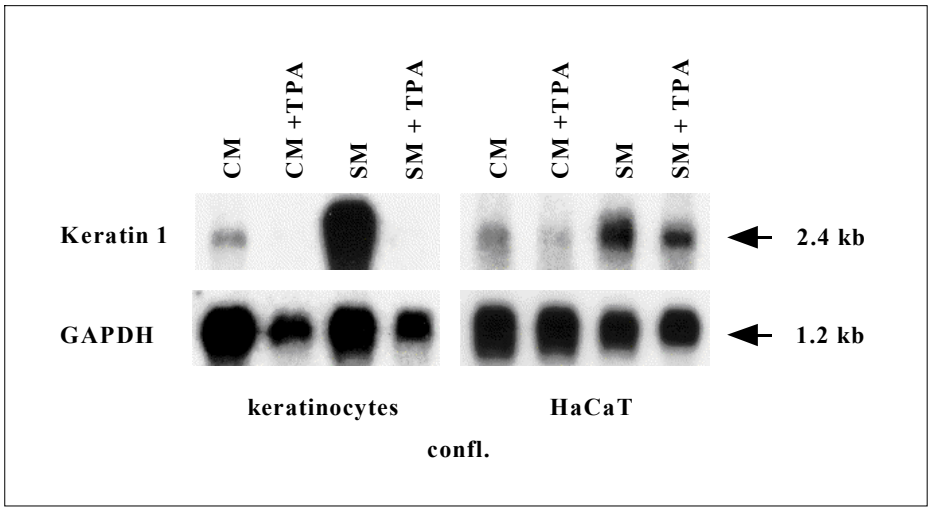


Figure 12. Keratin 1 mRNA expression suppressed by TPA in both, HaCaT and NHK. Normal human keratinocytes (NHK) and HaCaT were grown to confluence in complete medium (CM), switched or standard medium (SM), SM containing TPA (10 ng/ml), or CM containing TPA for 2 days. Poly(A)RNA was harvested and samples were analyzed by northern blot hybridization with the specific probes to K 1 or GAPDH.

These results suggest a similarly controlled regulation of keratin 1 expression of both cell types in response to TPA at culture confluence. However, this effect of TPA is rather modulative in NHK in contrast to HaCaT, since NHK become irreversibly committed to terminal differentiation after TPA treatment (Wille *et al.*, 1985).

3.3 Growth and differentiation characteristics of HaCaT cells transfected with dominant-negative c-jun

3.3.1 pmm 67 HaCaT express dominant-negative c-jun protein

pmm 67 HaCaT transfectants and the neo HaCaT control cell line were analyzed for TAM 67 protein expression by metabolic labeling followed by immunoprecipitation. The 29 kilodalton TAM 67 protein was expressed by pmm 67 HaCaT exclusively **(Figure 13)**.

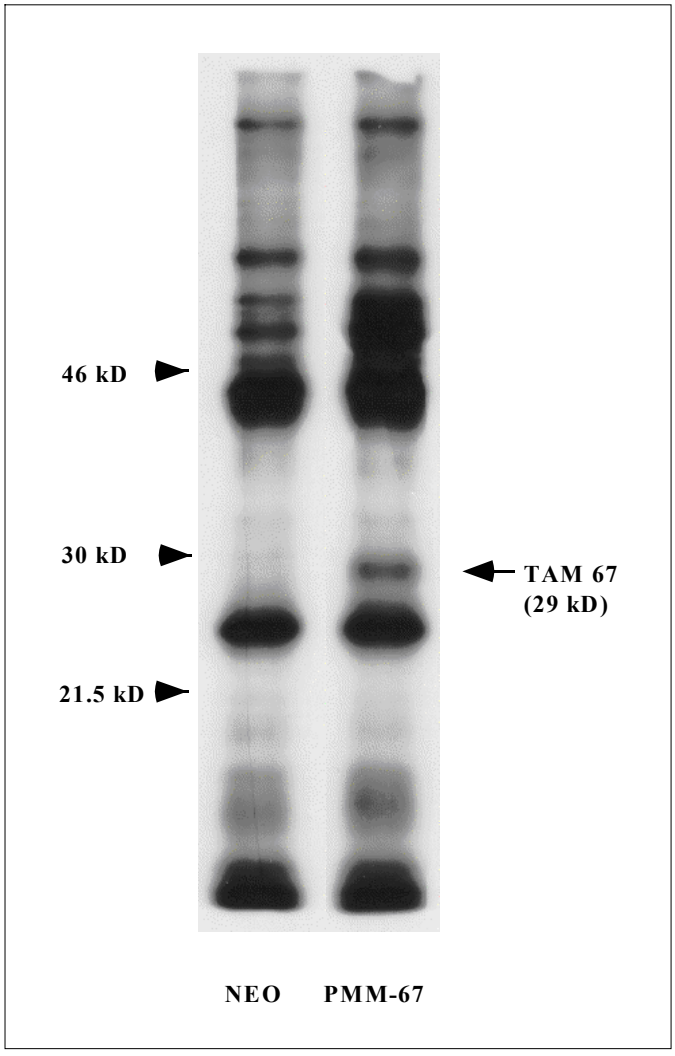


Figure 13. Truncated c-jun protein expression of pMEXMTX-TAM 67 HaCaT. ^{35}S-metabolically labeled cell extract from pmm 67 transfectants and vector only transfectants (neo), immunoprecipitated with c-jun/AP-1 antibody and separated by SDS-PAGE followed by autoradiography.

3.3.2 pmm 67 HaCaT and neo HaCaT control exhibit similar proliferation and differentiation characteristics

Proliferation kinetics of pmm 67 transfectants and the neo HaCaT control cell line were analyzed by cell enumeration. Both cell lines exhibited similar growth behavior in both, standard medium and complete medium (data not shown).

Northern Blot analysis of differentiated keratin 1 and 10 mRNA expression of both cell lines in standard- and complete medium revealed no differences (data not shown). These results suggest that dominant-negative mutant of c-jun fails to alter proliferation and differentiated keratin expression of HaCaT in both standard and complete medium.

3.3.3 TPA-mediated suppression of keratin 1 and 10 expression is abrogated in pmm 67 transfectants

The effects of TPA- and EGF-mediated suppression of keratin (K) 1 and 10 expression were examined in subconfluent pmm 67 transfectants in comparison with neo HaCaT control by Northern Blot analysis. EGF actively suppressed K 1 and K 10 mRNA levels in both cell lines at subconfluency **(Figure 14)**. However, TPA failed to suppress K 1 and K 10 mRNA expression in pmm 67 transfectants in contrast to neo HaCaT control. These results demonstrate that dominant-negative c-jun is sufficient to inhibit TPA-mediated suppression of K 1 and K 10 gene expression in subconfluent HaCaT cells in growth-factor depleted medium.

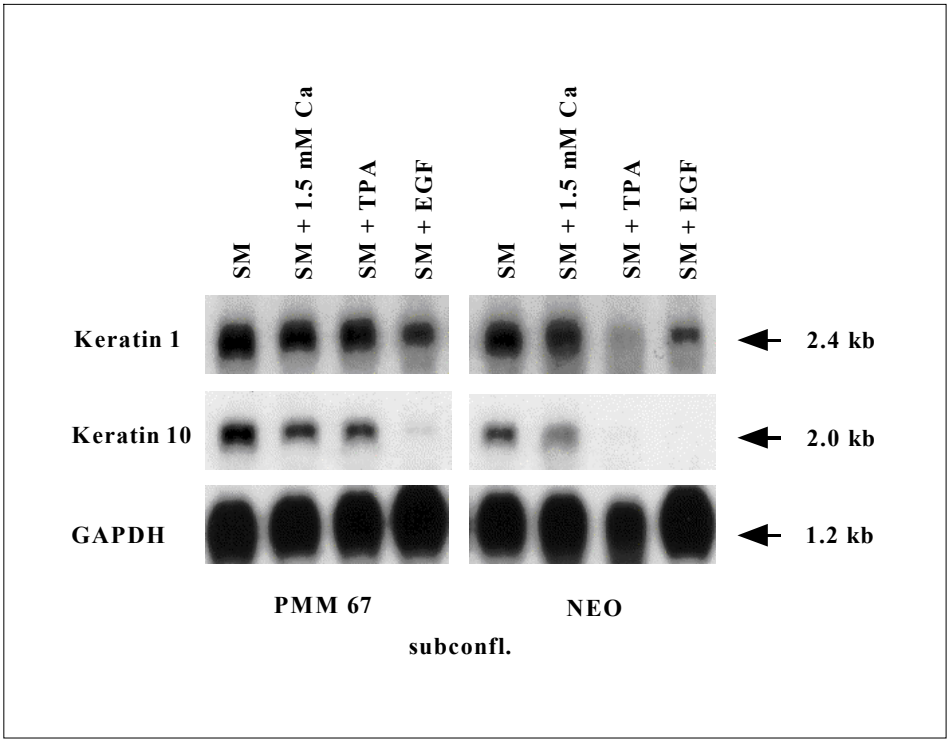


Figure 14. Effects of Ca^{2+}, TPA or EGF on K 1 and K 10 mRNA expression of pmm 67 HaCaT. pmm 67 or neo HaCaT expressing K 1 and K 10 at subconfluency were treated with 1.5 mM Ca^{2+} containing standard medium (SM), TPA (10 ng/ml) or EGF (10 ng/ml) for 24 hours. mRNA was harvested and subjected to Northern Analysis

4 DISCUSSION

4.1 HaCaT growth, differentiation and viability characteristics

Establishment of human and rodent keratinocytes in culture has provided useful models to examine cellular control of growth and differentiation and mechanisms of immortalization and transformation (Wille *et al.*, 1984; Shipley and Pittelkow, 1987; Watt, 1988; Fuchs, 1993; Fusenig *et al.*, 1995; Yuspa *et al.*, 1996). The HaCaT cell line is a well characterized epithelial cell type to investigate regulatory defects associated with immortalization (reviewed in Fusenig *et al.*, 1995; Fusenig and Boukamp, 1998). The present investigations report that HaCaT cells can be propagated in serum-free MCDB 153 culture medium with supplements. Growth and differentiation of HaCaT were examined under specific culture conditions similar to those reported for normal keratinocytes. These studies show that HaCaT are dependent on exogenous growth factors, specifically EGF or insulin, for viability and proliferation. HaCaT cells express differentiation-specific markers, including K 1, K 10 and involucrin upon growth factor withdrawal. These events accompany cell cycle growth arrest in the G1 phase that are coupled to irreversible loss of clonogenicity and commitment to terminal differentiation.

These findings contrast with rapidly growing, basal-like normal human keratinocytes that retain clonogenic potential in the presence or absence of exogenous growth factors at subconfluence in either low (0.1 mM) or high (1.5 mM) medium calcium concentrations (Poumay and Pittelkow, 1995). Sustained growth at subconfluent density in the absence of exogenous EGF or insulin has been termed “autonomous growth” (Pittelkow *et al.*, 1993). Transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding growth factor (HB-EGF) and other EGF-related ligands are the major autocrine factors that mediate autonomous proliferation in normal keratinocytes (Cook *et al.*, 1991b; Pittelkow *et al.*, 1993; reviewed in Piepkorn *et al.*, 1998).

The present study provides evidence that HaCaT cells proliferate autonomously, however their growth rate is markedly less compared to normal keratinocytes. Several different mechanisms may explain the differences observed for HaCaT versus normal keratinocytes. HaCaT might be defective in their regulation of autocrine growth

control. Game *et al.* (1992) have shown that HaCaT cells express TGF-α in serum-containing medium. Decreased expression or suppression of positive growth regulators (eg. TGF-α, AR, HB-EGF etc.) may follow withdrawal of exogenous growth factors in serum-free conditions. Either EGF or insulin restores exponential proliferation in MCDB 153 medium similar to growth in complete medium. The phenomenon of auto-induction and cross-induction of EGF-related growth factor expression has been observed in normal keratinocytes and other epithelial cells, some of which are immortalized or transformed (Coffey *et al.*, 1987; Barnard *et al.*, 1994).

Another mechanism may involve TGF-β, a potent negative growth regulator, that was shown to be constitutively expressed by HaCaT in serum-containing culture and was activated by physiological calcium concentration (Kato *et al.*, 1995). At low calcium concentration, in serum-free, growth factor-free medium, TGF-β may be activated and inhibit HaCaT proliferation.

HaCaT proliferation in serum-free culture medium may be dependent on EGF-receptor (EGF-R) activation. Normal human keratinocytes lose clonogenic potential and become committed to terminal differentiation when human EGF-R-1 (HER-1) is inhibited by a neutralizing monoclonal antibody (Mab), or HER-1 activation is blocked by the potent and specific tyrosine kinase inhibitor, PD 153035 (Peus *et al.*, 1997). These results suggest that keratinocyte growth and differentiation are mediated largely, if not solely, through HER-1. HER-1 expression and activity in HaCaT has not been thoroughly investigated. This study demonstrates that EGF is sufficient to stimulate HaCaT mitogenesis. However, for HaCaT, growth factor withdrawal might trigger down-regulation of EGF-R and induce terminal differentiation similar to HER-1 blockade by Mab or PD 153035 treatment of normal human keratinocytes.

Induction of terminal differentiation-specific markers and loss of clonal potential upon growth factor withdrawal at subconfluent density in HaCaT cultures is coupled to a decrease in BrdU labeling (21% vs 49 %) following a short pulse (2hour) exposure. Interestingly, a longer exposure of 26 hours labeled only 29 % of cells in standard medium vs. > 90 % in complete medium. A possible explanation for these findings is the preferential loss of cells that initiate DNA synthesis and incorporate BrdU label during culture in medium depleted of growth factors. These HaCaT cells may be selectively eliminated by programmed cell death.

Recent studies have shown that Bcl-X_L, a member of the Bcl-2 family, represses apoptosis and is expressed at high level in keratinocytes (Yin *et al.*, 1994; Chittenden *et al.*, 1995; Wrone-Smith *et al.*, 1995). Stoll *et al.* (1998) showed that both TGF-α and EGF increase the expression of Bcl-X_L in quiescent keratinocytes and HaCaT, and blockage of the EGF-R by PD 153035 induces apoptosis. Further studies by Jost *et al.* 1999 extend these findings and provide evidence that in contrast to downregulation of Bcl-X_L, pro-apoptotic members of the Bcl-2 family are not downregulated following EGF-R blockage in HaCaT cells. The present study shows that growth factor removal, alone, arrests HaCaT, induces differentiation, and increases the fraction of cells exhibiting features typical of apoptosis. Their finding that EGF-R inhibition induces apoptosis supports the observation in this study that HaCaT undergo apoptosis in standard medium. HaCaT express TGF-α and other EGF-related ligands (Game *et al.*, 1992; unpublished data from Dr. Pittelkow´s laboratory) that may be suppressed following growth factor removal, unlike normal keratinocytes (Cook *et al.*, 1991b; unpublished data). As a result, expression of anti-apoptotic factors, such as Bcl-X_L, may be down-regulated. The results presented in this study are also consistent with the finding that TGF-α upregulates suppressors of apoptosis that counteract TNF-α induced programmed cell death of HaCaT cells (Reinartz *et al.*, 1996).

Game *et al.* (1992) presented evidence that tumorigenic ras clones express higher mRNA levels of TGF-α than HaCaT cells while the number of high affinity EGF receptors increased with tumorigenic potential. In addition low levels of receptor-binding TGF-β were produced compared to non-ras-transformed HaCaT. Other studies revealed that through long-time culture of HaCaT (119 passages) this cell line has gained serum-independent growth potential in contrast to growth arrest in the absence of serum at low passages where non-tumorigenic behaviour was maintained (Boukamp *et al.*, 1997). In addition to the results presented in this investigation these findings suggest evidence that through long-time culture or through tumorigenic conversion by ras transfection, growth regulation of HaCaT is abrogated.

HaCaT have mutations in both alleles of *p53* (Lehmann *et al.*, 1993), similar to those found in a high percentage of skin carcinomas and premalignant lesions (Brash *et al.*, 1991; Ziegler *et al.*, 1993, 1994). These mutations are felt to be an initiating event in skin carcinogenesis (Boukamp *et al.*, 1995). With subsequent transformation events in

HaCaT, such as ras-mutations and chromosome loss, cells exhibit transformed phenotype and become tumorigenic. The immortalized HaCaT cells are still susceptible to growth control by exogenous factors and undergo apoptosis upon growth factor withdrawal. Immortalized HaCaT cells have recently been reported to become tumorigenic when platelet-derived growth factor B (PDGF-B) is overexpressed in HaCaT cells grafted to mice (Skobe and Fusenig, 1998). EGF-related growth factors or PDGF-B may play crucial roles in the regulation of apoptosis, proliferation and later events in conversion and progression to overt carcinoma.

The studies reported here demonstrate that HaCaT exhibit many features of normal keratinocytes when cultured in MCDB 153 medium which has been optimized for keratinocyte cultivation. Exogenous growth factor requirements are more stringent for HaCaT than normal keratinocytes, and HaCaT cells are more sensitive to induction of apoptosis following growth factor removal.
These culture conditions should be useful for future studies comparing HaCaT and normal keratinocytes and elucidating the cellular-molecular mechanisms that control growth, differentiation as well as immortalization and later events in epidermal carcinogenesis such a tumor-promotion by phorbolesters (discussed in 4.2).

4.2 The effects of the tumor-promoting phorbolester, 12-O-tetradecanoylphorbol-13-acetate (TPA) on HaCaT growth and differentiation

Establishment of skin multistage carcinogenesis models, based on *in vitro*- and *in vivo*-studies, has provided important clues for understanding the development of squamous cancer (reviewed in Yuspa *et al.*, 1996; Yuspa, 1998; Fusenig and Boukamp, 1998). During the process of multistep carcinogenesis, a cell is imagined to proceed through distinct stages which are associated with several genetic and epigenetic changes including morphological alterations, chromosomal aberrations and an altered control of growth and differentiation. HaCaT keratinocytes are considered an initiated cell and such represent an early event in the multistage *in vitro* carcinogenesis context (Fusenig *et al.*, 1995; Fusenig and Boukamp, 1998).
Several investigations report that tumorigenic conversion of HaCaT cells can be achieved by mutated-ras transfection, elevated temperature, culture stress through

serum withdrawal, or mesemchymal activation (reviewed in Fusenig and Boukamp, 1998). The mechanisms which are involved in distinct events of progression of immortalized HaCaT cells to malignant clones have been subsequently illustrated using a serum-containing culture system (reviewed in Fusenig and Boukamp, 1998).

The tumor-promoting phorbolester, 12-O-tetradecanoylphorbol-13-acetate (TPA) has long been known as a potent promoter of mouse skin carcinogenesis in the two-stage and multi-stage carcinogenesis systems (Boutwell, 1978; Yuspa *et al.*, 1981). However, HaCaT cells have not been exposed to TPA and responses examined in detail.

This part of the study was conducted to characterize proliferation and differentiation characteristics of HaCaT cells in response to the tumor-promoter TPA. HaCaT responses to TPA could be directly compared to these of normal human keratinocytes (NHK) which had previously been characterized in the same serum-free culture system (Wille *et al.*, 1985). The present studies report that TPA neither promotes nor inhibits growth of HaCaT cells. However, TPA promotes clonogenicity of HaCaT cells and is sufficient to increase colony-forming potential of HaCaT under culture conditions favoring loss of clonogenicity and commitment to terminal differentiation, such as culture in standard medium or culture confluence. Concomitant with the increase in clonogenicity of HaCaT cultured in standard medium, TPA suppresses expression of the early differentiation markers keratin 1, 10 and involucrin.

These results contrast with rapidly growing NHK which are growth-arrested after treatment with TPA. This growth arrest is irreversible in that treated keratinocytes lose their colony-forming potential and become committed to terminal differentiation (Wille *et al.*, 1985).

This investigation presents evidence that HaCaT differ from NHK in lacking the growth-inhibitory and commitogenic response to TPA. Several different mechanisms may explain the differences observed for HaCaT versus NHK. Growth arrest of NHK by TPA is associated with accumulation of cells in both the G1 and G2/M phases of the cell cycle (Wille *et al.*, 1985). In contrast, HaCaT cell cycle progression is only slightly inhibited by TPA and is associated with a marginal increase in the G1 phase of the cell cycle. HaCaT labeling index and proliferation kinetics, however, remain the same after TPA treatment. A possible explanation for this finding is that HaCaT cells may have reached a degree of transformation at which they may be at least in part defective in their ability to regulate their proliferation and commitogenic responses by

TPA-sensitive cell cycle-dependent mechanisms. Similarly, a human squamous carcinoma cell line SCC-25 was reported to be insensitive to the anti-proliferative effect of TPA in serum-free culture (Wille *et al.*, 1985).

One mechanism which may explain continuous proliferation of HaCaT cells after TPA exposure may involve c-myc which is an important growth controlling transcription factor (Bouchard *et al.*, 1998). TPA is known to suppress expression of c-myc in NHK (Younus and Gilchrest, 1992). However, c-myc expression may not be inhibited by TPA in immortalized HaCaT cells and thus growth may continue.

This study presents further evidence that TPA increases HaCaT clonal growth potential. An increased growth factor expression and secretion in response to TPA may explain the increase in clonal growth of HaCaT cells. A possible mechanism may be that TPA stimulates the expression and secretion of TGF-α in HaCaT similar to NHK (Pittelkow *et al.*, 1989). TGF-α is a potent mitogen for keratinocytes in clonal growth assays and, similar to EGF, may promote clonogenicity of HaCaT. TPA strongly inhibits the clonal growth of NHK (Wille *et al.*, 1985). However, HaCaT is an immortalized cell line that differs from normal keratinocytes in lacking the growth inhibitory and commitogenic response to TPA.

NHK are induced to express the early differentiation marker keratin 1 through attainment of culture confluence (Poumay and Pittelkow, 1995). This study shows that TPA suppresses differentiated keratin 1 mRNA in NHK at culture confluence similar to HaCaT. However, in NHK this event is closely associated with loss of clonogenicity and thus with irreversible commitment to terminal differentiation (Wille *et al.*, 1985). These results imply that regulation of differentiated keratin 1 expression in response to TPA is similarly controlled in both HaCaT and NHK. Therefore altered control of proliferation and commitment in HaCaT versus normal keratinocytes appears to be the major mechanism for the differential response to TPA. In accordance with the present studies Fusenig *et al.* (1995) report that HaCaT cells maintain differentiation characteristics throughout the process of tumor progression following ras-transfection but exhibit differences in growth behavior in serum-containing medium.

Immortalized, initiated cells similar to HaCaT may exist within otherwise normal epidermis. These cells may be unrecognizable until promoting events favor their outgrowth. This study demonstrates that the tumor promoter TPA fails to promote

HaCaT proliferation but induces HaCaT cells to resist commitogenic signals such as growth factor withdrawal or culture confluence. In contrast, NHK are irreversibly growth-arrested and committed in response to TPA. *In vivo* initiated cells may retain proliferative potential in response to TPA in contrast to normal keratinocytes. Evidence is emerging to indicate an important role for signaling through the epidermal growth factor receptor in tumor progression (reviewed in DiGiovanni, 1995; Dlugosz, 1996). Recent studies report that TPA is capable to induce expression of several epidermal growth factor receptor (EGF-R) ligands such as heparin-binding epidermal growth factor (HB-EGF) and amphiregulin (AR) in mouse epidermis (Kiguchi *et al.*, 1998). In addition, the levels of HB-EGF and AR transcripts were reported to be significantly increased in primary mouse skin tumors (papillomas and squamous cell carcinomas) induced by initiation-promotion protocols. Furthermore EGF-R ligands including TGF-α, AR, HB-EGF and betacellulin (BTC), have been described to be upregulated in v-Ha-ras keratinocytes (Dlugosz *et al.*, 1995). Other studies using the mouse skin multistep carcinogenesis model report that TPA is capable of inducing expression of granulocyte-macrophage colony-stimulating factor (GM-CSF) in mouse keratinocytes, which is also known to stimulate keratinocyte proliferation (Vasunia *et al.*, 1994). These findings indicate that several growth factors signaling directly or indirectly through the epidermal growth factor receptor play an important role in tumor progression as their expression is altered at different stages in carcinogenesis. The tumor-promoter TPA upregulates several of these growth factors and also GM-CSF in mouse skin and may have a similar effect on HaCaT cells. Continuous TPA exposure may lead to sustained upregulation of EGFR ligands *in vivo* and thus could favor the clonal outgrowth of initiated cells which retain proliferative potential in response to TPA.

Furthermore intercellular communication between initiated and normal cells may in part account for the clonal expansion of initiated cells *in vivo*. Recent studies by Karen *et al.* (1999) focused on mechanisms of clonal expansion of potentially malignant HaCaT-ras-clones in response to TPA. Using an organotypic culture model which contained mixtures of genetically marked low-grade malignant HaCaT keratinocytes (HaCaT-ras-II-4) and normal human keratinocytes (NHK), they showed that clonal expansion of HaCaT-ras-II-4 cells is associated with a marked decrease in proliferation of NHK after TPA treatment. Clonal expansion of HaCaT-ras-II-4 cells was moreover temporally linked to decreased expression of keratin 1 in adjacent NHK

and could not be demonstrated for HaCaT-ras-II-4 clones when they were cultured in the absence of NHK. This study suggests that TPA may stimulate clonal expansion of potentially malignant cells by modifying normal cells adjacent to them.
Consistent with the present studies, TPA inhibited K 1 expression of NHK in the studies of Karen *et al.* (1999). In addition, the present studies provide further evidence that non-ras-transfected HaCaT cells resist the growth-inhibitory and commitogenic response to TPA in contrast to NHK and thus retain proliferative potential. However TPA fails to promote proliferation of HaCaT similar to HaCaT-ras-II-4 clones. In an organotypic culture system as utilized by Karen *et al.* (1999), HaCaT cells may be capable to expand similar to HaCaT-ras-II-4 clones after TPA treatment and expansion may be similarly associated with growth arrest and decreased expression of the early differentiation marker K 1 in NHK. As discussed above several growth factors are expressed in response to TPA in NHK and mouse keratinocytes. Similar to HaCaT-ras-II-4 clones, HaCaT cells could be stimulated to expand through growth factors expressed and secreted by adjacent normal keratinocytes as well by growth factors expressed by HaCaT in response to TPA in a co-culture model. There is evidence to suggest that modulation of intercellular communication may be responsible for continuous tumor progression. Other epidermal or dermal cells, such as fibroblasts may also express growth factors in response to tumor-promoting events which in turn may provide a tissue environment conductive for the growth of initiated cells in the skin.

In the mouse skin multistep carcinogenesis system the initiated phenotype is described to result from intrinsic changes in intracellular signalling pathways, particularly those related to terminal differentiation. Mouse keratinocytes transduced with v-ras Ha oncogene are considered initiated cells and resist the induction of terminal differentiation by activators of protein kinase C (PKC), such as TPA. Thus the differential response to phorbol esters favors the growth of an initiated subpopulation (reviewed in Yuspa *et al.*, 1994). Similarly HaCaT cells are considered initiated and they differ from NHK in that they resist the growth-inhibitory and commitogenic effect of TPA. In accordance with studies on initiated mouse keratinocytes, these studies show that TPA exhibits a differential response in HaCaT versus normal keratinocyte proliferation and differentiation thus supporting the hypothesis that HaCaT are an initiated cell line.

These studies provide the first data regarding proliferation and differentiation responses of HaCaT cells to TPA in a serum-free culture system. HaCaT responses to TPA differ mainly from these of normal human keratinocytes in that particularly proliferation and commitogenic responses are differentially affected. Future studies using this serum-free culture system and comparing HaCaT and NHK with focus on tumor promotion as well as later events in epidermal carcinogenesis should be useful to elucidate cellular-molecular mechanisms that control growth and differentiation at these stages.

4.3 A possible mechanism for generation of initiated keratinocytes *in vivo* and therapeutic strategies to block carcinogenesis at an early stage

Several studies have provided a large body of knowledge about proliferation, differentiation and cell survival of normal human keratinocytes and HaCaT cells *in vivo* and *in vitro* (Boukamp *et al.*, 1988, 1990, 1994, 1995, 1997; Pittelkow, 1992; Pittelkow *et al.*, 1993; Poumay and Pittelkow, 1995; Peus *et al.*, 1997; reviewed in Fusenig *et al.*, 1995; Fusenig and Boukamp, 1998). Based on these studies one possible mechanism for generation of immortalized, initiated cells *in vivo* and a possible way to inhibit further expansion of these cells at an early stage of tumorigenesis are hypothesized in the following: UV-light is known to cause mutations in the *p53* gene which are considered to play a major role in keratinocyte immortalization (Lehmann *et al.*, 1993). Each sunburn is associated with exposure to UV-light and causes local heat development. After each sunburn genetic mutations may accumulate and a certain number of immortalized cells may be generated. Similarly the immortalized phenotype of HaCaT cells developed during prolonged cultivation of keratinocytes in primary culture at an elevated temperature which were obtained from histologically normal skin at the distant periphery of a melanoma indicating previous sun exposure (Boukamp *et al.*, 1988).

The present study shows that HaCaT cell proliferation and cell survival are highly dependent on growth factors. The immortalized cells generated by UV-light and heat *in vivo* may be similarly dependent on growth factors which in the *in vivo* setting could be provided by adjacent normal keratinocytes or other cells. Furthermore they may be susceptible to events such as tumor-promotion by TPA favoring these cells to expand.

Further elucidation of both, mechanisms involved in immortalization and the initiated phenotype and mechanisms involved in further progression of initiated cells, may serve to design inhibitory substances to block the process of tumorigenesis by inducing an environment in which initiated cells would not survive. During the transition from a normal cell to a benignant and finally to a malignant cell, the amount of chromosomal alterations increases and the cells exhibit an increased amount of regulatory defects (Yuspa *et al.* 1996; Fusenig and Boukamp, 1998). Disturbance with inhibitory substances at an early stage in tumorigenesis is more likely to be effective than at a later stage due to better preserved regulatory mechanisms. Particular pathways may be targeted specifically with inhibitory agents.

Based on the present knowledge one inhibitory mechanism to prevent survival and expansion of immortalized cells *in vivo* will now be hypothesized: an inhibitory agent which could block the growth factor supply to immortalized cells within the epidermis could be applied onto skin affected by sunburn causing specific growth inhibition and cell death of immortalized cells before further events such as tumor-promotion by TPA would impart these cells with a growth advantage.

For example, inhibition of EGF-receptor tyrosine kinase by PD 153035 has been shown to inhibit keratinocyte proliferation in a concentration- and time-dependent manner (Peus *et al.*, 1997). In addition, blocking of the EGF receptor pathway by PD 153035 induced apoptosis of both keratinocytes and immortalized HaCaT cells (Stoll *et al.*, 1998). PD 153035 may be used to inhibit growth and induce apoptosis of immortalized cells generated within the epidermis when used at concentrations and during time periods which may not affect growth of normal human keratinocytes. In such way the development of cancer may be inhibited at one of its earliest developmental stages.

4.4 Growth and differentiation characteristics of HaCaT cells transfected with dominant-negative mutant of c-jun

c-jun/AP-1 family of transcription factors is known to regulate events involved in proliferation, differentiation and transformation of keratinocytes (reviewed in Angel and Karin, 1991; Welter and Eckert, 1995). In this part of the study HaCaT cells were transfected with a dominant-negative c-jun, TAM 67, a deletion mutant lacking the transactivation domain but retaining DNA binding and dimerization domains, to elucidate the role of c-jun for HaCaT proliferation and differentiated keratin expression. The results of the study report that proliferation kinetics and differentiation characteristics of pmm 67 transfectants were largely similar to cells transfected with neo. However, dominant-negative c-jun abrogated TPA-mediated suppression of K 10 and K 1 expression in pmm 67 HaCaT in contrast to neo HaCaT at subconfluency.

Future studies focusing on AP-1 binding- and transactivation activities will have to be performed to thoroughly asses the effect of TAM 67 on HaCaT proliferation and differentiation. However, regardless of these studies, there is evidence to suggest that other AP-1 family members than c-jun could be involved in the regulation of proliferation and keratin 1 and 10 expression in HaCaT cells. The expression of these family members will also have to be examined.

Dominant negative c-jun inhibits TPA-mediated suppression of K 10 and K 1 expression in pmm 67 HaCaT in contrast to neo HaCaT. C-jun therefore may be involved in TPA-mediated suppression of K 1 and K 10 expression in HaCaT cells. Recent studies by Sudbeck *et al.* (1999) demonstrate that TPA fails to induce c-jun and c-fos mRNA expression in HaCaT cells. Similarly in normal human keratinocyte cultures, TPA does not induce c-jun mRNA expression (Younus and Gilchrest, 1992). Furthermore Sudbeck *et al.* (1999) report that c-jun and c-fos mRNAs are not constitutively expressed in unstimulated HaCaT cultures. Therefore it is possible that preexisting c-jun proteins may be involved in TPA-mediated suppression of K 1 and K 10 expression in HaCaT cells.

AP-1 binding sites are located within the keratin 1 promoter (Lu *et al.*, 1994). TPA is a well known activator of protein kinase C which in turn activates signalling in the mitogen activated protein kinase cascade that ultimately results in phosphorylation of AP-1 transcription factors (Cano and Mahadevan, 1995). Thus in HaCaT, c-jun may be a target of phosphorylation in response to TPA and could be involved in suppression of differentiated K 1 and 10 expression. Further studies will have to be

performed to more precisely examine the effect of dominant-negative c-jun on TPA-mediated suppression of K 1 and 10 expression in HaCaT cells.

HaCaT are immortalized cells which differ from normal keratinocytes in that they are more stringently dependent on exogenous growth factors for proliferation, differentiation and viability. In addition, HaCaT and normal keratinocytes exhibit differential responses to the tumor-promoter TPA. These differences in growth and differentiation characteristics may be associated with differences in AP-1 transcription factor expression and regulation. This culture system provides a suitable *in vitro* model for future studies focusing on AP-1 transcription factor expression and regulation in HaCaT cells in comparison to normal keratinocytes.

5 SUMMARY AND CONCLUSIONS

HaCaT, a human spontaneously immortalized keratinocyte cell line, is routinely cultured in serum-containing medium. This study established *in vitro* culture of HaCaT cells in serum-free medium containing MCDB 153 with supplements. Growth and differentiation characteristics were analyzed by cell enumeration, clonal growth assays, flow cytometry, bromodeoxyuridine (BrdU) incorporation, an apoptosis assay and Northern blot techniques and compared to those of normal human keratinocytes (NHK) which are also routinely cultured in this culture medium. In complete medium with epidermal growth factor (EGF), insulin and pituitary extract, the cell generation time was 26 hours. 90% of cells cycled as measured by steady state BrdU labeling. Initial removal of exogenous growth factors (GF) increased the cell cycle time to 40 hours. Subsequently proliferation decreased markedly and cells with apoptotic features developed. By 9 days of GF absence, S phase had decreased to ≤ 5%. EGF or insulin restimulated proliferation similar to growth in complete medium. Elevation of calcium (1.5 mM) in culture medium containing or depleted of GF did not affect proliferation kinetics but increased adherence. In contrast to NHK, subconfluent HaCaT cells cultured without GF were induced to express the early differentiation transcripts keratin 1 (K 1), keratin 10 (K 10) as well as involucrin. The majority of these cells lost the ability to form clones and became committed to terminal differentiation already at subconfluence. EGF suppressed K 1, K 10 and involucrin mRNA expression in both rapidly growing and confluent cells. 1.5 mM calcium concentration induced expression of K 1 at confluence and subconfluence in complete medium.
It may be concluded that HaCaT cells can be propagated in serum-free medium with preservation of several growth characteristics of NHK. However, proliferation and differentiation of HaCaT cells are more stringently dependent on exogenous GF, and GF depletion is associated with alterations in cell survival and induction of apoptosis.

HaCaT are immortalized cells and thus are considered initiated in the context of *in vitro* multistage carcinogenesis. The tumor-promoting phorbol ester 12-o-tetradecanoylphorbol-13-acetate (TPA) was used to determine HaCaT cell growth and differentiation responses which were assessed by cell enumeration, clonal growth assays, flow cytometry, BrdU incorporation and Northern blot techniques. TPA addition to complete medium did not significantly alter the generation time or decrease the labeling index of HaCaT cells in contrast to NHK. However, TPA increased clonal

growth potential of HaCaT cells in complete medium. TPA failed to stimulate proliferation of HaCaT cells propagated in standard medium without exogenous GF but was sufficient to increase HaCaT cell clonal growth potential under these conditions. Thus at least some HaCaT cells retained proliferative potential under culture conditions favoring loss of clonogenicity and commitment to terminal differentiation. Concomitant with an increase in clonogenicity, TPA actively suppressed differentiated K 1 (similar to NHK),K 10 and involucrin mRNA expression at both, subconfluency and confluency.

These findings demonstrate that TPA-mediated effects on HaCaT cell growth and differentiation contrast distinctly to those of NHK. In particular, HaCaT cells differ from NHK in lacking the growth-inhibitory and commitogenic response to TPA. These studies indicate that TPA protects HaCaT cells from commitogenic signals such as GF withdrawal.

jun and fos of the AP-1 transcription factor family regulate genes expressed during proliferation, differentiation and tumor progression. Growth and differentiation characteristics were analyzed in HaCaT cells transfected with a dominant-negative c-jun, TAM 67 (cell line designated pmm 67 HaCaT), a deletion mutant lacking the transactivation domain but retaining DNA binding and dimerization domains and compared with cells transfected with neo (control cell line designated neo HaCaT). TAM 67 protein expression was confirmed by metabolic labeling. Proliferation kinetics and differentiated keratin 1 and keratin 10 expression were analyzed by cell enumeration and Northern Blot analysis. pmm 67 HaCaT proliferation and differentiation characteristics were largely similar to those of HaCaT cells transfected with neo. However, TPA failed to suppress K 1 and K 10 mRNA expression at subconfluency in growth factor-deficient medium of pmm 67 HaCaT compared to neo HaCaT.

These studies provide evidence that c-jun may not be involved in HaCaT cell regulation of proliferation and differentiated keratin 1 and keratin 10 expression. However, c-jun is likely involved in TPA-mediated suppression of K 1 and K 10 expression of HaCaT cells at subconfluent cell densities.

Serum-free cultivation represents an *in vitro* model for direct comparison of growth and differentiation of both immortalized HaCaT cells and NHK in which deletion or addition of certain chemicals can be performed easily and in a well-defined fashion. In this manner, the response of keratinocytes can be readily and more precisely monitored. These culture conditions should be useful for future studies comparing HaCaT cells and normal keratinocytes and elucidating the cellular-molecular mechanisms that control growth, differentiation as well as immortalization and later events in epidermal carcinogenesis.

6 ABBREVIATIONS

AP-1	Activator protein–1
AR	amphiregulin
BPE	bovine pituitary extract
BSA	bovine serum albumin
BrdU	bromodeoxyuridine
BTC	betacellulin
CM	complete medium
DAPI	4,6-Diamidin-2-phenylindoldihydrochloride
DEPC	diethylpyrocarbonate
dFBS	dialyzed fetal bovine serum
DMBA	dimethylbenzanthracene
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
FACS	fluorescence-activated cell sorter
FGF	fibroblast growth factor
GAPDH	glyceraldehyde phosphate dehydrogenase
GF	growth factor
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
G_T	cell generation time
HER1	human EGF receptor 1
HAA	high amino acids
HB-EGF	heparin binding epidermal growth factor
K 1	keratin 1

neo	pMexMTX-neo (plasmid)
neo HaCaT	designated cell line transfected with neo
NHK	normal human keratinocytes
PBS	phosphate buffered saline
PDGF-B	platelet-derived growth factor B
PI	propidium iodide
PKC	protein kinase C
pmm 67	pMexMTX-neoTAM 67 (plasmid)
pmm 67 HaCaT	designated cell line transfected with pmm 67
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SM	standard medium
TAM 67	transactivation domain mutant 67
TCA	trichloracetic acid
TEMED	N,N,N',N' Tetramethylethylenediamine
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
TNF-α	tumor necrosis factor α
TPA	12-0-tetradecanoylphorbol-13-acetate
TRE	TPA-responsive element
TSP-1	thrombospondin-1

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8 CURRICULUM VITAE

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Date / Place of birth	April 5th, 1972 / Kattowitz	
Citizenship	German	
School	1983 - 1992	High school, Düsseldorf
	May 1992	A-level
Medical Training	1993 - 2000	Heinrich-Heine-University, Düsseldorf
	Clinical electives	
	July - Sept 1995	Surgery (Thusis, Switzerland)
	Aug 96	Dermatology (Mayo Clinic, USA)
	Sept 96	Internal Medicine (Mayo Clinic, USA)
	Feb - March 98	Pediatrics (University of Capetown, South Africa)
	Research Year	
	Oct 95 - Sept 96	Mayo Clinic (Rochester, USA)
	Practical Year	
	Oct 99 - Jan 00	Surgery (University of Basel, Switzerland)
	Feb 00 - May 00	Pediatrics (University of Düsseldorf, Germany)
	May 00 - Aug 00	Internal Medicine (Queen Mary and Westfield College University of London)
Scholarships	Jan - Oct 96	scholar of the Heinrich-Hertz-Foundation of the Nordrhein-Westfalen Governments Ministry of Research, Germany
	Feb - March 98	scholar of the Dr. Carl Duisberg Foundation of the Bayer Leverkusen pharmaceutical company, Germany

Presentations	April 96, 97	poster-presentations at the meetings of the Society for Investigative Dermatology (SID) in Washington D.C., USA
Languages	English-fluent in speaking and writing German-Mother tongue Polish-Mother tongue French-for conversation Danish-for conversation	

9 SUMMARY IN GERMAN

Wachstums- und Differenzierungsverhalten einer spontan immortalisierten Keratinozytenzellinie (HaCaT) in einem definierten, serumfreien Zellkultursystem

HaCaT Zellen sind humane, spontan immortalisierte Keratinozyten, die bisher nur in serumhaltigem Medium kultiviert und charakterisiert wurden. In dieser Arbeit ist es gelungen, HaCaT Zellen in serumfreiem MCDB 153-Medium mit Zusätzen zu etablieren. HaCaT Zellen wurden hinsichtlich Proliferations- und Differenzierungskriterien analysiert und mit Normalen Humanen Keratinozyten (NHK) verglichen. Die Methoden Zellzahlbestimmung, Durchflußzytometrie, klonaler Wachstumsassay, Bromodeoxyuridine (BrdU)-Inkorporation, Apoptoseassay, Northernblot-Analyse, metabolische Markierung und Transfektion wurden hierzu in drei verschiedenen Projekten eingesetzt.

Im Kompletten Medium (KM) mit epidermalem Wachstumsfaktor (EGF), Insulin und bovinem Hypophysenextrakt betrug die Zellgenerationszeit 26 Stunden. 90% aller Zellen befanden sich im Wachstumspool, wie durch Langzeit-BrdU-Markierung gezeigt werden konnte. Nach Entzug der exogenen Wachstumsfaktoren (WF) betrug die Zellgenerationszeit 40 Stunden, das Wachstum der Zellen ging schnell zurück und es entstanden Zellen mit apoptotischen Merkmalen. Nach 9 Tagen ohne WF befanden sich weniger als 5% der Zellen in der S-Phase des Zellzyklus. Durch Zusatz von EGF oder Insulin wurde das Wachstum ähnlich wie durch KM stimuliert. Eine Erhöhung der Kalziumkonzentration auf 1.5 mM im Kulturmedium (mit oder ohne exogene WF) hatte keinen Einfluß auf das Wachstumsverhalten, führte jedoch zu einer erhöhten Adhäsion der Zellen. Im Gegensatz zu NHK exprimierten HaCaT Zellen im Kulturmedium ohne exogene WF die frühen Differenzierungsmarker Keratin 1 (K1), Keratin 10 (K10) und Involucrin. Ein großer Teil dieser Zellen verlor die Eigenschaft, Zellklone zu bilden und leitete somit die terminale Differenzierung bereits unter subkonfluenten Wachstumsbedingungen ein. EGF supprimierte die K1, K10 und Involucrin mRNA-Expression in subkonfluenten und konfluenten Zellkulturen. Bei Erhöhung der Kalziumkonzentration auf 1.5 mM exprimierten HaCaT Zellen K1 in subkonfluenten und konfluenten Zellkulturen in KM. Aus diesen Ergebnissen kann geschlossen werden, daß HaCaT Zellen in serumfreiem Medium kultiviert werden können, wobei sie viele Wachstums- und Differenzierungseigenschaften von NHK beibehalten. Allerdings sind HaCaT Zellen im Vergleich zu NHK stärker von Wachstumsfaktoren abhängig, denn deren Entzug resultiert in einem Wachstumsrückgang, der Expression von Differenzierungsmarkern und dem Auftreten von apoptotischen Zellen.
HaCaT Zellen sind immortalisierte Zellen. Dadurch werden sie im Sinne der In-Vitro-Mehrstadien-Tumorgenese im Initiationsstadium eingestuft. Die Auswirkung des Tumor-Promotors 12-o-tetradecanoyl-13-acetat (TPA) auf Wachstum und Differenzierung von HaCaT Zellen wurde im weiteren untersucht. Im Gegensatz zum zellwachstumsinhibierenden Effekt auf NHK hatte TPA keinen Einfluß auf die Zellgenerationszeit und den BrdU-Markierungsindex von HaCaT Zellen. Allerdings erhöhte TPA das klonale Wachstumspotential von HaCaT Zellen in KM. TPA konnte das Wachstum von HaCaT Zellen im Kulturmedium ohne exogene WF nicht steigern, jedoch erhöhte es auch hier das klonale Wachstumspotential. Somit konnten zumindest einige HaCaT Zellen auch nach dem Entzug von WF ihr proliferatives Potential erhalten. TPA supprimierte darüber hinaus die mRNA-Expression von K1, K10 und Involucrin im Kulturmedium ohne WF. Diese Ergebnisse zeigen, daß sich HaCaT Zellen von NHK durch ihr Ansprechverhalten auf TPA unterscheiden. TPA hatte auf HaCaT Zellen keinen zellwachstumsinhibierenden Effekt und verhinderte die Einleitung der terminalen Differenzierung in einigen HaCaT Zellen.
jun und fos aus der Activator Protein 1 (AP-1) Familie der Transkriptionsfaktoren regulieren Gene, die während des Wachstums, der Differenzierung und der Tumor-Progression exprimiert werden. HaCaT Zellen wurden mit einem dominant-negativen Deletionskonstrukt von c-jun (Transactivation domain mutant 67 (TAM 67)) transfiziert. Diese Zellreihe wurde pmm 67 HaCaT genannt. Als Kontrollzellreihe dienten HaCaT Zellen, die mit dem neo-Vektor transfiziert wurden (neo HaCaT). Die TAM 67-Proteinexpression wurde durch metabolische Markierung nachgewiesen. Viele der Wachstums- und Differenzierungskriterien der pmm 67 HaCaT Zellreihe waren mit denen der Kontrollzellreihe neo HaCaT vergleichbar. Allerdings konnte TPA die mRNA-Expression von K1 und K10 in der pmm 67 HaCaT Zellreihe im Vergleich zur neo HaCaT Zellreihe bei Subkonfluenz nicht unterdrücken. Diese Ergebnisse deuten an, daß c-jun wahrscheinlich nicht an der Wachstumsregulation, sowie der K1- und K10-Expression in HaCaT Zellen beteiligt ist. Wahrscheinlich spielt jedoch c-jun in der TPA-mediierten Suppression der K1- und K10-Expression in subkonfluenten HaCaT Zellkulturen eine wichtige Rolle.

Düsseldorf, 30.11.2000 gez.: Prof. Dr. Dr. h. c. T. Ruzicka