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Activin and TGF-β regulated genes in keratinocytes and their role in cutaneous wound repair

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1 Zusammenfassung

Die Wachstums- und Differenzierungsfaktoren Activin und TGF-β, zwei Mitglieder der TGF-β Superfamilie, spielen in der Wundheilung der Haut eine wichtige Rolle. Beide Faktoren sind in der Lage, den Wundheilungsprozess zu beschleunigen. Während bereits zahlreiche Funktionen von TGF-β während des Reparaturprozesses bekannt sind, wie z.B. die Steuerung der Synthese extrazellulärer Matrixproteine oder die Regulation der Zellmigration, ist bisher nur wenig über die Funktion von Activin bekannt.

Sowohl Activin als auch TGF-β beeinflussen den Reepithelialisierungs-Prozess. *In vitro* Experimente haben gezeigt, dass beide Faktoren unterschiedliche Effekte auf Keratinozyten ausüben. Während Activin hauptsächlich die Differenzierung dieser Zellen fördert, ist es die Hauptaufgabe von TGF-β, ihr Zellwachstum zu stoppen. Um die molekulare Wirkungsweise dieser beiden Faktoren in Keratinozyten genauer zu untersuchen, war es daher Ziel dieser Arbeit, Gene zu identifizieren, die in den Epithelzellen durch Activin oder TGF-β reguliert werden.

Hierfür wurden drei verschiedene Ansätze gewählt. Es wurden die *Differential Display Reverse Transcription* (DDRT) *PCR*-Technik verwendet und es wurde ein käuflich erwerblicher Atlas-Filter hybridisiert. Weiterhin wurden verschiedene Differenzierungsmarker auf Expressionsänderungen nach Wachstumsfaktorbehandlung untersucht. Um eine mögliche Regulation der identifizierten Zielgene durch Activin und TGF-β *in vivo* zu bestimmen, wurde deren Expression anschliessend während des Wundheilungsprozesses analysiert.

Mit Hilfe der DDRT-PCR-Technik konnte das *Keratin 15* Gen als TGF-β reguliertes Gen identifiziert werden. Keratin 15 ist ein Bestandteil des Cytoskeletts der Basalzellen vielschichtiger Epithelien. Die Expression von *Keratin 15* mRNA wurde in Keratinozyten durch TGF-β und TNF-α, wie auch in geringem Masse durch KGF und EGF reprimiert. Im Gegensatz dazu wurde die Expression von *Keratin 14*, dem bedeutendsten Typ I Keratin der Basalzellen, durch TGF-β induziert. Übereinstimmend mit den *in vitro* Daten wurde die *Keratin 15* Expression während des

Wundheilungsprozesses reprimiert, dagegen aber die *Keratin 14* Expression induziert. Mittels Immunfluoreszenz konnte gezeigt werden, dass Keratin 15 in allen, an die Wunde angrenzenden Basalzellen der Epidermis exprimiert wird, nicht aber in dem hyperproliferativen Epithel über dem Granulationsgewebe. Diese Ergebnisse zeigen, dass Keratin 15, möglicherweise als Folge der erhöhten Wachstumsfaktorexpression in der verwundeten Haut, nicht in den aktivierten Keratinozyten des verdickten Wundepithels exprimiert wird.

Die Analyse verschiedener Differenzierungsmarker nach Wachstumsfaktor-behandlung ergab, dass Mad1, der Antagonist des Transkriptionsfaktors c-Myc, durch Activin und TGF-β in Keratinozyten induziert wird. Mittels eines *solid phase DNA binding assays* konnte gezeigt werden, dass nach Wachstumsfaktor-Behandlung die Menge an DNA-gebundenem Mad1 ansteigt. In Übereinstimmung mit den *in vitro* Daten wird die *Mad1* Expression während des Wundheilungs-prozesses deutlich induziert. *In situ* Hybridisierung ergab, dass *mad1* in polymorphkernigen Leukozyten und in den suprabasalen Keratinozyten des hyperproliferativen Epithels exprimiert wird. Grössere Mengen an *mad1* mRNA konnten weiterhin in dem hyperproliferativen Epithel von Psoriasis-Patienten detektiert werden. Da Mad1 das Zellwachstum und/oder die Differenzierung verschiedener Zelltypen reguliert, sprechen diese Ergebnisse dafür, dass die Zellwachstums-inhibierenden und/oder Differenzierungs-induzierenden Funktionen von Activin und TGF-β zumindest partiell durch Mad1 vermittelt werden.

Mittels des käuflich erwerblichen Atlas-Filters konnten vier potentielle Activin-regulierte Gene identifiziert werden. Die meisten dieser Gene spielen in der Zellwachstums- und/oder Differenzierungsregulation von Keratinozyten eine Rolle. Eines dieser Gene kodiert für Id-1, ein Protein der Id-Familie. Id-Proteine fungieren als negative Regulatoren der *basic helix-loop-helix* (bHLH) Transkriptionsfaktoren. Sie fördern das Zellwachstum und inhibieren die Differenzierung der Keratinozyten. Es konnte gezeigt werden, dass die Expression der *Id-1* mRNA sowohl durch TGF-β als auch durch Activin reprimiert wird.

Mittels der DDRT-Technik wurde *Leu-13* als falsch positives TGF–β reguliertes Gen identifiziert. Hierbei handelt es sich um ein durch Interferon induzierbares Oberflächenprotein, das das Zellwachstum und die Zelladhäsion verschiedener Zellarten reguliert. Untersuchungen ergaben, dass *Leu-13*, das bisher nicht als in Keratinozyten exprimiertes Gen beschrieben wurde, in diesen Zellen durch wachstumsinhibierende Cytokine wie TNF-α induziert wird. Um eine mögliche Regulation von *Leu-13* durch Cytokine *in vivo* zu bestimmen, wurde die *Leu-13* Expression während des Wundheilungsprozesses analysiert. Sie stieg nach Verwundung signifikant an. Mittels *in situ* Hybridisierung wurde deutlich, dass *Leu-13* mRNA in den Entzündungszellen des Granulationsgewebes zu finden ist. Untersuchungen der *Leu-13* Expression in der entzündlichen Darmerkrankung Morbus Crohn ergaben, dass *Leu-13* sehr stark in den, in das kranke Gewebe einwandernden, Entzündungszellen exprimiert wird. Diese Ergebnisse weisen daraufhin, dass die *Leu-13* Expression ein Merkmal inflammatorischer Prozesse ist und möglicherweise als Entzündungsmarker verwendet werden kann.

Zusammenfassend kann man sagen, dass Activin, im Gegensatz zu TGF-β, in Keratinozyten nur eine geringe Zahl an Genen reguliert. Fast alle der identifizierten Gene kodieren für Proteine, die die Proliferation und Differenzierung von Keratinozyten beeinflussen. Dies weist darauf hin, dass die Regulation der Differenzierung und/oder Proliferation die Hauptaufgabe von Activin in diesem Zelltyp ist. TGF-β dagegen kontrolliert die Expression zahlreicher Proteine, die verschiedenste Funktionen übernehmen und scheint daher eine deutlich umfassendere Aufgabe in Keratinozyten zu übernehmen.

2 Summary

Activin and TGF-β, two members of the TGF-β superfamily, are important modulators of tissue repair. Both can accelerate the wound healing process. Whereas various functions of TGF-β during the repair process are known, including for example promotion of extracellular matrix synthesis and regulation of cell migration, little is as yet known about the function of activin.

Both growth factors are important for the reepithelialization process of wounds. However, *in vitro* experiments have revealed differences in the action of activin and TGF-β on keratinocytes. Activin mainly induces the differentiation of keratinocytes, whereas TGF-β stops the proliferation of these cells. To get more information about the molecular mechanisms of activin and TGF-β action in keratinocytes, I attempted to identify genes, which are regulated by these two growth factors. For this purpose, three methods were used: the differential display RT-PCR technology and the hybridization of a cDNA array. Furthermore, the expression of known differentiation markers was analyzed after growth factor treatment. To determine a potential role of activin and TGF-β in the regulation of these genes *in vivo*, we analyzed their expression during the wound healing process where high levels of both growth factors are present.

Using the differential display RT-PCR technology, we identified *keratin 15* as a gene that is downregulated by TGF-β1. Keratin 15 is a component of the cytoskeleton of basal cells in stratified epithelia. Expression of *keratin 15* was also suppressed by TNF-α and to a lesser extent by EGF and KGF. In contrast, the major basal type I keratin K14 was upregulated by TGF-β and not regulated by the other growth factors. Consistent with the *in vitro* data we found a suppression of *keratin 15* during the wound healing process, whereas *keratin 14* expression was weakly upregulated. Immunostaining revealed the presence of K15 in all basal cells of the epidermis adjacent to the wound, but not in the hyperproliferative epithelium above the granulation tissue. These results demonstrate that *keratin 15* expression is excluded from the activated keratinocytes of the hyperthickened

wound epithelium, possibly as a result of increased growth factor expression in injured skin.

Analyzing various differentiation markers for their growth factor regulation, we identified Mad1, the antagonist of the transcription factor c-Myc, as the product of an activin A and TGF-β1 upregulated gene. Expression and activity of Mad1 was strongly induced by both factors, although the intensity of the induction was different for activin and TGF-β. To determine a possible role of both factors in the regulation of *mad1* expression *in vivo*, we analyzed *mad1* expression during cutaneous wound repair when high levels of these factors are present. Expression of *mad1* mRNA and protein, but not of other *mad* genes, increased significantly after skin injury, particularly in polymorphonuclear leukocytes and in suprabasal keratinocytes of the hyperproliferative epithelium. Elevated levels of *mad1* mRNA were also detected in the hyperproliferative epithelium of patients suffering from the inflammatory and hyperproliferative skin disease psoriasis. Since Mad1 regulates proliferation and/or differentiation of various cell types, our results indicate, that it mediates, at least in part, the proliferation-inhibiting and/or differentiation-inducing activities of activin and TGF-β.

Hybridization of a cDNA array with cDNAs from quiescent and activin A-regulated keratinocytes revealed four potentially activin regulated genes. Most of them are involved in the regulation of proliferation and/or differentiation of keratinocytes. We particularly analyzed the expression of Id-1, a protein of the Id-protein family. Id proteins are negative regulators of basic helix-loop-helix (bHLH) transcription factors and, therefore, promote cell growth and inhibit differentiation of various cell types. We could demonstrate a downregulation of *id-1* mRNA expression by activin A and TGF-β1 in keratinocytes.

Using the differential display RT-PCR technology, we identified *leu-13* as a false positive TGF-β regulated gene. Leu-13 is an interferon-induced cell surface protein which triggers growth inhibition and cell adhesion of various cell types. During our studies it turned out that *leu-13* is expressed by keratinocytes, in particular upon

treatment of these cells with TNF-α and interferon-α. This indicates that Leu-13 plays a role in the inhibition of keratinocyte proliferation. To analyze a potential regulation of *leu-13* by cytokines *in vivo*, we investigated *leu-13* expression during the tissue repair process. *Leu-13* expression was significantly upregulated after skin injury. *In situ* hybridization revealed expression of *leu-13* in inflammatory cells of the granulation tissue. Furthermore, we investigated the expression of *leu-13* in the inflammatory bowel disease Crohn's disease. We could demonstrate an expression of *leu-13* mRNA in the infiltrating inflammatory cells of the gut.

Our studies demonstrate a strong upregulation of *leu-13* expression in inflammatory processes, suggesting that *leu-13* expression can be used as an inflammatory marker.

In summary, the results presented in this thesis demonstrate that activin regulates, very few genes in keratinocytes. Most of these genes are involved in the regulation of proliferation and differentiation of this cell type. This result indicates that the major function of activin in keratinocytes is the regulation of differentiation and/or proliferation. By contrast, TGF-β was shown to regulate a large number of genes which encode proteins involved in various cellular processes, indicating that TGF-β regulates multiple functions in keratinocytes.

3 Introduction

3.1 Structure and wound repair of the skin

3.1.1 The structure of the skin

The skin (*Cutis)* is the largest organ of the human body and comprises an area of about 2 m^2 in the adult. It is composed of two tissue layers, the dermis and the epidermis. The function of the skin is the protection against mechanical and physical damage, water loss and germs, but it is also responsible for the regulation of the temperature, the sense of touch, the sense of temperature and the secretion of different substances via glands.

The **epidermis** is a stratified, squamous epithelium. It mainly consists of keratinocytes, but also contains melanocytes (pigment cells), Langerhans cells, which are cells of the immune system, and Merkel cells, which are derivatives of the nervous system. The epidermis undergoes continuous renewal. Only keratinocytes of the basal, innermost epidermal layer have the capacity to synthesize DNA and to undergo mitosis. After an as yet unknown signal, a basal cell starts a journey to the surface of the epidermis. During this journey it undergoes a series of morphological and biochemical changes. At the end of this process, it is dead, flattened and enucleated. It will be sloughed from the surface and replaced by an inner cell, which also runs through this program. The whole process, which lasts about 30 days, is called "terminal differentiation".

The epidermis can be subdivided into four layers (Fig.1). Keratinocytes in each layer have a characteristic differentiation status, which is reflected by the expression pattern of keratins. Keratins are part of the cytoskeleton and their major function is the mechanical stabilization of the epidermis. Cells of the basal layer express the keratins K5 and K14. The next layer, the suprabasal, *spinous layer* (prickle cell layer), contains postmitotic cells which are still metabolically active.

The keratinocytes express the keratins K1 and K10. Hallmarks of the basal and spinous cells are desmosomes, which are calcium-activated membrane junctions. They interconnect the cells into a three-dimensional lattice.

Afterwards cells reach the *granular layer*. They stop the synthesis of keratins and make their final change in protein expression. They now produce filaggrin, loricrin and various other proteins that form the cornified envelope. The keratinocytes start to loose their nuclei and their cytoplasmatic organelles.

The most upper layer is the *stratum corneum.* This layer contains terminally differentiated keratinocytes. The cells are sealed together by lipids, forming an impermeable, insoluble and highly protective layer, which keeps microorganisms out and body fluids in (Fuchs, 1990).

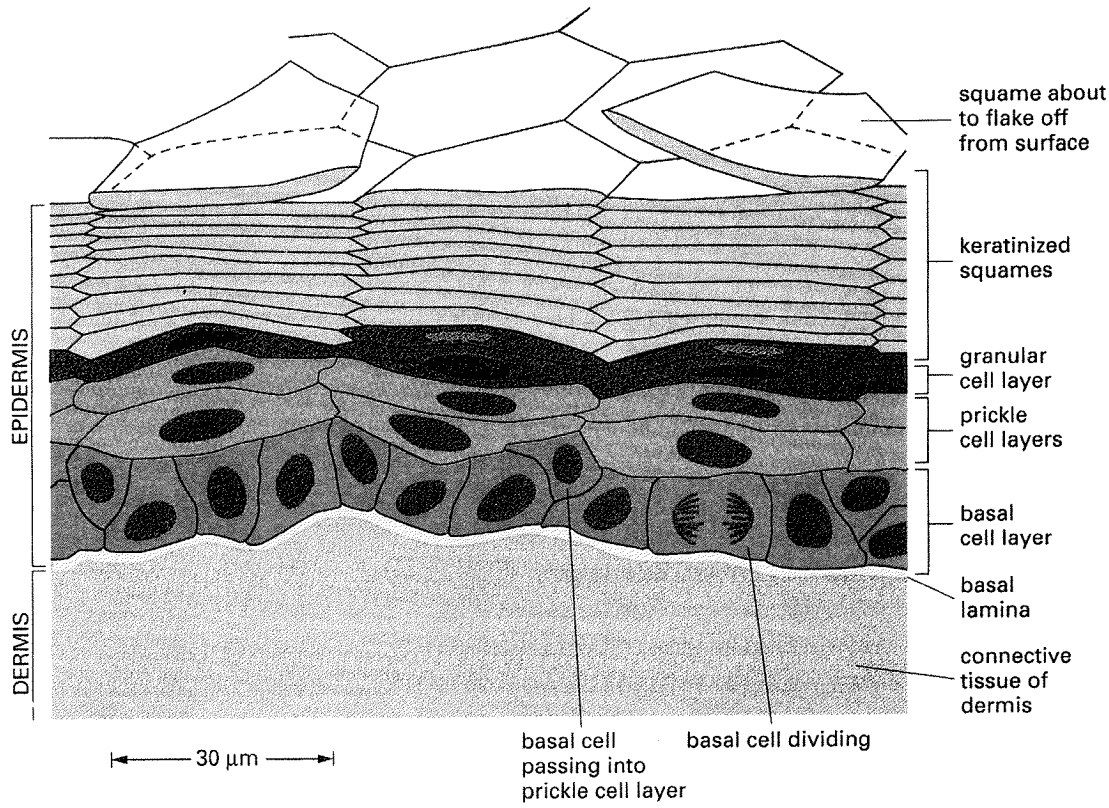

Fig. 1 Structure of the epidermis (from Alberts *et al.*, 1994)

The **dermis**, which is separated from the epidermis by a basal lamina, is a collagen-rich connective tissue. Besides the collagen bundles it contains a network

of elastic fibers, which gives the skin the resilience and tensile strength. The major cell population of the dermis are fibroblasts, which produce most of the extracellular matrix molecules. The dermis also contains blood and lymph vessels, nerve cell axons, mast cells, macrophages and cutaneous appendages that are originally derived from the epithelium. Appendages are hair follicles including the sebaceous glands, sweat glands and claws or nails. A loose connective tissue (subcutaneous fat layer) connects the dermis to the underlying tissue.

3.1.2 Cutaneous wound repair

The skin functions as a protective barrier against the environment. If an injury occurs, it needs to be rapidly and efficiently repaired. Wound repair is a complex process, which requires the interaction of many different cell types. A temporary wound closure is achieved by formation of a clot, which plugs the wound. Afterwards the tissue is regenerated in several steps, which last several days. The wound repair process can be temporally subdivided into three phases: the inflammatory phase, the phase of new tissue formation and the tissue remodelling phase which are partially overlapping (Clark, 1996, Martin, 1997).

Inflammatory phase

Most injuries to the skin cause blood vessel disruption and a leakage of blood constituents into the tissue. Blood coagulation generates a clot, which plugs and protects the wound area and can function as a matrix for cell migration. The clot is a network composed mainly of fibrin which is generated by thrombin through cleavage of fibrinogen. It also contains small amounts of plasma fibronectin, vitronectin and thrombospondin. Platelets are embedded in the mesh. When they degranulate they release multiple mediators, including growth factors and cytokines. These factors initiate the wound healing process by attracting inflammatory cells, such as neutrophils and monocytes, to the wound area. Neutrophils have the function to clean the wound from foreign particles, including bacteria. Furthermore, they are a source of pro-inflammatory cytokines, which

activate fibroblasts and keratinocytes (Hübner *et al.*, 1996c). The infiltrated monocytes become activated in the tissue and become macrophages. They phagocytose cell debris and the neutrophils that have undergone apoptosis. Activated macrophages also release various growth factors and cytokines (reviewed by Clark, 1996).

Phase of new tissue formation

One or two days after injury the epidermal keratinocytes at the wound edge as well as the keratinocytes from injured hair follicles start to migrate into the wound area and subsequently proliferate (Fig. 2). The stimulus for these processes is as yet unclear. It is speculated that the absence of neighbouring cells at the wound margin triggers the proliferation and migration ("free-edge effect"). Another possibility is that locally released growth factors induce the epidermal proliferation and migration. Indeed, the expression of various growth factors including EGF, TGF-α, KGF, TGF-β and activin is upregulated in the wound area (reviewed by Martin, 1997), and at least the inhibition of KGF signalling was shown to delay reepithelialization (Werner *et al.*, 1994).

In unwouded skin, basal keratinocytes are anchored to the basal lamina via hemidesmosomes. Before the migration process starts, the hemidesmosomes have to be dissolved. Furthermore, the leading-edge keratinocytes have to express new cell adhesion molecules including specific integrins, which help them to migrate over the dermis and the provisional wound matrix. During their migration process keratinocytes have to cut a path through the fibrin clot. Therefore, the expression of matrix-metalloproteinases and tissue-type plasminogen activator is upregulated in the wound-edge keratinocytes. The latter cleaves plasminogen within the clot, generating the fibrinolytic enzyme plasmin (reviewed by Martin, 1997).

When the wound surface is covered by keratinocytes, epidermal cells stop to migrate and a new stratified epidermis with underlying basal lamina is created. Suprabasal cells cease to express integrins and basal keratins and start their standard differentiation program.

A new stroma, often called granulation tissue, begins to form 3-4 days after injury. The granulation tissue consists of new blood vessels, inflammatory cells, fibroblasts and loose connective tissue. Soon after injury, the fibroblasts at the wound edge start to proliferate. 3-4 days after injury they begin to migrate into the provisional wound matrix of the clot. Thereby they synthesize their own collagen-rich matrix. Growth factors present at the wound site, like PDGF and TGF-β can act as mitogens or as a chemoattractants for the wound fibroblasts (Erkisson *et al.*, 1992, Heldin and Westermark, 1996, Frank *et al.*, 1996, Roberts and Sporn, 1996).

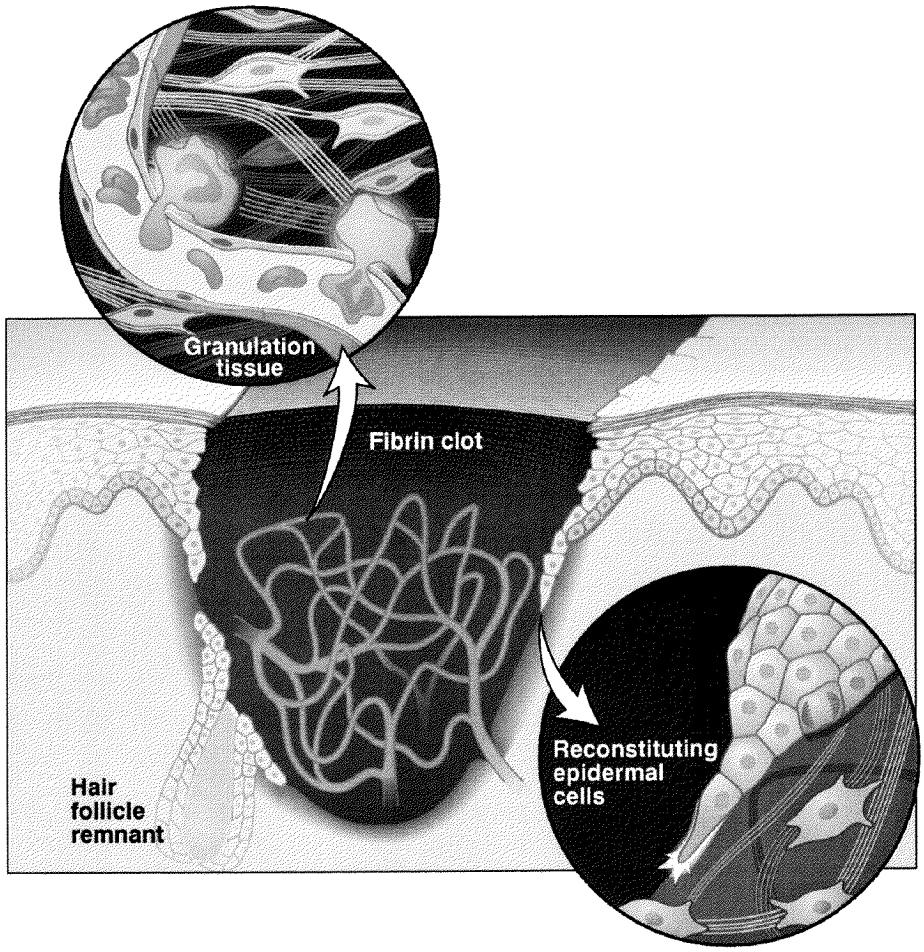

Fig. 2 Cartoon illustrating the key players in the healing of the wound (Martin, 1997).

Two different growth factors, bFGF and VEGF mainly induce new blood vessel formation (angiogenesis). bFGF is released by damaged endothelial cells and

macrophages, whereas VEGF (Vascular endothelial growth factor) is expressed by activated keratinocytes at the wound edge and also by macrophages (Broadley *et al.*, 1989, Abraham and Klagsbrun, 1996). To allow the migration of endothelial cells into the wound area, the cells have to upregulate the expression of special cell-adhesion molecules such as integrins.
Furthermore, the fibrin clot and the new connective tissue need to be partially degraded.

Tissue remodelling phase
About one week after injury, the wound is filled with granulation tissue. Then the fibroblasts transform, triggered by growth factors like TGF-β but also by mechanical cues, into myofibroblasts, which resemble smooth muscle cells. They have the capacity to generate strong contractile forces. Their function is to contract the wound. If the wound is closed, the resisting forces decrease. Most of the fibroblasts return into a quiescent state, the remaining cells undergo apoptosis. During the following weeks and months the extracellular matrix is reorganized (Desmoulière *et al.*, 1993 and 1995, Desmoulière and Gabbiani, 1996).

Key players of the wound healing process are growth factors and cytokines which regulate various steps of this complex system. Two important modulators during tissue repair are the growth and differentiation factors activin and TGF-β which will be introduced in the following chapter.

3.2 Activin and TGF-β - members of the TGF-β superfamily

3.2.1 The transforming growth factor-β (TGF-β) superfamily

The TGF-β superfamily consists of a large variety of factors with diverse activities. It can be grouped into four families, including the TGF-β family (TGF-βs 1-5), the inhibin family (inhibins and activins), the DPP/VG1 family (bone morphogenetic proteins (BMPs) and others) and the Mullerian Inhibiting Substance family (MIS)

(reviewed by Massague *et al.*, 1990). The TGF-β superfamily members trigger cellular responses through binding to heteromeric complexes of specific type I and type II serine/threonine kinase receptors. Up to now five type II receptors and seven type I receptors have been identified. Ligand binding mediates the formation of heteromeric receptor complexes. Type II receptors are constitutively active kinases and, after ligand binding, they phosphorylate specific serine and threonine residues of the type I receptor, leading to type I receptor activation. The activated type I receptor phosphorylates a receptor-regulated Smad-protein (R-Smad), which recruits a common-partner Smad (Co-Smad). The Smad complex enters the nucleus, binds directly or indirectly through other DNA-binding proteins to DNA and regulates transcription of target genes (reviewed by Itoh *et al.*, 2000). Each specific TGF-β superfamily member binds to specific type I and type II receptors and the receptor activation leads to phosphorylation of specific Smad-proteins.

3.2.2 Activin

Activin consists of two polypeptide chains which are disulfide linked. Three isoforms of activin, the homodimeric activin A ($\beta_A\beta_A$) and activin B ($\beta_B\beta_B$), and the heterodimeric activin AB ($\beta_A\beta_B$) have been described, and, recently, three other β-chains, β_C, β_D, and β_E, have been identified (for review see Yu and Dolter, 1997). Activin was first described as a protein of gonadal origin, which stimulates the production of the follicle stimulating hormone (FSH) from pituitary cells (Ling *et al.*, 1985). Independently, it was isolated as an erythroid differentiation factor from human monocytic leukemia cells (Eto *et al.*, 1997). Today, it is known that activin is produced by a lot of different cell types and influences proliferation and differentiation of various target cells (Vale *et al.*, 1990). Activin plays an important role during early embryogenesis where it acts as a mesoderm-inducing factor (reviewed by Ariizumi and Asashima, 1995). Furthermore it could be shown, by generating mice lacking activin or its receptors, that activin has multiple functions in

organogenesis (Feijen *et al.*, 1994, Verschueren *et al.*, 1995 and Matzuk *et al.*, 1995a).

Up to now, two inhibitors of activins are known. Activins are antagonized by inhibins, which counteract activin action, and by the soluble activin-binding protein follistatin, which binds to activin and thereby inhibits the activation of the signalling receptor (reviewed by Massagué, 1990).

3.2.2.1 Activin action on keratinocytes

In vitro experiments have shown that activin influences proliferation and differentiation of keratinocytes. Addition of activin A to cultured keratinocytes inhibited keratinocyte proliferation (Shimizu *et al.*, 1998, Seishima *et al.*, 1999), although high concentrations were required for this inhibitory effect compared to TGF-β. Activin A also induced differentiation of cultured keratinocytes. Application of activin A lead to upregulation of differentiation markers, including transglutaminase 1, loricrin, keratin 1 and involucrin (Seishima *et al.*, 1999).

3.2.2.2 Activin in the skin

Both activin chains, β_A and β_B, are expressed in murine skin. Activin β_A mRNA was detected at low levels in the dermis, but it is not expressed in the epidermis. The expression of the β_B mRNA is significantly higher than the expression of the β_A mRNA. β_B mRNA was found at highest levels in the dermis and at lower levels in the epidermis (Hübner *et al.*, 1996).

The importance of activin expression in the skin was demonstrated by different knockout and transgenic mouse models. Besides various defects which caused death within the first 24h after birth, mice deficient in activin βA lacked whiskers and the whisker follicles were abnormal (Matzuk *et al.*, 1995a). Mice lacking follistatin have an abnormal whisker development and hyperkeratotic skin (Matzuk *et al.*, 1995b). Activin β_A transgenic mice, which overexpress the β_A chain in the

epidermis under the control of a keratin 14 promoter, showed severe abnormalities in the skin. The epidermis of these mice was remarkably thickened, mainly due to an increased spinous layer. The epidermal architecture was disorganized. In the dermis, subcutaneous fatty tissue was replaced by connective tissue. Furthermore, the transgenic mice showed increased proliferation and abnormal differentiation of the keratinocytes (Munz *et al.*, 1999). Mice overexpressing follistatin under the control of the keratin 14 promoter showed an opposite phenotype to the activin β_A overexpressing mice. The epidermis and the dermis of these mice were slightly thinner in comparison to control mice. In addition the coat appeared rough and irregular (Wankell *et al.*, submitted). Mice overexpressing follistatin under the control of a metallothionein I-promoter showed gross hair abnormalities. Their hair was shiny and irregular. However, the histological analysis of the skin did not reveal any obvious reasons for this phenotype (Guo *et al.*, 1998).

3.2.2.3 The role of activin in wound healing

Activin expression is strongly upregulated during the wound healing process. The mRNA expression of both activin chains, β_A and β_B, is induced, but the time course of expression is different for both chains and they are expressed in different wound areas. In mice, the expression of the β_A chain is upregulated one day after wounding and declines at day 5 to day 7 after injury to its basal level. The mRNA is expressed in mesenchymal cells of the granulation tissue adjacent to the hyperproliferative epithelium and below the eschar. The activin β_B mRNA is upregulated one day after wounding, but it has still not returned to its basal level at day 13 after injury. The mRNA of the β_B chain is expressed in the hyperproliferative epithelium and in the migrating epithelial tongue (Hübner *et al.*, 1996). The reason for the upregulation of activin has not clearly been identified. *In vitro* studies showed that serum growth factors and cytokines induce activin expression in cultured keratinocytes and fibroblasts. Thus it is possible, that serum growth factors, derived from the blood upon hemorrhage, and macrophage-derived

proinflammatory cytokines, are at least partially responsible for the activin induction *in vivo* (Hübner and Werner, 1996). The function of activin during the wound healing process has recently been explored. Munz *et al.* (1999) demonstrated, that mice, overexpressing activin β_A in the epidermis under the control of a keratin 14 promoter showed accelerated wound healing. 5-day wounds of these mice have a remarkably larger amount of granulation tissue and show increased reepithelialization. Interestingly, mice overexpressing follistatin in the epidermis under the control of a keratin 14 promoter showed the opposite phenotype. They have a severely impaired wound healing. 5-day wounds of these animals have less granulation tissue and a thinner wound epidermis (Wankell *et al.*, submitted). These data suggest an important role of endogenous activin in granulation tissue formation and reepithelialization.

3.2.3 Transforming growth factor-β (TGF-β)

Today five TGF-βs (TGF-β 1-5) are known. Three of them (TGF-β1-3) are found in mammals (Assoian *et al.*, 1983; Cheifetz *et al.*, 1987; Seyedin *et al.*, 1987; ten Dijke *et al.*, 1988a, *Derynck et al.*, 1988), whereas TGF-β4 (Jakowlew *et al.*, 1988a, b) and TGF-β5 (Kondaiah *et al.*, 1990) have only been identified in chicken or *Xenopus laevis* respectively. TGF-βs consist of two identical amino acid chains, which are disulfide linked. Each chain is synthesized as a 390 amino acid precursor, which contains a secretory polypeptide and a hydrophobic signal sequence for the translocation across the endoplasmatic reticulum (Derynck *et al.*, 1985). After secretion, the cleaved pro-region remains associated with the TGF-β dimer and they form a latent complex. The proteolytic processing of the latent form releases bioactive TGF-β. TGF-β has a lot of different functions in many cell types and tissues. It can either inhibit or stimulate the proliferation of different target cells (Trucker *et al.*, 1984, Moses *et al.*, 1985, Roberts *et al.*, 1985). TGF-β also plays an important role in the regulation of cell-adhesion. It upregulates cell-cell and cell-matrix adhesion molecules. Furthermore, it induces the synthesis and prevents the

degradation of extracellular matrix molecules (reviewed by Massague, 1990). TGF-β regulates the differentiation of target cells. It acts as a positive or negative regulator on the differentiation of various types of cells (Green and Meuth, 1974, Seyedin *et al.*, 1987).

The importance of TGF-β for the mammalian organism was demonstrated by the generation and analysis of knockout mice lacking the different isoforms. TGF-β1 knockout animals are viable, but three weeks after birth, they develop a lymphocyte-based inflammatory process, which shortly afterwards leads to death (Kulkarni *et al.*, 1995, Diebold *et al.*, 1995). TGF-β2 knockout mice die during or immediately after birth. Their early death is caused by heart and blood vessel abnormalities (Sanford *et al.*, 1997). Lack of TGF-β3 leads to neonatal lethality, caused by abnormal lung development and cleft palate (Kaartinen *et el.*, 1993, Proetzel *et al.*, 1995).

3.2.3.1 TGF-β action on keratinocytes

In vitro experiments have shown that TGF-β regulates the proliferation of keratinocytes, but its role in differentiation has yet not been defined. Using the epithelial cell line BALB/MK, it was shown that addition of TGF-β1 reversibly inhibits growth of these cells (Coffey *et al.*, 1988a). The suppression of cell growth by TGF-β can be at least partially explained by a downregulation of proliferation inducing proteins, like c-Myc or KC (Coffey *et al.*, 1988b). The role of TGF-β in the differentiation process of keratinocytes remains up to now unclear. Saunders and Jetten (1994) showed a suppression of differentiation by TGF-β, whereas Mansbridge *et al.* (1988) suggested that TGF-β might be important for regulating the balance between cell growth and maturation.

3.2.3.2 TGF-β and the skin

All three mammalian isoforms of TGF-β are expressed in the skin. In mouse skin TGF-β1 and TGF-β2 are strongly expressed in the dermis and to a much lesser extent in the epidermis. In contrast, TGF-β3 is only expressed in the dermis and its expression level is much lower than the levels of the other two TGF-β isoforms (Frank *et al.*, 1996).

The importance of TGF-β expression in the skin was demonstrated by different transgenic mouse models. Transgenic mice which overexpress TGF-β1 in the epidermis under the control of the keratin 1 promoter showed skin abnormalities, which lead to death during the first 24h after birth. The skin of the mice appeared shiny and tautly stretched. Histological analysis demonstrated that the transgenic animals have less hair follicles, a slightly thinner interfollicular epidermis and they reveal orthohyperkeratosis. Furthermore, suppression of keratinocyte proliferation and an alternative differentiation were detected in the TGF-β1 overexpressing mice (Sellheyer *et al.*, 1993). These results were confirmed with transgenic mice, which inducibly expresses TGF-β1 in the epidermis. The inducible system prevented the transgenic mice from early death and allowed long-term studies (Wang *et al.*, 1999). However, contradictory results were obtained when TGF-β1 was over-expressed under the control of a keratin 10 or keratin 6 promoter. These mice showed an increased epidermal mitotic rate, but no histological abnormalities (Cui *et al.*, 1995, Frowlis *et al.*, 1996).

3.2.3.3 The role of TGF-β during the wound healing process

TGF-β is one of the most important modulators of the tissue repair process. It is stored at high levels in platelets and is expressed by several cell types in the wound area, including activated macrophages, keratinocytes and fibroblasts (Assoian *et al.*, 1983, Assoian *et al.*, 1987, Kane *et al.*, 1991, Levine *et al.*, 1993, Nath *et al.,* 1994, Tsnunawaki *et al.*, 1998*).* All three mammalian isoforms of TGF-β

are upregulated during the wound healing process. In mice, TGF-β1 mRNA is strongly upregulated at 1 day after wounding and remains high for several days after injury. TGF-β2 and TGF-β3 mRNA upregulation occurs mainly during the phase of granulation tissue formation and reepithelialization. TGF-β2 was upregulated at day 5 after wounding and remained high for several days, the maximal induction of TGF-β3 was observed at day 7 after injury (Frank *et al.*, 1996). The expression pattern of all three isoforms in the wound is different. TGF-β1 mRNA is mainly expressed by migrating fibroblasts and macrophages at the wound edge and in the granulation tissue. TGF-β2 mRNA expression can be detected in the granulation tissue and TGF-β3 mRNA is mainly found in the hyperproliferative epithelium of the wound (Frank *et al.*, 1996).

There are several known functions of TGF-β during the repair process. It functions as a promoter of extracellular matrix deposition and regulates cell migration. TGF-β can act as a chemoattractant for monocytes (Wahl *et al.*, 1987) and fibroblasts (Postlethwaite *et al.*, 1987). Furthermore, Roberts *et al.* (1986) demonstrated, that TGF-β induces new blood vessel formation.

The importance of the TGF-βs for the wound healing process was demonstrated in different expriments. Application of TGF-β to wounds accelerated wound healing in normal and of healing-impaired rats (Mustoe *et al.*, 1987, Pierce *et al.*, 1989, Beck *et al.*, 1991 and 1993). Furthermore, Frank *et al.* (1996) demonstrated, that mice, which have wound healing defects caused by glucocorticoid treatment, show an aberrant expression of the TGF-β genes. Disturbing the ratio of the three TGF-β isoforms during the wound healing process by injection of ligands or neutralizing antibodies, had a significant influence on the repair process. TGF-β1 and -β2 induced connective tissue formation and scarring, wheras TGF-β3 caused the opposite phenotype (Shah *et al.*, 1994, Shah *et al.*, 1995). Furthermore, wound healing studies were performed with different knockout and transgenic mice. TGF-β1 knockout mice die 3 weeks after birth because of a lymphocyte-based inflammatory process. Cutaneous wounds on 10-day old TGF-β1-deficient mice healed normally until the inflammatory disease interferred with the wound healing

process (Brown *et al.*, 1995). Therefore, immunodeficient TGF-β1 knockout mice (TGF-β1-/- Scid-/-) were generated, which also allowed to study the later stages of the wound healing process. These mice showed a delay in each of the three major phases of the repair process (Crowe *et al.*, 1999). Surprisingly, mice lacking Smad 3, an intracellular mediator of TGF-β function, had accelerated wound healing, characterized by increased reepithelialization and a reduced infiltration of monocytes (Ashcroft *et al.*, 1999). Furthermore, mice overexpressing TGF-β1 under the control of the albumin promotor were generated to study the effect of elevated circulating TGF-β1 levels on the wound healing process. Interestingly, these mice showed reduced scarring and developed a better architecture of the neodermis (Shah *et al.*, 1999).

3.2.4 Comparison of activin and TGF-β

As described above a series of *in vitro* and *in vivo* experiments have been performed to investigate the function of activin and particularly of TGF-β on keratinocytes in normal skin and during the wound healing process. Besides some similarities, various differences could be demonstrated. *In vitro* experiments showed that activin strongly induces the differentiation but has a minor influence on proliferation of keratinocytes (Shimizu *et al.*, 1998, Seishima *et al.*, 1999). In contrast, TGF-β strongly inhibited the proliferation of these cells (Coffey *et al.*, 1988a). Its role in keratinocyte differentiation is, however, still unclear. A significant difference in activin and TGF-β action in the skin could be demonstrated through the generation of transgenic mice, which overexpress activin A or TGF-β1 in the epidermis. Activin A transgenic mice showed increased keratinocyte proliferation, and the epidermis of the skin was thickened (Munz *et al.*, 1999). TGF-β1 overexpressing cells had an opposite phenotype. Keratinocyte proliferation was suppressed and the epidermis was hyperkeratotic (Sellheyer *et al.*, 1993). Activin and TGF-β actions during the repair process are complex and have only partially been explored. The temporal and spatial expression pattern of these factors is

different in the wound. Increased levels of activin in the skin accelerated the healing process (Munz *et al.*, 1999), whereas the results obtained with TGF-β are controversial. In most cases, a stimulatory effect of TGF-β on repair was described (Mustoe *et al.*, 1987, Pierce *et al.*, 1989, Beck *et al.*, 1991 and 1993). However, a direct comparison of both factors in the same experimental setup is still missing.

3.3 Questions and aims of this thesis

Activin and TGF-β play an important role during the tissue repair process. Although they differently affect proliferation and differentiation of keratinocytes, they both accelerate the wound healing process. Therefore, it would be interesting to learn more about the function of activin and TGF-β during tissue repair and to reveal similarities and differences in activin and TGF-β action.

To gain insight into the molecular mechanisms of activin and TGF-β action on keratinocytes, I attempted to identify genes which are regulated in keratinocytes by activin A and TGF-β1. This was first done in cell culture experiments using the HaCaT-cell line, a human immortalized, but not transformed keratinocyte line. The identification of activin A or TGF-β1 regulated genes was performed using the DDRT-PCR technology or by hybridization of an Atlas cDNA array.

Afterwards the expression of the identified genes during the tissue repair process was analyzed using RNase protection assay, immunofluorescence and *in situ* hybridization.

4 Materials and Methods

4.1 Material

4.1.1 Chemicals and consumables

Acetic anhydride	Fluka Chemie AG, Buchs
Acetic acid	Fluka Chemie AG, Buchs
Acrylamide/bisacrylamide	Roth, Karlsruhe, Germany
Agarose SeaKem ME	Biozym, FMC, Rockland, USA
Ammonium persulfate (APS)	Sigma, Munich, Germany
Ampicillin	Sigma, Munich, Germany
Aprotinin	Sigma, Munich, Germany
[α^{32}P]-dATP (3000 Ci/mmol, 10 mCi/ml)	Amersham, Braunschweig, Germany
[α^{32}P]-rUTP (800 Ci/mmol; 20 mCi/ml)	Amersham, Braunschweig, Germany
[α^{32}S]-rUTP (1000 Ci/mmol; 10 m Ci/ml)	Amersham, Braunschweig, Germany
Bacto agar	Difco, Detroit, USA
BactoTryptone	Roth, Karlsruhe, Germany
Boric acid	Roth, Karlsruhe, Germany
5-Bromo-6-chloro-3-indolyl-β-D-galactoside (X-Gal)	Roth, Karlsruhe, Germany
Bromophenol blue	Sigma, Munich, Germany
BSA (Bovine serum albumin)	Sigma, Munich, Germany
Cell culture plastic materials	Nunc, Roskilde, Denmark
Chloroform	Fluka Chemie AG, Buchs
Chromatography paper (3MM)	Whatman, Maidstone, GB

Cryotubes (for freezing cells)	Nunc, Roskilde, Denmark
Denhardt`s solution (50x)	Sigma, Munich, Germany
Dextran sulfate	Sigma, Munich, Germany
Diethylpyrocarbonate (DEPC)	Sigma, Munich, Germany
D, L-Dithiothreitol (DTT)	Sigma, Munich, Germany
DNA marker	MBI Fermentas, St. Leon-Rot, Germany
dNTPs	Roche, Rotkreuz
Dulbecco`s *Modifies Eagle`s Medium* (DMEM)	Gibco BRL, Paisley, Scotland
EDF/EDP photochemicals	Kodak, Rochester, USA
Eosin	Sigma, Munich, Germany
Ethanol	Fluka Chemie AG, Buchs
Ethidium Bromide (EtBr)	Sigma, Munich, Germany
Ethylenediamine tetraacetic acid (EDTA)	Roth, Karlsruhe, Germany
Fetal calf serum (FCS)	Gibco BRL, Paisley, Scotland
Formaldehyde	Fluka Chemie AG, Buchs
Formamide	Fluka Chemie AG, Buchs
Gene Screen Membrane	NEN, Boston, USA
Glycerol	Merck, Darmstadt, Germany
Guanidine thiocyanate (GSCN)	Sigma, Munich, Germany
[γ^{32}P]-ATP	Hartmann Analytics, Braunschweig, Germany
Hematoxylin solution	Sigma, Munich, Germany
Hybond^{TM}-N^{+}-membrane	Amersham, Braunschweig
Hydroxychinolin	Roth, Karlsruhe, Germany
Isopropylthiogalactoside (IPTG)	Roth, Karlsruhe, Germany
β-Mercaptoethanol	Fluka Chemie AG, Buchs
Mowiol	Hoechst, Frankfurt, Germany
Nitrocellulose Transfer Membrane (Protran)	Schleicher & Schuell GmbH,

	Dassel, Germany
NTB-2 film emulsion	Kodak, Rochester, USA
Nucleoside triphosphate	Roche, Rotkreuz
Paraformaldehyde (PFA)	Sigma, Munich, Germany
Penicillin	Sigma, Munich, Germany
Phenol	Fluka Chemie AG, Buchs
Phenylmethylsulfonylfluoride (PMSF)	Sigma, Munich, Germany
Piperazine-N,N'-bis[2-ethenesulfonic acid] (PIPES)	Sigma, Munich, Germany
Ponceau S solution	Sigma, Munich, Germany
Potassium chloride	Merck, Darmstadt, Germany
Protein marker (broad range)	Bio-Rad, Richmond, USA
RNasin	Promega, Madison, USA
Scintillation solution	Packard, Groningen, Netherlands
Scott water	Sigma, Munich, Germany
Sephadex G-50-columns (nick column™)	Pharmacia, Uppsala, Sweden
Sephadex G-50-columns (MicroSpin™)	Pharmacia, Uppsala, Sweden
Slides Super Frost	Shandon, Frankfurt, Germany
Sodium azide	Sigma, Munich, Germany
Sodium chloride	AnaLaR, Poole, UK
Sodium citrate	Fluka Chemie AG, Buchs
Sodiumhydroxide (NaOH)	Fluka Chemie AG, Buchs
Sodium N-lauroylsarcosine	Sigma, Munich, Germany
Sodium phosphate	Fluka Chemie AG, Buchs
S/P Accu Mount 60 mounting medium	Baxter, Riverdale, USA
Streptomycin	Sigma, Munich, Germany
N,N,N',N'-Tetramethylendiamine (TEMED)	Bio-Rad, Richmond, USA
Tissue freezing medium	Jung, Nussloch, Germany
Tris[hydroxymethyl]aminomethane	Sigma, Munich, Germany
Triton X-100	Sigma, Munich, Germany

t-RNA (from *E.coli* RNase free)	Roche, Rotkreuz
Tween20	Sigma, Munich, Germany
Urea	AnaLaR, Poole, UK
Xylene cyanol	Sigma, Munich, Germany
Xylene	Synopharm, Ofingen
Yeast extract	Difco Laboratories, Detroit, USA

Chemicals not listed above came from Fluka Chemie, Merck, or Sigma.

4.1.2 Enzymes

DNA polymerase I (Klenow fragment)	Roche, Rotkreuz
DNA restriction endonucleases	Roche, Rotkreuz
DNase RQ1	Promega, Madison, USA
Pronase E	Sigma, Munich, Germany
Proteinase K	Roche, Rotkreuz
Reverse transcriptase (Superscript Plus™)	Gibco BRL, Paisley, Scotland
RNA polymerases (T3, T7 and SP6)	Roche, Rotkreuz
RNase A	Roche, Rotkreuz
RNase T1	Roche, Rotkreuz
Taq DNA polymerase	Pharmacia, Uppsala, Sweden
T4 DNA ligase	Roche, Rotkreuz

4.1.3 Antibodies

anti-Keratin 6 (rabbit polyclonal)	BabCo, Richmond, USA
anti-Keratin 14 (mouse monoclonal)	Serotec, Oxford, UK
anti-Keratin 15 (rabbit polyclonal)	Dr. T. Magin, Bonn, Germany
anti-Tec 3 (rat polyclonal)	Dianova, Hamburg, Germany
anti-Mad1 (rabbit polyclonal)	Santa Cruz Biotechnology Inc.,

	USA
anti-guinea pig IgG-Texas Red	Dianova, Hamburg, Germany
anti-mouse IgG-FITC	Sigma, Munich, Germany
anti-rabbit IgG-FITC	Roche, Rotkreuz
anti-rabbit IgG-HRP	Promega, Madison, USA
anti-rat IgG-FITC	Dianova, Hamburg, Germany

4.1.4 Growth factors and cytokines

Activin A	Dr. Denis Gospodarowicz, Chiron Corporation, Emeryville
Epidermal growth factor (EGF)	Roche, Rotkreuz
Interferon-α	Pepro Tech EC LTD., London, England
Keratinocyte growth factor (KGF)	Roche, Rotkreuz
Transforming growth factor beta 1 (TGF-β1)	Roche, Rotkreuz
Tumor necrosis factor alpha (TNF-α)	Roche, Rotkreuz

4.1.5 Oligodesoxynucleotides and plasmids produced during this work

4.1.5.1 Universal primers

Oligo $d(T)_{15}$-Primer (for cDNA-synthesis)	Promega, Madison, USA
Universal primer (for sequencing of pBlue-script KS/SK constructs)	Pharmacia, Uppsala, Sweden
Reverse primer (for sequencing of pBlue-script KS/SK constructs)	Pharmacia, Uppsala, Sweden
Differential Display RT PCR Primer C1 and N7 – N10	described by Bauer *et al.* (1994)

4.1.5.2 Special primers

These primers were used for the amplification of cDNA-fragments using PCR. The obtained fragments were cloned into the transcription vector pBluescript KSII(+).

Primers for amplification of cDNA fragments of cytokeratins:

human keratin 14:

hK14-1 5'-AGCTCCATGAAGGGCTCCTG-3'

hK14-2 5'-CATGGTCACCTTCTCACTGC-3'

The obtained fragment corresponds to nucleotides 94-417 of the published sequence (Marchuk *et al.*, 1985).

human and mouse keratin 15:

hK15-1 5'-GGAGGTGGAAGCCCAAGTAT-3'

hK15-2 5'-GAGAGGAGACCACCATCGCC-3'

The obtained fragment corresponds to nucleotides 173-366 of the published human sequence (Leube *et al.*, 1988). The mouse fragment corresponds to nucleotides 1457-1632 of the published sequence (Nozaki *et al.*, 1994), except 1621, where A is changed against T.

Primers for amplification of a cDNA fragment of Leu-13:

mouse Leu-13:

mLeu13-1 5'-CGTCTAGACAGAAGATGCACAAGGAGGA-3'

mLeu13-2 5'-GGACCAGACGACATGGTCGG-3'

The sequence for mouse *leu-13* is unknown. The primers were chosen based on the published human sequence (Deblandre *et al.*, 1995).

Primers for amplification of cDNA fragments of Max-dimerization partners:

human mad1:

hmad1-1 5'-GCATCGGCTCCACCGTCTCC-3'

hmad1-2 5'-AAGGAGACAGCCGCAGTGCC-3'

The amplified fragment corresponds to nucleotides 581-840 of the published sequence (Ayer *et al.*, 1993). The plasmid was kindly provided by Dietmar Beer.

mouse mad1:

mmad1-1 5'-CATACAGCAAGGACAGAG-3'

mmad1-2 5'-AGCTGGTCTATTTGGTGG-3'

The amplified fragment corresponds to nucleotides 153-411 of the published cDNA sequence (Västrik *et al.*, 1995).

human mad2/mxi1:

hmad2-1 5'-GAGGACATTGGAGATGCC-3'

hmad2-2 5'-GGATTGATCATCCACAGG-3'

The amplified fragment corresponds to nucleotides 1190-1425 of the published cDNA sequence (Zervos *et al.*, 1993).

mouse mad2/mxi1:

mmad2-1 5'-AGATGGAGCGGATACGAA-3'

mmad2-2 5'-TCCGCCCTGCACTGTTAG-3'

The amplified fragment corresponds to nucleotides 472-735 of the sequence published in the database (accession number: NM_010847).

mouse mad3:

mmad3-1 5'-TGCATATCCAGAAGCTGG-3'

mmad3-2 5'-GAGCTCTGTTTCAGTCCC-3'

The amplified fragment corresponds to nucleotides 311-558 of the published cDNA sequence (Hurlin *et al.*, 1995).

human mad4:

hmad4-1 5'-GAGCAGCTCAAGCAACTG-3'

hmad4-2 5'-AACTCCATGCCCTCTATG-3'

The amplified fragment corresponds to nucleotides 232-510 of the sequence published in the database (accession number: NM_006454).

mouse mad4:

mmad4-1 5'-GCCCGAACAACAGGTCTT-3'

mmad4-2 5'-CGCACACGCTCTACACTC-3'

The amplified fragment corresponds to nucleotides 152-425 of the published cDNA sequence (Hurlin *et al.*, 1995).

human and mouse mnt/rox:

mmnt-1 5'-GGCAAGCGCAGCAACAAC-3'

mmnt-2 5'-GCAGGGGTGGCAAGTGGT-3'

The amplified human fragment corresponds to nucleotides 256-481 of the published cDNA sequence (Meroni *et al.*, 1997). The amplified mouse fragment corresponds to nucleotides 395-608 of the published cDNA sequence (Meroni *et al.*, 1997).

mouse mmip1:

mmmip1-1 5'-TGAATCTTCCTGGAGAGG-3'

mmmip1-2 5'-TCGATGGCTGACTTGATC-3'

The amplified fragment corresponds to nucleotides 308-555 of the published cDNA sequence (Gupta *et al.*, 1998).

mouse mmip2:

mmmip2-1 5'-TGCACTCGGTGTGGAAGG-3'

mmmip2-2 5'-CGACATTACAGCTGATGG-3'

The amplified fragment corresponds to nucleotides 223-558 of the published cDNA sequence (Yin *et al.*, 1999).

human c-myc:

h-myc-1 5'-ATGCACCAGCCCCAGGTCCT-3'

h-myc-2 5'-CCTTACGCACAAGAGTTCCG-3'

The amplified fragment corresponds to nucleotides 1581-1861 of the published cDNA sequence (Watt *et al.*, 1983). The plasmid was kindly provided by Dietmar Beer.

mouse c-myc:

m-myc-1 5'-GTGCTCCAGCCCCAGGTCCT-3'

m-myc-2 5'-GTTTATGCACCAGAGTTTCG-3'

The amplified fragment corresponds to nucleotides 1629-1928 of the published cDNA sequence (Stanton *et al.*, 1984). The plasmid was kindly provided by Heike Steiling.

Primers for amplification of cDNA fragments of Id-family members:

human Id-1H:

hId-1-1 5'-TGTCTGTCTGAGCAGAGC-3'

hId-1-2 5'-GATTCCGAGTTCAGCTCC-3'

The amplified fragment corresponds to nucleotides 121-356 of the published cDNA sequence (Hara *et al.*, 1994).

human Id-2H:

hId-2-1 5'-CATGAAAGCCTTCAGTCC-3'

hId-2-2 5'-ATCTGCAGGTCCAAGATG-3'

The amplified fragment corresponds to nucleotides 96-308 of the published cDNA sequnce (Hara *et al.*, 1994).

human Id-3:

hId-3-1 5'-CATGAAGGCGCTGAGCCC-3'

hId-3-2 5'-CTGGCTAAGCTGAGTGCC-3'

The amplified fragment corresponds to nucleotides 738-936 of the published cDNA sequence (Deed *et al.*, 1994).

Primers for amplification of cDNA fragments of potentially activin A regulated genes:

human interferon regulatory factor 1 (IRF1):

hIRF1-1 5'-ACTCCCTGCCAGATATCG-3'

hIRF1-2 5'-GCATGTAGCCTGGAACTG-3'

The amplified fragment corresponds to nucleotides 454-708 of the published cDNA sequence (Maruyma *et al.*, 1989).

4.1.6 Plasmids

4.1.6.1 Vectors

pBluescript II KS(+), transcription vector Stratagene, La Jolla, USA

4.1.6.2 Other plasmids

In addition to the plasmids described in 4.1.5.2, pBluescript KSII(+)-based plasmids were generated which included the sequences obtained by DDRT-PCR or which were kindly provided by other research groups.

DDRT-PCR fragments:

pBKS(+)hk15:

Using the DDRT-PCR technology we obtained a 285 bp fragment which consists of 75 nucleotides of an unknown sequence, 91 nucleotides which correspond to nucleotides 425-515 of the published sequence and 119 nucleotides which correspond to nucleotides 1587-1705 of the published sequence (Marchuk *et al.*, 1985).

pBKS(+)hLeu13:

The fragment, obtained by differential display, corresponds to nucleotides 465-647 of the published cDNA sequence (Deblandre *et al.*, 1995).

provided plasmids:

mouse keratin 6:

290-bp-fragment, which corresponds to the 3'-untranslated region of the murine K6 mRNA. The plasmid was kindly provided by Dr. S. Yuspa, NIH, Bethesda, MD, USA.

mouse keratin14:

450-bp fragment, which corresponds to the 3'-untranslated region of the murine L14 mRNA. The plasmid was kindly provided by Dr. S. Yuspa, NIH, Bethesda, MD, USA.

CMV-Mad1 (Sommer *et al.*, 1998):

The plamid was kindly provided by Dr. B. Lüscher, University of Hannover, Germany.

4.1.7 Kits

ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit	PE Applied Biosystems, Rotkreuz
Atlas cDNA Expression Array	Clontech Laboratories, Palo Alto, CA, USA
Bio-Rad Protein Assay	Bio-Rad, Richmond, USA
ECL kit	Amersham, Braunschweig, Germany
m-RNA Separator Kit	Clontech Laboratories, Palo Alto, CA, USA
QIAGEN Plasmid Maxi Kit	Diagen, Düsseldorf, Germany
QIAexII Kit	Diagen, Düsseldorf, Germany
QIAquick Extraction Kit	Diagen, Düsseldorf, Germany
QIAquick PCR Purification Kit	Diagen, Düsseldorf, Germany
Rediprime Random Primer Labeling Kit	Amersham, Braunschweig, Germany
T7 Sequencing Kit	Pharmacia, Uppsala, Sweden

4.1.8 Bacterial strains

E.coli XI1-Blue MRF'	Stratagene, La Jolla, CA, USA
E.coli DH5α	Gibco BRL, Paisley, Scotland

4.1.9 Eukaryotic cell lines

COS	adult monkey, kidney cells	*American Type Culture Collection*, Rockville, USA
HaCaT	human adult keratinocytes (Boukamp *et al.*, 1988)	

4.1.10 Standard buffers and solutions

PBS:
140 mM NaCl
30 mM KCl
6.5 mM Na_2HPO_4
1.5 mM KH_2PO_4

TBE:
90 mM Tris/HCl (pH 8.0)
80 mM Boric acid
3 mM EDTA

20xSSC:
3 mM NaCl
0.3 mM Na_3citrate

Proteinase K:
The lyophilized enzyme was dissolved in distilled water, incubated for 30 minutes at 37°C, aliquoted and stored at –20°C.

RNase A:
100 µg enzyme were dissolved in 10 ml water, heated to 95°C for 15 min, aliquoted and stored at –20°C.

4.2 Methods

4.2.1 Cell Culture

4.2.1.1 Cultivation and storage of eukaryotic cells

HaCaT and COS cells were grown in Dulbecco`s Modified Eagle`s Medium (DMEM) containing penicillin/streptomycin (each 100 U/ml) and 10 % fetal calf serum (FCS). They were incubated at 37°C, 95 % relative humidity and 5 % CO_2. Cells were passaged after they reached confluency. For passaging cells, they were washed with sterile PBS. The cell-cell and cell-matrix contacts were disrupted by incubation with Trypsin/EDTA. The cells were resuspended in fresh medium and 5-10 % were seeded for further cultivation.

Trypsin/EDTA:	0,05 % (w/v)	Trypsin
	0.02 M	EDTA
	1 x	PBS

For long-term storage cells were trypsinized, centrifuged and resuspended in freezing medium. They were slowly frozen (ca. 1°C/min) in cryotubes and stored in liquid nitrogen.

Freezing medium:	1 x	DMEM
	20 %	FCS
	8.7 % (v/v)	glycerol

4.2.1.2 Transient transfection of COS cells

COS cells were transfected using the calcium phosphate transfection method (Chen *et al.*, 1987). $5x10^5$ cells were seeded in a 6 cm petri dish. After 12 hours the cell culture medium was changed. 4 µg of the purified plasmid was mixed with

200 µl 2x HeBS and dropwise with 200 µl $CaCl_2$ (250 mM). The mixture was added dropwise to the medium. After a 5 h incubation cells were washed with HEPES buffer and cultured for 48 hours.

2x HeBS buffer (pH 7.1):	274 mM	NaCl
	42 mM	HEPES
	9.6 mM	KCl
	1.5 mM	Na_2HPO_4
1x HEPES buffer (pH 7.3):	142 mM	NaCl
	10 mM	HEPES
	6.7 mM	KCl

4.2.1.3 Growth factor treatment of HaCaT keratinocytes

HaCaT keratinocytes were grown to confluency in Dulbecco's modified Eagle's medium (DMEM) containing 1 % Penicillin/Streptomycin and 10 % fetal calf serum (FCS). After reaching confluence the medium was removed, serum-free medium was added and cells were rendered quiescent by incubation for 16 h to 32 h. The medium was replaced by fresh serum-free DMEM and after 1/2 h the growth factor was added. Cells were harvested at different time points after growth factor addition, and used for RNA isolation or preparation of cell lysates.

The cells were treated with the following concentrations of growth factors/ cytokines :

Activin A	18 ng/ml
EGF	20 ng/ml
KGF	10 ng/ml
Interferon-α	200 U/ml

TGF-β1	1ng/ml
TNFα	300 U/ml

4.2.2 Microbiological Methods

4.2.2.1 Cultivation and storage of *E.coli* strains

E.coli suspensions were grown overnight in LB-medium (containing 100 µg/ml Ampicillin) at 37°C under shaking.

LB-medium:	1 % (w/v)	Bacto tryptone
	1 % (w/v)	Yeast extract
	0.5 % (w/v)	NaCl

E.coli cultures on LB agar plates were also incubated overnight at 37°C. For preparing the plates, 1.5 % (w/v) agar was added to the liquid LB-medium before autoclaving. After cooling down the autoclaved LB-medium to 50°C, ampicillin was added and the medium was poured into sterile 10 cm petri dishes. The plates were stored at 4°C.

If a plasmid was used, which allows to check the insertion efficiency of a DNA fragment in the plasmid by "Blue/White Screening" (for example pBluescript KSII(+)), each LB-plate was treated with 100 µl X-Gal- and 40 µl IPTG-solution before plating the bacteria.

X-Gal-solution:	2 % (w/v) X-Gal (5-Bromo-6-chloro-3-indolyl-beta-D-galactoside) in 100 % dimethylformamide (DMF)
IPTG-solution:	0.1 M IPTG in H_2O

4.2.2.2 Preparation of transformation-competent *E.coli* cells

For the preparation of transformation-competent *E.coli*-cells (DH5α and XL1-Blue) a fresh overnight culture was diluted 1:100 with LB-medium and shaken at 37°C until an absorption at 620 nm of 0.4 to 0.5 was reached. After centrifugation (2500 rpm, 10 min at 4°C) the bacterial pellet was carefully resuspended in a quarter of the original volume of cold, sterile 0.1 M magnesium chloride solution. The mixture was incubated on ice for 30 minutes, centrifuged (2500 rpm, 10 min at 4°C) and the bacterial pellet was resuspended in a fiftieth of the original volume of cold, sterile 0.1 M calciumchloride solution. The mixture was incubated 3-4 hours on ice. Cold, sterile glycerol (87 %) was added to a final concentration of 30 %. The cells were aliquoted and stored at –80°C.

4.2.3 Molecular methods

4.2.3.1 Transformation of *E. coli*–cells with plasmid DNA

200 µl transformation-competent *E. coli* cells (4.2.2.2) were mixed with a suitable amount of plasmid-DNA or a ligation mix (4.2.3.6.3) and incubated on ice for 60-90 minutes. After a heat shock (4 minutes at 37°C) the transformation mix was directly put on ice and incubated for 10 minutes. 500 µl LB-medium was added to the mixture and shaken for 30 minutes at 37°C. The bacteria were plated on 2 LB ampicillin plates (which were treated with X-Gal and IPTG if necessary (4.2.2.1)) and incubated overnight at 37°C.

4.2.3.2 Purification of nucleic acids via phenol extraction and ethanol precipitation

To purify nucleic acids from proteins, lipids and other components a phenol extraction and a following ethanol precipitation were performed.

For the phenol extraction an aqueous DNA- or RNA- solution was mixed 1:1 with phenol/chloroform (pH 7.5). The phases were separated by centrifugation (13000 rpm, 1min, RT) and the residual phenol was removed by a chloroform extraction. The nucleic acids of the aqueous phase were precipitated with 2.5 volumes of ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) at –20 °C. The precipitate was sedimented by centrifugation (13000 rpm, 15 min, 4°C).

Phenol/chloroform (pH 7.5):	4.5 volumes	phenol
	4.5 volumes	chloroform
	1 volume	1 M Tris/HCl, pH 9.5

Alternative to the phenol extraction, DNA was purified with the QIAquick-kit (Diagen, Düsseldorf).

4.2.3.3 Measurement of the concentration of nucleic acids

The concentration of DNA, RNA and oligodesoxynucleotides, dissolved in water, was analyzed by measurement of the absorbtion at 260 nm. The measurement was performed in an Eppendorf Biophotometer using a quartz cuvette and ddH_2O as a blank.

The following approximations are valid for DNA-, RNA- and oligodesoxynucleotide-concentrations:

DNA:	$OD_{260} = 1 \cong 50\mu g$ DNA/ml
RNA:	$OD_{260} = 1 \cong 40\mu g$ RNA/ml
Oligodesoxynucleotide:	$OD_{260} = 1 \cong 33\mu g$ DNA/ml

The relationship of the ODs at 260 nm and 280 nm is an indicator for the purity of the nucleic acid solution. The optimal value is 1.8 for DNA and 2.0 for RNA.

4.2.3.4 Preparation of DNA

4.2.3.4.1 Small scale preparation of plasmid DNA

This method was used for the rapid preparation of small amounts of plasmid DNA. 2 ml overnight bacterial culture were centrifuged (13000 rpm, 30 sec, RT) and the bacterial pellet was resuspended in 100 µl solution I. 100 µl of solution II was added and the mixture was carefully inverted. For neutralisation, 100 µl of solution III was added. Bacterial chromosomal DNA aggregates with proteins and lipids to an insoluble precipitate. In contrast, the plasmid DNA can, because of its "supercoil"-structure, renature, and dissolve in the aqueous phase. The precipitate was removed after centrifugation (13000 rpm, 5 min, 4°C). The supernatant was purified by phenol/chloroform extraction and the DNA precipitated with ethanol. After centrifugation (13000 rpm, 15 min, 4 °C) the precipitated DNA was dissolved in 100 µl aqueous RNase A solution (100 µg/ml). 10 µl of this solution was used for analytic restriction digests.

Solution I:	50 mM	glucose
	10 mM	EDTA
	25 mM	Tris/HCl, pH 8.0
Solution II:	1 %	SDS
	0.2 M	NaOH
Solution III:	3 M	sodium acetate, pH 4.8

4.2.3.4.2 Plasmid preparation with the QIAGEN Plasmid Maxi Kit

Plasmid DNA in larger amounts was isolated from 100 ml overnight culture using the *Plasmid Maxi Kit* (Diagen, Düsseldorf) following the instructions of the manufacturer.

4.2.3.5 Agarose gel electrophoresis of nucleic acids

4.2.3.5.1 Analytic agarose gel electrophoresis

The separation of DNA-fragments was performed with 1-2 % agarose gels (SeaKem ME) in TBE. For the separation of total cellular or polyadenylated RNA 1 % gels were used. The agarose gels contained 1 µg ethidium bromide per ml gel. The samples were mixed with loading buffer and loaded on the gel. The electrophoresis was performed by 80-140 V depending on the gel size and the concentration of the agarose. The cells were visualized on a UV-light box and photographed.

10x loading buffer:	40 % (w/v)	saccharose
	0.25 % (w/v)	bromophenol blue
	0.25 % (w/v)	xylene cyanole

4.2.3.5.2 Preparative agarose gel electrophoresis

DNA fragments were isolated from agarose gels using the *QIAexII-Kit* (Diagen, Düsseldorf) or the *QIAquick-Kit* (Diagen, Düsseldorf) following the instructions of the manufacturer.

4.2.3.6 *In vitro* reactions with DNA

4.2.3.6.1 Digestion by restriction endonucleases

Digestion of DNA by restriction endonucleases was performed under the conditions described by the manufactures (Roche, Rotkreuz). Usually, 0.5-1 units of the corresponding restriction endonuclease were used per µg DNA.

4.2.3.6.2 Filling the recessed 3'-termini by Klenow polymerase

Cloning of DNA fragments with recessed 3'-termini in blunt-end vectors requires a filling of these termini. Therefore, 1-5 µg DNA was mixed in a final volume of 30 µl with 3 µl 10x reaction buffer, 0.5 µl dNTP mix (2.5 mM each) and 0.5 µl Klenow enzyme. The mixture was incubated for 1 h at 37°C.

4.2.3.6.3 Ligation

For an efficient ligation the optimal stoechiometric relation of vector to insert is 1:3 to 1:4 for cohesive termini and 1:10 for blunt-end termini. The vector and the purified DNA fragment were mixed with 10x ligation buffer and 1 U T4 DNA ligase. The reaction mix was incubated overnight at room termperature.

4.2.3.6.4 Polymerase chain reaction (PCR)

The polymerase chain reaction allows the amplification of any DNA fragment from a template DNA in the presence of suitable oligodesoxynucleotide primers. The reaction was performed in a volume of 20-50 µl.

Reaction mix:	2.0 µl	DNA template (plasmid- or cDNA)
	5.0 µl	10x buffer (Pharmacia)

8.0 µl	dNTP-mix (2.5 mM each)
1.0 µl	5`-primer (50 µM)
1.0 µl	3`-primer (50 µM)
0.3 µl	Taq DNA-Polymerase (1.5 U)
32.7 µl	ddH_2O

The reaction was performed in a PCR machine (Eppendorf *Mastercycler gradient* or MWG-Biotech *Primus 96*) following different temperature programs:

Denaturation:	4 min	94°C
"Annealing" of the primers:	30-60 sec	45-60°C
Polymerisation:	30-60 sec	72°C

Dependent on the concentration of the DNA template 30-40 cycles were performed. The polymerization time was adapted to the size of the product, per 1000 bp we programmed 1 min polymerization time. After the last cycle the mixture was incubated for 10 min at 72°C to fill the termini of incompletely synthesized fragments and then cooled down to 4°C.

4.2.3.6.5 DNA sequencing

Double stranded DNA was sequenced using the *ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit* following the instructions of the manufacturer.

4.2.3.6.6 Radioactive labeling of DNA fragments via "Random primer extension"

[^{32}P]-dCTP-labeled DNA fragments were synthesized using the *Rediprime Random Primer Labeling Kit* (Amersham) following the instructions of the

manufacturer. The labeled DNA fragment was separated from the non-incorporated nucleotides by gel filtration using Sephadex G-50 columns (*nick™ Column*, Pharmacia) following the instructions of the manufacturer.

4.2.3.6.7 End-labeling of free 5'-OH termini (for gel shift assay)

50 ng of two complementary oligonucleotides were mixed with 2 µl 10x reaction buffer, 0.7 µl polynucleotide kinase (PNK) and 3 µl [γ^{32}P]-ATP, filled to a volume of 20 µl with ddH_2O and incubated for 10 min at 37°C. For hybridization of the complementary oligonucleotides, the mixture was incubated for 5 min at 95°C. Afterwards the heating block was switched off and cooled to RT. The labeled oligonucleotide was separated from non-incorporated nucleotides by gel filtration using Sephadex G-50 columns (MicroSpinTM, Pharmacia) following the instructions of the manufacturer.

4.2.3.7 RNA methods

4.2.3.7.1 General rules for working with RNA

All experiments with RNA were performed using sterile plastic or baked (200°C, 8 h) glass vessels. All solutions (except solutions containing Tris/HCl) were pre-treated with diethylpyrocarbonate (DEPC) to remove RNases. For this purpose they were mixed with 0.1 % (v/v) DEPC and stirred for 16 hours at RT. The solutions were autoclaved to destroy the DEPC. Solutions which contain Tris/HCl were produced using DEPC-treated water in baked vessels.

4.2.3.7.2 Isolation of total cellular RNA

Isolation of total cellular RNA was performed as described by Chomczynski and Sacchi (1987). Cells were lysed by using a detergent and the chaotropic

guanidinum isothiocyanate, which acts simultaneously as an RNase inhibitor. The chromosomal DNA was removed by an extraction with acidic phenol.

Isolation of total cellular RNA from cultured eukaryotic cells

Adherent cells were washed twice with PBS. 400 µl guanidinum isothiocyanate solution (GSCN) was added per 10 cm dish and equally distributed. The solution was scraped off the dish and transferred to a 1.5ml reaction tube. After addition of 40 µl 2M sodium acetate (pH 4.0), 400 µl H_2O-saturated phenol, and 120 µl chloroform the solution was vigorously mixed. The mixture was incubated for 15 minutes on ice and centrifuged (13000 rpm, 4°C, 15 min) afterwards. The aqueous solution, which contains the RNA, was mixed with 1 ml ethanol. After centrifugation (13000 rpm, 4°C, 15 min) the RNA pellet was dissolved in 400 µl DEPC-H_2O, extracted first with phenol/chloroform (ph 7.5), second with chloroform and precipitated again with ethanol. The RNA pellet was dissolved in a suitable volume of DEPC-H_2O and heated for 5 min to 65°C in order to completely dissolve the RNA. The quality of the RNA preparation was checked on an analytical agarose gel. The RNA was stored in 20 µg RNA aliqouts in 0.3 M NaOAc/2.5 volumes ethanol at –20°C.

GSCN-solution:	50 %(w/v)	Guanidinumisothiocyanate
	15 mM	Sodium citrate (pH 7.0)
	0.5 %(w/v)	Sodium lauroylsarcosine
	0.7 % (v/v)	β-Mercaptoethanol

Isolation of total cellular RNA from tissues

Tissues for RNA isolation were snap frozen in liquid nitrogen immediately after preparation and stored at –80°C. The samples were transferred into a suitable amount of cold GSCN-solution (see above) and homogenised with an Ultra Turrax.

Insoluble particles were removed by centrifugation (3500 rpm, 10 min, 4°C). The supernatant was transferred to a new tube and treated as described above for isolation of RNA from cultured cells.

4.2.3.7.3 Isolation of polyadenylated RNA

The preparation of poly(A)+-mRNA from total cellular RNA was performed using the mRNA Separator Kit (Clontech, Palo Alto, CA, USA) following the instructions of the manufacturer. The poly(A)+-mRNA was separated from the residual RNA by affinity chromatography using oligo-d(T)-cellulose columns. The isolated poly(A)+-mRNA was used for the hybridization of an Atlas cDNA-array (4.2.3.8.2).

4.2.3.7.4 Northern Blot

20 µg total cellular RNA was dried, dissolved in 20 µl sample buffer and incubated for 5 min at 80°C. Afterwards the samples were put on ice, mixed with 4 µl stop-mix and loaded on a 1% agarose gel, which contains RB-buffer and 6.6 % formaldehyde. The electrophoresis was performed at 100-150V (30 mA) for 3-4 hours. Afterwards the gel was incubated in ddH_2O for 5 min and in 20x SSC for 10 min. The RNA was transferred overnight from the gel to a Gene Screen Plus nylon membrane using capillary forces. To remove agarose residues the membrane was washed shortly with 2x SSC after the transfer. The RNA was fixed on the membrane by UV-light (for 3min) or by baking the membrane at 80°C (for 3h).

sample-buffer:	50 %	Formamide
	2x	RB
	6.6 %	Formaldehyde

stop-mix:	25 %	Ficoll 400
	0.25 %	Bromophenol blue
10x RB-buffer, pH 7.0 (1l):	25 ml	2 M NaOAc, pH 7.0
	41.86 g	MOPS
	2 ml	0.5 M EDTA, pH 8.0
	17 ml	4 N NaOH

The RNA was stained by incubation of the membrane in 5 % acetic acid for 5 min and in the methylene blue staining solution (0.5 M NaOAc pH 5.2, 0.04 % methylene blue) for 1-5 min. Afterwards the membrane was washed with water and the 18S- and 28S-RNAs were marked for subsequent use as a size marker.

To block unspecific binding sites, the membrane was incubated for 6h in hybridization solution at 65°C in a rocking water bath. The hybridization was performed overnight at 55°C. Therefore, 10^6 cpm/ml [^{32}P]-dCTP-labeled DNA fragment (4.2.3.6.6) was added to the hybridization solution. Afterwards the membrane was washed twice for 20 min with washing-solution I, and once with washing-solution II at 50°C. Finally the membrane was wrapped in Saranwrap and exposed to X-ray film. The hybridized, radiolabeled DNA fragment was removed by boiling the membrane for 5 min in H_2O.

Hybridization solution:	50 % (v/v)	deionised formamide
	1 % (w/v)	sodium lauroylsarcosine
	10 % (w/v)	dextrane sulfate
	100 µg/ml	heat denaturated salmon sperm DNA

Washing-solution I:	2x	SSC
	0.5 %	SDS

Washing-solution II:	0.1x	SSC
	0.1 %	SDS

4.2.3.7.5 RNase Protection Assay

The RNase protection assay is a highly sensitive method, which allows to detect down to 0.1 pg of a specific transcript from a pool of total cellular RNA. A radioactively labeled RNA probe, which is complementary to the specific transcript, is synthesized by *in vitro* transcription and hybridized with the total cellular RNA under high stringently conditions. The complementary areas of the RNA probe and the cellular mRNA form RNA-RNA hybrids, which are protected against the following RNase digestion. The remaining RNA fragments are separated by polyacrylamide gel electrophoresis and visualized by autoradiography. Laser densitometric analysis allows the quantification using the NIH image program.

Synthesis of a [^{32}P] -rUTP labeled riboprobe by *in vitro*-transcription

The RNA probe, which is complementary to the cellular transcript, was synthesized by *in vitro* transcription and radioactively labeled by incorporation of [α-^{32}P]-rUTP. The DNA template, which was used, was a transcription vector, which contains at least one RNA-polymerase recognition site (usually pBluescript II KS(+)). The vector, which contains at least 150 bp of the cDNA of interest, was linearised by restriction digest using a restriction site, which is 140-400 bp upstream of the 3'-end of the cDNA.

In vitro transcription:	3.0 µl	DEPC-H2O
	1.0 µl	NTP-mix (5mM ATP, CTP and GTP)
	1.0 µl	10x transcription buffer
	0.5 µl	RNAsin (40 U/µl)

1.0 µl	RNA-polymerase (T3-, T7, or SP6-polymerase, 20 U/µl)
2.5 µl	[α-^{32}P]-rUTP (800 Ci/mmol)
1.0 µl	linearised and denaturated (5 min, 65°C) template (1 µg/µl)

The reaction mix was incubated for 1 h at 37°C.

Purification of the riboprobe

After addition of 10 µl FLB100 to the reaction mix, the solution was loaded on a denaturing polyacrylamide gel to purify the transcript. The quality of the transcription was checked by autoradiography. The position of the transcript was determined and it was excised from the gel. The riboprobe was eluted from the gel by shaking the gel in 150-300 µl elution buffer for 2 h. After centrifugation (13000 rpm, 5min) the supernatant was transferred to a fresh tube and the activity of 1 µl of the eluate was determined in a scintillation counter in the Czerenkov channel.

FLB100:	100 % (v/v)	deionised formamide
	0.5 % (w/v)	bromophenol blue
	0.5 % (w/v)	xylene cyanol
Acrylamide/urea gel:	5 %	acrylamide/bisacrylamide (29:1)
	7 M	urea
	1x	TBE
Elution buffer:	0.1x	TBE
	0.2 %	SDS

Hybridization of the total cellular RNA with the [^{32}P]-rUTP labeled riboprobe

10-20 µg total cellular RNA and 50 000 to 100 000 cpm of the labeled riboprobe were precipitated with ethanol. To avoid that large amounts of SDS disturb the hybridization, the volume of the probe solution was restricted to 10 µl. After precipitation (30 min, -80°C) and centrifugation (13000 rpm, 15 min, 4°C) the supernatant, which should not contain any radioactive components, was removed. The RNA precipitate was resuspended in 30 µl FAB buffer, denatured for 15 min at 85°C and incubated overnight at 42°C.

FAB buffer:	80 % (v/v)	deionised formamide
	400 mM	NaCl
	40 mM	PIPES, pH 6.4
	1 mM	EDTA

RNase digestion of single-stranded RNA molecules

After hybridization 300 µl RNase A/T_1 mix was added to the samples and they were incubated for 45 min at 30°C. Afterwards the RNases were digested by addition of 6.6 µl 10 % SDS and 4.4 µl proteinase K (10 µg/µl). The mixture was incubated for 15 min at 42°C. The remaining RNA was purfied by phenol/chloroform extraction and precipitated with 1.5 µl tRNA and 880 µl ethanol for 30 min at –80°C. After centrifugation (13000 rpm, 15 min, 4°C) the RNA pellet was resuspended in 30 µl FLB80 buffer.

RNase A/T_1 mix:	300 µl	RNase buffer
	1 µl	RNase A (10 µg/µl)
	2 µl	RNase T_1 (100 U/µl)

RNase buffer:	0.3 M	Sodium acetate, pH 7.0
	10 mM	Tris/HCl, pH 7.5
	5 mM	EDTA, pH 8.0
FLB 80 buffer:	80 % (v/v)	deionised formamide
	1x	TBE
	1 mM	EDTA
	0.05 %(w/v)	bromophenol blue
	0.05 % (w/v)	xylene cyanol

Gel electrophoresis of the RNA fragments

The dissolved RNA fragments were denatured for 5 min at 95°C and separated on a denaturing polyacrylamide gel. Gels were dried for 2 h at 80°C in the vacuum and afterwards exposed to X-ray film.

4.2.3.7.6 *In situ* hybridization with tissue sections

In situ hybridization allows to identify cells within a tissue, which express a specific gene. Specific mRNA of the tissue hybridizes with a complementary, radioactively labelled *antisense* RNA-probe. The resulting RNA-RNA hybrids are protected against the following RNase digestion. For detection of mRNA expression sections were covered in emulsion, incubated for 3-5 weeks and subsequently developed. Thus, where the emulsion has been exposed to the radiolabeled probe, a black signal is observed, which results from the silver grains.

Synthesis of a [α^{35}S]-rUTP labeled riboprobe by *in vitro* transcription

In vitro transcription:	100 µCi	[α^{35}S]-rUTP, lyophilised
	5.5 µl	DEPC-H_2O
	1.0 µl	10x transcription buffer
	1.0 µl	NTP mix (5mM ATP, CTP and GTP)
	0.5 µl	RNasin
	1.0 µl	RNA-polymerase (T3- or T7-polymerase)
	1.0 µl	linearised and denaturated (5 min, 65°C) template (1 µg/µl)

The reaction mix was incubated for 90 min at 37°C.

After the transcription the DNA template was digested by addition of 1µl (1 unit) RNase-free RQ1-DNase. The mixture was incubated for 15 min at 37°C. 90 µl DEPC-H_2O was added to the RNA solution and proteins were removed from the solution by phenol/chloroform extraction. 1 µl of the aqueous solution was mixed with 9 µl FLB80 buffer (4.2.3.7.5) and loaded on an acrylamide/urea minigel. The quality of the transcription was checked by autoradiography. The residual RNA solution was precipitated with 0.3 M NaOAc and 2.5 volumes of ethanol and stored at –20°C.

4.2.3.7.6.1 *In situ* hybridization with frozen sections

Preparation of frozen sections

Frozen sections were dried and fixed for 1-2 h at 55°C. Afterwards the sections were fixed for 10 min in a 4°C cold paraformaldehyde solution (4 % (w/v) in PBS)

and washed for 5 min in 0.5x SSC. The tissue was incubated for 10 min in proteinase K solution and washed for 5 min in 0.5x SSC.

Proteinase K solution:	500 mM	NaCl
	10 mM	Tris/HCl, pH 8.0
	1 µg	Proteinase K

Hybridization of the tissue sections with the [^{35}S]-rUTP labeled riboprobe

After washing in 0.5x SSC the sections were covered with 100 µl rHB2 buffer and incubated in a humid chamber (covered with Whatman 3MM paper soaked in box buffer) for prehybridization for 1-2 h at 55°C.

The *in vitro* synthesized transcripts were centrifuged (13000rpm, 15 min, 4°C) and dissolved in 50 µl 10 mM DTT. The activity of 1 µl was measured in the scintillation counter in the sulphur channel. The riboprobe was diluted with 10 mM DTT to a final concentration of 300.000 cpm/µl. 100 µl of the dilution was mixed with 850 µl rHB2 buffer and 50 µl aqueous tRNA-solution (50 µg/µl). The mixture was incubated for 30 min at 80°C and then placed into a 65°C heating block. 20 µl of the mixture was applied to the sections, which were covered with rHB2. Sections were incubated overnight at 55°C in the humid chamber.

rHB2 buffer:	50 % (v/v)	formamide
	0.3 M	NaCl
	20 mM	Tris/HCl, pH 8.0
	5 mM	EDTA, pH 8.0
	1x	*Denhardt's* solution
	10 % (w/v)	dextran sulfate
	10 mM	DTT

box buffer:	5x	SSC
	20 % (v/v)	formamide

RNase digest and washing

The sections were washed for 10 min at RT in wash solution I and incubated for 30 min at 37°C in a RNase solution (20 µg/ml RNase A in RNase buffer) to remove the remaining single-stranded RNA molecules. Afterwards the sections were washed twice in washing solution I for 10 min at RT, followed by three times washing in the stringent washing solution II (10 min, 55°C). Finally, the sections were incubated for 10 min in 0.5x SSC and afterwards dehydrated by passing the slides through an increasing ethanol series (30 %, 60 %, 80 %, 95 % ethanol/0.3 M ammonium acetate, 2x 100 % ethanol). The sildes were dried for 2 h at 30°C. Then the sections were dipped in film emulsion (prewarmed to 44°C) and stored light-protected at 4°C together with silica gel. The sections were developed (3 min developer, 20 sec H_2O, 3 min fixer, 5 min H_2O) and counterstained with hematoxylin/eosin (4.2.5.3.3).

Washing solution I:	2x	SSC
	10 mM	β-mercaptoethanol
	1 mM	EDTA, pH 8.0
Washing solution II:	0.1x	SSC
	10 mM	β-mercaptoethanol
	1 mM	EDTA, pH 8.0
RNase buffer:	0.5 M	NaCl
	10 mM	Tris/HCl

4.2.3.7.6.2 *In situ* hybridization with paraffin sections

Pretreatment of the paraffin sections

For fixation the sections were incubated overnight at 50°C. Afterwards they were dewaxed and rehydrated:

Deparaffinisation:	xylene	15 min
	xylene	15 min
Rehydration:	100 % ethanol	2 min
	100 % ethanol	10 sec
	100 % ethanol	10 sec
	95 % ethanol/0.9 % NaCl	10 sec
	85 % ethanol/0.9 % NaCl	10 sec
	60 % ethanol/0.9 % NaCl	10 sec
	30 % ethanol/0.9 % NaCl	10 sec
	0.9 % NaCl	5 min
	PBS	5 min

The sections were fixed for 10 min in 4 % paraformaldehyde/PBS and washed twice for 5 min in PBS. Afterwards they were incubated for 12 min in pronase buffer, washed for 5 min in PBS, refixed for 10 min in 4 % paraformaldehyde/PBS and washed again for 3 min in PBS. They were then treated twice with 2.5 % acetic anhydride/0.1 M triethanolamine for 5 min. The treatment leads to acetylation of hydroxyl groups and should reduce unspecific binding of the riboprobe. Finally, the sections were dehydrated by an increasing ethanol series and air-dried for 2 h.

Pronase buffer:	50 mM	Tris/HCl, pH 7.5
	5 mM	EDTA, pH 8.0
	20 µg/ml	pronase

Hybridization of the tissue sections with the [^{35}S]-rUTP labeled riboprobe

The radiolabeled riboprobe was centrifuged (13000 rpm, 15 min, 4°C) and dissolved in 50 µl 10 mM DTT. The activity of 1µl was measured in the scintillation counter in the sulphur channel. The riboprobe was diluted with hybridization mix to a final concentration of 10 000-20 000 cpm/µl and denaturated for 3 min at 80°C. DTT was added to a final concentration of 10 mM and the mixture was then placed into a 65°C heating block. An appropiate volume of the hybridization solution was applied to the sections and they were incubated in a humid chamber (covered with Whatman 3MM paper soaked in box buffer) for at least 16 h at 52°C.

Hybridization mix:	50 % (v/v)	deionised formamide
	0.3 M	NaCl
	20 mM	Tris/HCl, pH 8.0
	5 mM	EDTA, pH 8.0
	10 mM	sodium phosphate, pH 8.0
	10 % (w/v)	dextran sulfate
	1x	*Denhardt's* solution
	0.5 mg/ml	tRNA
box-buffer:	5x	SSC
	50 % (v/v)	formamide

RNase digest and washing

The sections were briefly washed in washing solution I (prewarmed to 55°C). To remove unspecifically bound riboprobe they were washed for 25 min at 55°C in washing solution II. Afterwards the slides were washed three times for 10 min at 37°C in RNase buffer and incubated for 30 min at 37°C in RNase A solution (20 µg/ml in RNase buffer) to remove the remaining single-stranded RNA molecules.

Subsequently, the sections were washed in RNase buffer (15 min, 37°C) and in washing solution II (25 min, 55°C). To avoid salt depositions the sections were incubated for 15 min at 37°C in 2x SSC and in 0.1x SSC. Finally they were dehydrated by passing through an increasing ethanol series (30 %, 60 %, 80 %, 95 % ethanol/0.3 M ammonium acetate, 2x 100 % ethanol). The sildes were dried for 2 h at 30°C, dipped in film emulsion (prewarmed to 44°C), and stored light-protected at 4°C together with silica gel. The sections were developed (3 min developer, 20 sec H_2O, 3 min fixer, 5 min H_2O) and counterstained with hematoxylin/eosin (4.2.5.3.3).

Washing solution I:	5x	SSC
	10 mM	DTT
Washing solution II:	50 %(v/v)	formamide
	2x	SSC
	0.1 % (v/v)	β-mercaptoethanol
RNase buffer:	0.5 M	NaCl
	10 mM	Tris/HCl, pH 8.0
	5 mM	EDTA, pH 8.0

4.2.3.7.7 Generation of cDNA by reverse transcription

cDNA from eukaryotic cells or tissue was generated by reverse transcription of total cellular RNA. 5 µg RNA was dissolved in 7 µl DEPC-H_2O, mixed with 1 µl (0.5 µg) Oligo $d(T)_{15}$ primer, incubated for 10 min at 70°C and directly put on ice. Afterwards the RT mix was added, the mixture was incubated for 1 h at 37°C and then heated for 5 min to 95°C.

RT mix:	4 µl	5x first strand buffer
	2 µl	DTT (0.1 M)
	1 µl	RNAsin
	4 µl	dNTP mix (2.5 mM each)
	1 µl	reverse transcriptase (200 U) (*Super-script Plus*, Gibco)

For PCR amplification of cDNA fragments 2 µl of the cDNA solution was added to 50 µl reaction mixture.

4.2.3.8 Analyis of differential gene expression

4.2.3.8.1 Differential Display RT PCR (DDRT-PCR)

The "Differential Display RT PCR"-technology (Liang and Pardee, 1992) allows to identify differentially expressed genes in cells and tissues.
cDNA was generated by reverse transcription of the total cellular RNAs of interest. The cDNAs were then used in PCR reactions with randomly chosen primers.
DDRT-PCR was carried out according to Bauer *et al.*, 1994.

Reverse transcription

cDNA was generated by reverse transcription of a defined amount of HaCaT RNA. For the preferential amplification of mRNA, which constitutes only 1-2 % of the total cellular RNA, a modified Oligo d(T) primer was used. The primer should be complemetary to the poly(A)-tail, which is located at the 3'-end of most mRNA species. To reduce the number of amplified cDNAs the modified Oligo d(T) primers contained at the 3'-end two randomly chosen nucleotides (the base last but one had to be different from T). During this thesis the C1 [$d(T)_{12}CG$] primer, described by Bauer *et al.*, 1994 was used.

Reverse transcription:	300 ng	total cellular RNA (dissolved in 3 µl DEPC-H_2O
	3.0 µl	C1 primer (25 µM)
	7.5 µl	DEPC-H_2O

To denature the RNA and the primer, the mixture was incubated for 10 min at 70°C and directly placed on ice. 16.5 µl cDNA master mix was added and the solution was incubated for 2 min at RT. After the addition of 1.5 µl (100 U) Super Script Reverse transcriptase the mixture was incubated for 8 min at RT and afterwards for 1 h at 37°C. To inactivate the enzyme the reaction mix was incubated for 5 min at 95°C.

cDNA master mix (for 4 reactions):	25 µl	5x first strand buffer
	12.5 µl	0.1 M DTT
	25 µl	dNTP (100 µM each)
	3.2 µl	RNasin (40 U/µl)
	3.2 µl	DEPC-H_2O

PCR reaction and isolation of the fragments

The PCR reaction was used to amplify specific DNA fragments of the generated cDNA. Therefore, the same modified Oligo d(T) primer and a decameric 5'-primer were used. Calculations show, that the size of the about 150 products should be between 150-500 nt. Fragments of this size can be separated on a standard sequenzing gel. 24 upstream primers, which are chosen by defined criteria, are described by Bauer *et al.*, 1994. They allow to cover all transcripts of eukaryotic cells or tissue.

PCR mix (for 9 reactions):	23.4 µl	10x PCR buffer
	9.0 µl	[α^{35}S]-dATP (10 mCi/ml)
	4.7 µl	dNTP (100 µM each)
	2.2 µl	Taq polymerase (11 U)
	101 µl	DEPC-H_2O
PCR reaction (20 µl):	12 µl	PCR mix
	2 µl	C1 primer (25 µM)
	1 µl	cDNA
	5 µl	upstream primer (2 µM)

The PCR reaction was performed following the described temperature program:

3 min	94°C
40 cycles:	
30 sec	94°C
1 min	42°C
30 sec	72°C
5 min	72°C

After the PCR reaction the samples were dried in a speedvac, dissolved in 2 µl H_2O and mixed with 3µl formamide loading buffer (T7 *sequencing kit*, Pharmacia). The samples were denatured for 2 min at 95°C and loaded on a denaturing polyacrylamide gel (Ultrapure Sequagel Sequencing system, National Diagnostics, Atlanta, GA, USA). After autoradiography bands of interest were excised from the gel, mixed with 100 µl H_2O and incubated for 10 min at RT and for 15 min at 95°C.

The DNA was purified by phenol/chloroform extraction and precipitated with 0.3 M NaOAc, 2.5 volumes ethanol and 20 µg tRNA. The DNA pellet was dissolved in 100 µl H_2O.

Reamplification and cloning of the cDNA fragments

The amplification of the cDNA fragments was performed following the PCR reaction described above. The same temperature program (30-35 cycles) and the same primers were used.

30 µl	purified cDNA fragment
5 µl	10x PCR buffer
5 µl	dNTP (500 µM each)
5 µl	C1 primer (2 µM)
5 µl	upstream primer (2 µM)
0.2 µl	Taq polymerase (20 U)

10 µl of the reaction mix were loaded on an agarose gel; the residual 40 µl were treated with Klenow polymerase, purified by phenol/chloroform extraction and precipitated with ethanol. The cDNA fragment was cloned into the transcription vector pBluescript KSII(+) and sequenced. The differential gene expression was confirmed by RNase protection assay.

4.2.3.8.2 Hybridization of cDNA arrays

The hybridization of commercially availabe cDNA arrays is a possibility to investigate differential gene expression. Several hundred cDNAs are dotted on a membrane. A system of coordinates is used to identify the position of the cDNAs. Hybridization of such membranes with two different, radioactively labeled cDNA populations offers the possibility to determine complete gene expression profiles. During this thesis the *Atlas human cDNA expression array I* (Clontech Laborato-

ries, Palo Alto, CA, USA) was hybridized with radioactively labeled cDNA from quiescent and activin A treated (for 5 h and 8 h) HaCaT cells.
The Atlas cDNA array hybridization was performed following the instructions of the manufacturer. The required poly(A)-RNA was generated as described in 4.2.3.7.3.
To confirm the results obtained upon hybridization of this cDNA array, cDNA fragments of the identified genes were amplified by RT-PCR of HaCaT cDNA and cloned into the transcription vector pBluescript KSII(+). Afterwards, differential expression was verified by RNase protection assay.

4.2.4 Protein methods

4.2.4.1 Preparation of protein lysates from cultured cells and tissues

4.2.4.1.1 Preparation of protein lysates from cultured eukaryotic cells

Preparation of total cell lysates

Adherent cells were washed twice with PBS. 500µl lysis buffer was added to a 10 cm petri dish and incubated for 10 min at 4°C on a rocker. Subsequently the cells were scraped off the dish and the suspension was sonicated. Afterwards the lysate was centrifuged (13000 rpm, 10 min, 4°C). The supernatant was transferred to a fresh tube, the lysates were snap frozen and stored at –80°C.

1x lysis buffer:	1 % (v/v)	Triton X-100
	20 mM	Tris/HCl, pH 8.0
	137 mM	NaCl
	10 % (v/v)	glycerol
	2 mM	EDTA

The following protease and phosphatase inhibitors were added directly before use:

1 mM	PMSF
0.15 U/ml	Aprotinin
1 % (v/v)	Leupeptin
1 % (v/v)	Pepstatin
1 mM	$Na_2P_2O_7$
1 mM	Na_3VO_4

Separation of nuclear and cytosolic fractions

To separate the cytosolic fraction from the nuclei, cells were incubated for 10 minutes in lysis buffer (see above). The supernatant (cytosolic fraction) was removed, centrifuged (13000 rpm, 10 min, 4°C), snap frozen and stored at –80°C. To isolate the nuclear fraction 200 µl lysis buffer were added to the remaining nuclei on the petri dish. They were srcaped off the dish and the suspension was sonicated. Afterwards the nuclear lysate was centrifuged (13000 rpm, 10 min, 4°C), the supernatant transferred to a fresh tube, snap frozen and stored at –80°C.

Preparation of total cellular protein lysates using Frackelton buffer

Adherent cells were washed twice with PBS. 300 µl Frackelton buffer was added to the petri dish. The cells were scraped off the dish. The suspension was incubated for 10 min on ice, vortexed for 45 sec and cleared by centrifugation (13000 rpm, 4°C). The protein lysates were snap frozen and stored at –80°C.

Frackelton buffer:		
	10 mM	Tris/HCl, pH 7.05
	50 mM	NaCl
	5 µM	$ZnCl_2$
	30 mM	$Na_2P_2O_7$

1 % (v/v)	Triton X-100

The following protease inhibitors were added directly before use:

1 mM	PMSF
0.15 U/ml	Aprotinin
1 % (v/v)	Pepstatin
1% (v/v)	Leupeptin
1 mM	Na_3VO_4

This method was used for preparation of nuclear lysates used in solid phase DNA binding assays.

4.2.4.1.2 Preparation of protein lysates from tissue

Tissue, which had previously been stored at –80°C, was homogenised in 2x lysis buffer (4.2.4.1.1) using an *Ultra Turrax*. The supernatant was cleared by centrifugation and diluted 1:1 with ddH_2O. The protein lysate was snap frozen and stored at –80°C.

4.2.4.2 Determination of the protein concentration

The protein concentration was determined by the *Bradford* method. The Bio-Rad protein assay was used, following the instructions of the manufacturer.

4.2.4.3 Tris/glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed using the buffer system of Laemmli (1970, glycine as a zwitter ion). Gel electrophoresis was carried out in vertical direction in 1.5 mm thick gels. The protein gel was composed of an approximately 1.5 cm long stack gel and a 8 cm long separating gel.

The samples were mixed with a suitable volume of sample loading buffer (5x concentrated), denaturated for 5 min at 95°C and loaded on the gel. Gel electrophoresis was performed at 200 V.

Stack gel:	acrylamide/bisacrylamide (30:0.8)	2.6 ml
	1 M Tris/HCl, pH 6.8	1.9 ml
	10 % (w/v) SDS	0.15 ml
	H_2O	10.2 ml
	10 % (w/v) APS	0.15 ml
	TEMED	15 µl
Separating gel (12 %):	acrylamide/bisacrylamide (30:0.8)	16 ml
	1 M Tris/HCl, pH 6.8	15 ml
	10 % (w/v) SDS	0.4 ml
	H_2O	8.2 ml
	10 % (w/v) APS	0.4 ml
	TEMED	16 µl

SDS-PAGE running buffer:	25 mM	Tris HCl
	192 mM	glycine
	0.1 %	SDS
sample loading buffer (5x):	50 %(v/v)	glycerol
	7.5 g/l	SDS
	250 mM	Tris HCl, pH 8.0
	0.5 mg/ml	bromophenol blue
	12.5 %	β-mercaptoethanol

4.2.4.4 Immunological methods

4.2.4.4.1 Western Blot

The western blot allows the detection of specific proteins using antibodies. Proteins were separated by SDS-PAGE and transferred from the gel to a nitrocellulose membrane. Afterwards unspecific binding sites of the membrane were blocked with a highly concentrated protein solution. The membrane was incubated with the first antibody, which recognizes the protein of interest. The secondary antibody, which is directed against the first one, is coupled to an enzyme, which catalyzes a detection reaction.

Protein transfer by semi-dry blotting

6 sheets of Whatman 3MM paper were soaked in transfer buffer and placed on the anode side of the blotting chamber. The nitrocellulose membrane (soaked in transfer buffer) and the separating gel were put on top of the Whatman 3MM paper. Finally, another 6 sheets of Whatman 3MM paper were soaked in transfer buffer and placed on top of the gel and connected with the cathode. Blotting was performed at 1 mA/cm^2.

Transfer buffer:	25 mM	Tris
	192 mM	glycine
	20 %	methanol

The efficiency of the blotting was controlled by staining of the membrane with Ponceau S solution (Sigma, Munich, Germany). For this purpose the membrane was shaken for 10 min in Ponceau S solution and afterwards destained with water until the protein bands became visible.

Incubation of the membrane with the antibodies

All incubation steps were performed at RT.

Blocking:	5 % milk powder in TBST; 30 min
First antibody:	suitable dilution in 5 % milk powder in TBST; 1h
Washing:	3x5 min in TBST
Secondary antibody:	suitable dilution in 5 % milk powder in TBST; 30 min
Washing:	3x5 min in TBST

TBST:	0.15 M	NaCl
	10 mM	Tris/HCl, pH 8.0
	0.05 % (v/v)	Tween 20

Detection of proteins using enhanced chemoluminescence (ECL)

The secondary antibody is coupled to a horseradish peroxidase. This enzyme catalyses the oxidation of luminol (3-aminophthalhydrazide) to 3-aminophthalic acid and nitrogen by hydrogen peroxide. During this reaction energy is released in form of light (425 nm), which can be visualized by an X-rax film.
The ECL kit (Amersham, Braunschweig, Germany) was used following the instructions of the manufacturer.

4.2.4.4.2 Immunofluorescence on tissue sections

For the localisation of a specific protein in tissue sections immunofluorescence staining was performed. For this purpose the unfixed cryosections were fixed for 10 min at –20°C in acetone. Afterwards the sections were briefly washed in PBS. The first antibody was diluted in PBS/0.1 % BSA and applied to the sections. After a 1 h

incubation at RT the sections were washed three times with PBS for 10 min at RT. The secondary antibody conjugated with a fluorochrome (FITC or Texas-Red) was diluted in PBS/0.5 % BSA and applied to the sections. After the addition of the secondary antibody further steps should be performed under light exclusion. The sections were incubated with the secondary antibody for 0.5 h at RT, washed as described above and mounted with Mowiol.

4.2.4.4.3 Solid-phase-DNA binding assay

The solid-phase DNA-binding assay was performed as described by Larsson *et al.* (1997). It is a combination of immunoprecipitation and DNA-binding assay.
The oligonucleotide was labeled and hybridized as decribed in 4.2.3.6.7. The protein lysates were isolated using Frackelton buffer as described in 4.2.4.1.1.
For immunoprecipitation, the protein lysates were incubated with a suitable amount of antibody for 2 h at 4°C. A 1:1 suspension of protein A sepharose in Frackelton buffer was added to the protein lysates and the mixture was incubated for 4 h at 4°C. After centrifugation (13000 rpm, 4°C, 1 min) the sediment was washed three times with Frackelton buffer and once with gelshift buffer. Afterwards a DNA binding assay was performed. 30 µl gelshift buffer, 1 µl salmon sperm DNA (1 µg/µl) and 150000cpm labeled oligonucleotide were added to the pellet and incubated for 25 min at 25°C. After centrifugation (13000 rpm, 4°C, 1 min) the precipitate was washed three times with gel shift buffer and the activity was determined in a scintillation counter in the Czerenkov channel.

Frackelton buffer:	10 mM	Tris/HCl, pH 7.05
	50 mM	NaCl
	5 µM	$ZnCl_2$
	30 mM	$Na_2P_2O_7$
	1 % (v/v)	Triton X-100

The following protease or phosphatase inhibitors were added directly before use:

	1 mM	PMSF
	0.15 U/ml	Aprotinin
	1 % (v/v)	Leupeptin
	1 % (v/v)	Pepstatin
	1 mM	Na_3VO_4
Gelshift buffer:	20 mM	HEPES, pH 7.3
	50 mM	KCl
	3 mM	$MgCl_2$
	1 mM	EDTA
	8 %	glycerol
	1 mM	β-mercaptoethanol
	10 mM	dithiothreitol

4.2.5 Animal experiments

4.2.5.1 Wounding

All wound healing-experiments were performed by Prof. Dr. Sabine Werner, who has an authorization for these animal experiments.

BALB/c mice (8-12 weeks of age) were anesthetized with a single intraperitoneal injection of ketamine/xylazine. The hair on the animals' back was shaved and the skin was wiped with 70 % ethanol. Four full-thickness excisional wounds (5 mm diameter, 3-4 mm apart) were generated on the back of each animal by excising skin and *panniculus carnosus* as described by Werner *et al.* (1994). Animals were sacrificed at different time points after injury. The wounds including 2 mm of the epidermal margins were excised and immediately frozen in liquid nitrogen until used for RNA or protein isolation. Tissue, which was used for immunohistochemistry, was treated as decribed in 4.2.5.3.1.

Ketamine/xylazine solution:	25 µl	ketamine
	6.25 µl	xylazine
	269 µl	0.9 % NaCl-solution

4.2.5.2 Glucocorticoid-treatment of mice

For the glucocorticoid-treatment 3 month old, female mice were injected daily subcutaneously with dexamethasone (1 mg per kg body weight). Mice injected with phosphate-buffered saline were used as a control. Seven days after the first injection full-thickness excisional wounds were generated on the back of the animals. After wounding, the daily injection with dexamethasone or PBS was continued until the mice were sacrificed and the wounds excised.

4.2.5.3 Histological methods

4.2.5.3.1 Preparation of cryosections

Embedding of the tissue

Tissue, which should be used for *in situ* hybridization, was fixed overnight at 4°C in 4 % PFA/PBS. Afterwards the tissue was incubated in 15 % sucrose/PBS until it has dropped to the bottom of the vessel (2-3 h). It was subsequently frozen on dry ice in tissue freezing medium.

For immunohistochemistry unfixed sections were used. Therefore, complete wounds were isolated, bisected and directly frozen in tissue freezing medium.

The cryoblocks were stored at –80°C.

Preparation of sections

Cryosections of 6 µm thickness were prepared at –20°C using the cryostat. The sections were mounted on coated slides (Superfrost Plus, Menzel) and dried at RT. They were stored at –80°C.

4.2.5.3.2 Preparation of paraffin sections

Embedding of the tissue

The tissue was fixed overnight at 4°C in 4 % PFA/PBS. After different washing and eliquilibration steps at 4°C (PBS, 0.9 % NaCl, 50 % ethanol in 0.9 % NaCl, 70 % ethanol in 0.9 % NaCl, 30 min each), the tissue was embedded in paraffin as described by Hogan *et al.* (1994).

Preparation of sections

Paraffin sections of 6 µm thickness were prepared using the microtome. The sections were mounted on coated slides (Superfrost Plus, Menzel) and stored at 4°C.

4.2.5.3.3 Hematoxylin/eosin staining

The hematoxylin/eosin staining was used as a counterstaining for *in situ* hybridization. Hematoxylin stains the cell nuclei in blue and eosin stains cytoplasmic and extracellular matrix components in pink.
The sections were treated as follows:

Hematoxylin-solution (4 g/l)	45 sec
ddH_2O	10 sec

ddH_2O	10 sec
ddH_2O	10 sec
Scott-water	30 sec
ddH_2O	10 sec
70 % ethanol	10 sec
eosin solution (1 % in 90 % ethanol)	10 sec
80 % ethanol	10 sec
80 % ethanol	10 sec
95 % ethanol	10 sec
95 % ethanol	10 sec
100 % ethanol	10 sec
100 % ethanol	10 sec
xylene	10 min
xylene	10 min

Afterwards the sections were mounted with S/P™ Accu Mount 60™.

5 Results

To learn more about the function of activin and TGF-β and to gain insight into their mechanisms of action, we searched for genes, which are regulated by activin A and/or TGF-β1 in keratinocytes. For this purpose we used the immortalized, but non-transformed HaCaT keratinocyte cell line (Boukamp *et al.*, 1987). These cells were stimulated with activin A or TGF-β1 and the isolated RNA was analyzed for differential gene expression. Three different approaches were used to identify activin/TGF-β-regulated genes: a.) Differential Display Reverse Transcription PCR, b.) hybridization of a commercially available cDNA array, and c) the direct analysis of known differentiation markers by RNase protection assay. Following these *in vitro* studies, we determined a possible *in vivo* regulation of the identified genes by activin and TGF-β, by analyzing their expression in wounded skin where high levels of these growth factors are present.

5.1 Identification of activin A and TGF-β1 regulated genes using the *differential display reverse transcription* (DDRT)-PCR technology

To identify activin A and TGF-β1 regulated genes in keratinocytes, we used the DDRT-PCR technology. For this purpose, we isolated RNA from quiescent and activin A or TGF-β1 treated cells and generated cDNAs from these RNAs. Using the primers described in *Materials and Methods* for DDRT-PCR, we identified two genes which appeared to be regulated by TGF-β1, but not by activin A. Subsequent cloning and sequencing of the obtained fragments revealed that these genes encode keratin 15 and Leu-13.

5.1.1 Suppression of keratin 15 expression by TGF-β1 *in vitro* and by cutaneous injury *in vivo*.

5.1.1.1 Various Keratin 15 transcripts are downregulated by TGF-β1

Using the DDRT-PCR technology, we obtained a 285 bp fragment which was significantly less abundant after amplification of cDNA from TGF-β1-stimulated cells compared to quiescent cells. This band was eluted from the gel, reamplified and cloned into the transcription vector pBluescript KSII+. Sequence analysis revealed that nucleotides 76-166 and 167-285 of this fragment correspond to nucleotides 425-515 or 1587-1705, respectively, of the keratin 15 (K15) cDNA (Marchuk *et al.*, 1985). Thus, 1071 nucleotides of the K15 cDNA (nt 516-1586) are missing, possibly as a result of alternative splicing (Fig.3). Surprisingly, the first 75 nucleotides of our cDNA did not correspond to the published K15 sequence and did not reveal homology to any known sequence. To determine whether our cloned cDNA corresponds to a naturally occurring RNA we performed RNase protection assays under conditions where every single mismatch can be detected. As shown in Fig. 4, a weak signal was detected below the hybridization probe (labeled "1" in Fig. 4). The size difference between the probe and the protected fragment corresponds to the vector sequences present in our transcript. Therefore, this protected fragment seems to correspond to the complete cDNA template (Fig. 3 (1)), demonstrating that the fragment that we identified is part of a naturally occurring mRNA. Furthermore, the generation of this mRNA is not the result of a chromosomal rearrangement in our HaCaT cell line, since the same protected fragment was also obtained with RNA from primary keratinocytes (data not shown). Complete sequencing of the cloned fragment demonstrated the lack of an open reading frame in the unknown 5'-sequence and the lack of an ATG in the K15 part. Therefore, this mRNA does obviously not encode a protein. It might either be derived from a pseudogene or it could be the result of alternative splicing, generating a non-functional mRNA.

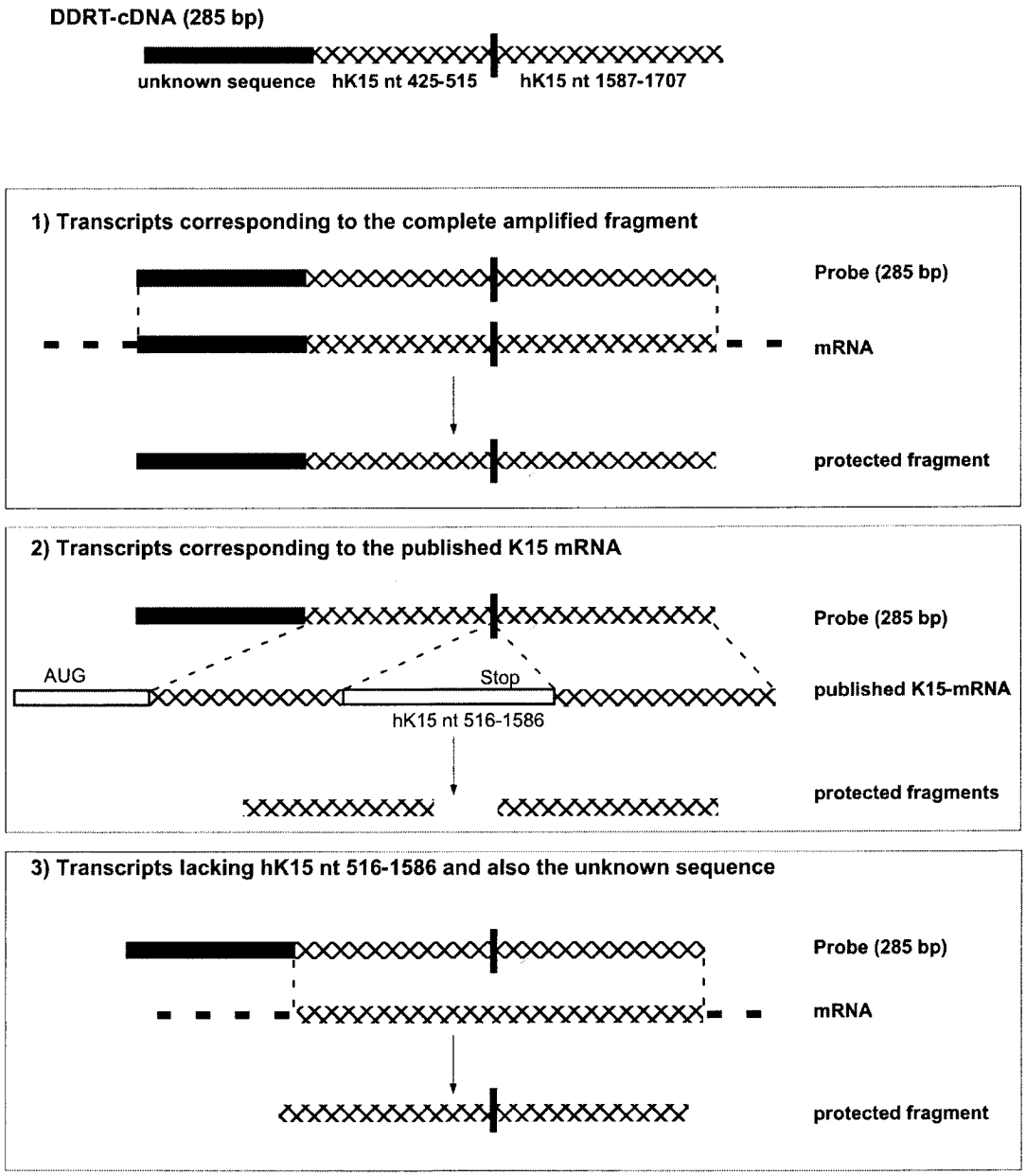

Fig. 3 Different keratin 15 transcripts are present in HaCaT cells. Schematic representation of the DDRT-PCR fragment, the K15 mRNAs present in HaCaT cells and the different protected fragments generated by these mRNAs using the DDRT-PCR fragment as a probe for RNase protection assay. The DDRT-PCR fragment is shown on the top. The unknown sequence is indicated as a black solid bar. K15 specific sequences are indicated by „xxx". A protected fragment corresponding to the complete DDRT-PCR fragment is generated by RNA including the unknown sequence but lacking nucleotides 516-1586 of the K15 mRNA (1). Dotted areas represent unknown sequences. The published keratin 15 mRNA gives rise to two protected fragments, most likely corresponding to nucleotides 425-515 and 1587-1707 of the K15 mRNA (2). The locations of the AUG and the stop codon within the published K15 mRNA are indicated. K15 specific

sequences not included in the DDRT-PCR fragment are indicated with white bars. Finally, transcripts lacking the unknown sequence and also the middle part of the K15 mRNA give rise to a protected fragment of 210 bp (3).

In addition to the full-length protected fragment, we obtained three strong bands in our RNase protection assay. The two lowest bands of approximately 90 and 120 bp (labeled „2" in Fig.4) are likely to correspond to the published version of K15 (Fig. 3 (2)). The additional strong band of 210 bp (labeled „3" in Fig.4) might be derived from a mRNA which lacks the unknown sequence of the 5'-end but also nucleotides 516-1586 of the K15 cDNA (Fig. 3, (3)).

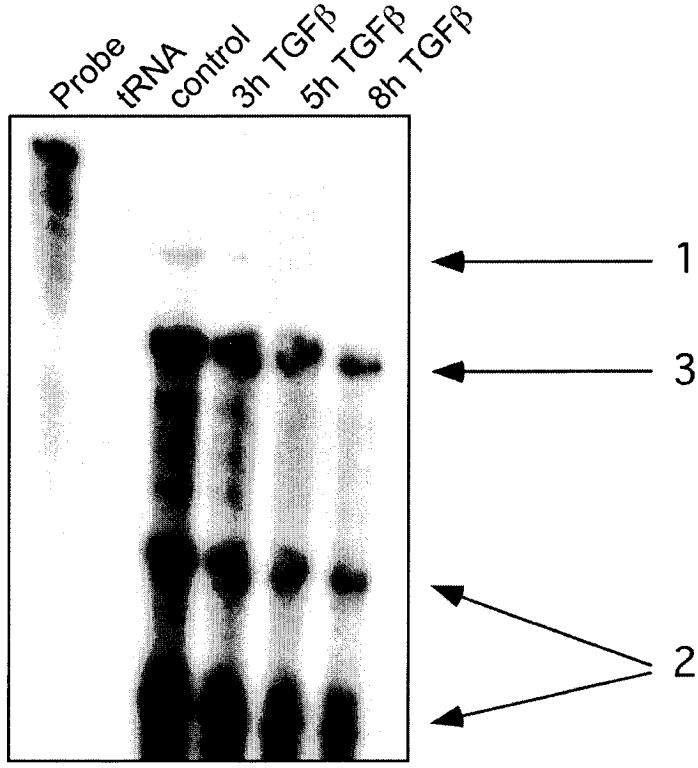

Fig. 4 RNase protection assay demonstrating the negative regulation of different K15 variants by TGF-β1. HaCaT cells were rendered quiescent as described in *Materials and Methods* and treated with 1 ng/ml TGF-β1. RNA was isolated from these cells at different time points after addition of the growth factor. 20 µg total cellular RNA were analyzed by RNase protection assay using a ^{32}P-labeled riboprobe corresponding to the fragment obtained by DDRT-PCR. The time after addition of TGF-β1 is indicated on top of the figure. 50 µg tRNA were used as a negative control. 1000 cpm of the hybridization probe were loaded in the lane labeled „probe" and used as a size marker. Protected fragments generated by three different transcripts (1, 2, 3; see below) are indicated with arrows.

Taken together, these results demonstrate the existence of different K15 variants. Most importantly, all mRNA species were present at higher levels in non-treated keratinocytes compared to TGF-β1-treated cells. Therefore, we were able to verify our DDRT-PCR result by RNase protection assay.

5.1.1.2 Expression of K15 is suppressed by TGF-β1 in cultured keratinocytes

To confirm the regulation of the gene encoding the published full-length K15 by TGF-β1, we amplified a cDNA corresponding to nt 173-366 of the published K15 cDNA. This sequence is not included in our DDRT-PCR fragment. This fragment was used as a template for all experiments shown in Figs. 5-7.

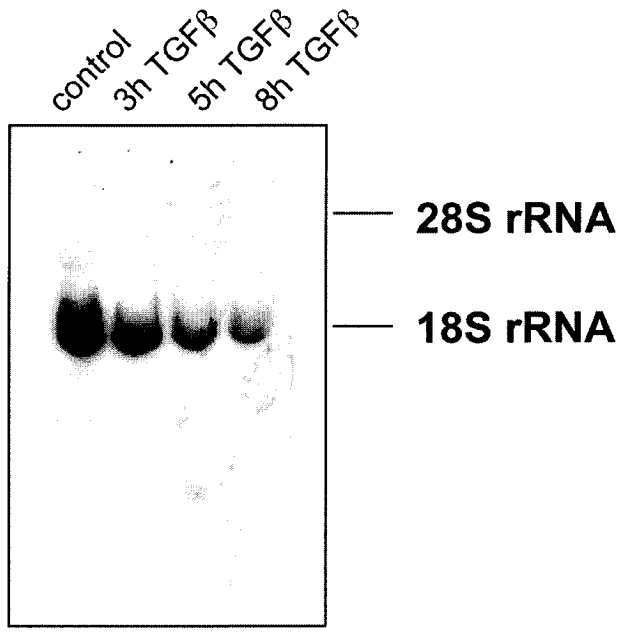

Fig. 5 Expression of the published K15 variant is downregulated by TGF-β1 in cultured HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation. They were subsequently treated with TGF-β1 as indicated. Total RNA was harvested after 3, 5 and 8 hours. 20 µg total cellular RNA was analyzed by Northernblotting for the expression of K15 mRNA. A ^{32}P-labeled cDNA fragment corresponding to the 5'-end of the published K15 cDNA was used as a probe. The locations of the 28s and 18s rRNAs are indicated. Note the presence of a single band of the expected size and the downregulation by TGF-β1.

Using this probe, we confirmed the negative regulation of K15 expression by TGF-β1 as shown in the Northern blot of Fig. 5 and in the RNase protection assay of Fig. 6. A 70 % reduction in the K15 mRNA level was seen within 8 hours after addition of this factor. A similar effect was seen with TNF-α (Fig. 6) and a 40 % reduction was observed after KGF or EGF treatment (Fig.7).

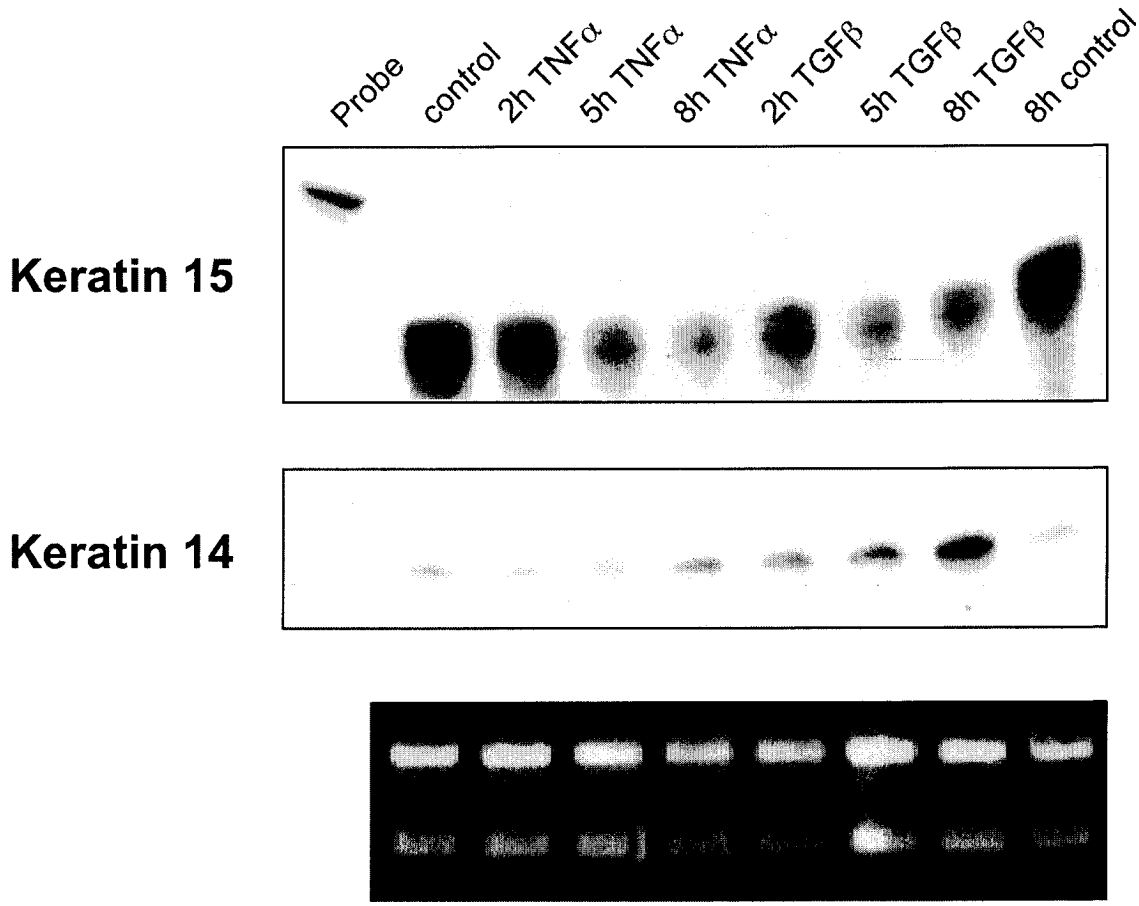

Fig. 6 K15 and K14 expression is differentially regulated by TGF-β1 and TNF-α in HaCaT keratinocytes. Cells were rendered quiescent by serum starvation. They were subsequently treated with TGF-β1 or TNF-α. Total RNA was harvested after 2, 5 and 8 hours as indicated. 20 µg total cellular RNA was analyzed by RNase protection assay for the expression of K15 and K14. 1000 cpm of the hybridization probes were loaded in the lanes labeled "probe" and used as size markers. 1 µg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide (shown below the protection assays).

Importantly, only a single band of the expected size of approximately 1.7 kb was obtained in the Northern blot (Fig.5), indicating the existence of a single keratin 15 transcript which includes the regular 5'-end of the coding sequence. By contrast, no shorter transcripts were detected, suggesting that shorter splice variants which include the published 5'-end do not exist. The lack of such variants was also confirmed by RT-PCR using a 5'-primer overlapping the ATG of the published K15 gene and 3'-primers, which overlap the stop codon or which correspond to the 3'-end of the DDRT-PCR fragment. In both cases only one band of the expected size was obtained (data not shown).

5.1.1.3 Differential regulation of K15 and K14 expression in cultured keratinocytes

K15 is expressed in the basal keratinocytes of stratified epithelia (Moll *et al.*, 1982a and Moll *et al.*, 1982b). The major keratins of these cells, however, are keratins 14 and 5 (reviewed by Fuchs, 1990). Since K15 is a type I keratin like K14 we compared the regulation of both keratins by different growth factors and cytokines. As shown in Fig. 6, TGF-β1 treatment of HaCaT cells did not reduce the expression levels of K14 mRNA but caused even a weak stimulation within 8 hours after addition of the growth factor. By contrast, EGF, KGF and TNF-α had no effect (Fig.7). Therefore, all tested growth factors and cytokines affect the ratios between K14 and K15 mRNAs, particularly TGF-β1.

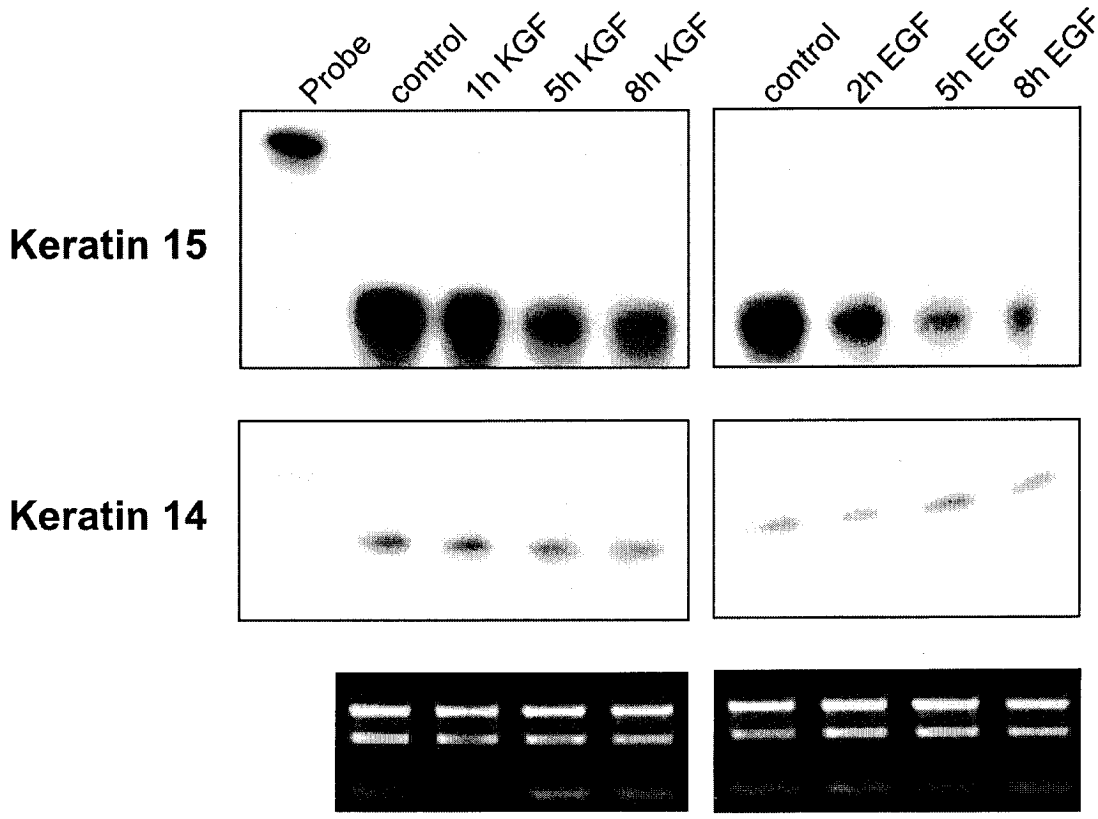

Fig. 7 K15 but not K14 expression is downregulated by KGF and EGF in HaCaT keratinocytes. Cells were rendered quiescent by serum starvation. They were subsequently treated with KGF or EGF. Total RNA was harvested after 1, 2, 5 and 8 hours as indicated. 20 µg total cellular RNA was analyzed by RNase protection assay for the expression of K15 and K14. 1000 cpm of the hybridization probes were loaded in the lanes labeled "probe" and used as size markers. 1 µg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide (shown below the protection assays).

5.1.1.4 Differential regulation of K15, K14 and K6 expression during cutaneous wound repair

To determine a potential regulation of K15 expression by TGF-β*1 in vivo*, we analyzed the expression of this keratin under conditions where high levels of TGF-β1 are present, such as cutaneous wound repair. For this purpose we first amplified a murine cDNA probe corresonding to the human probe located at the 5'-end of the published K15 mRNA. Full-thickness excisional wounds were generated on the back of mice and the wound tissue was isolated at different time points after

injury and used for RNA isolation. As shown in the RNase protection assay of Fig. 8, expression of K15 was detected in non-wounded skin.

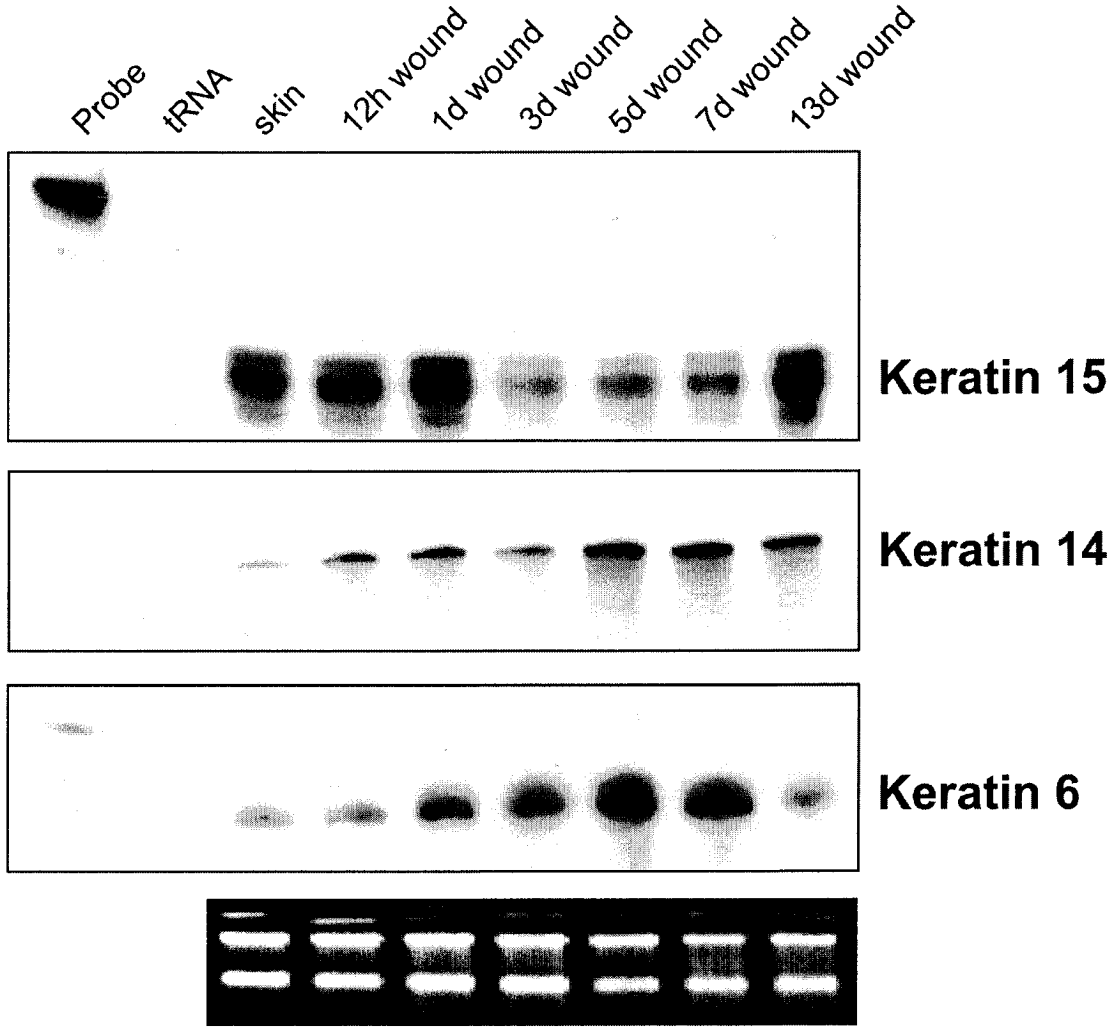

Fig. 8 Expression of K15, K14 and K6 mRNAs during cutaneous wound repair in mice. Mice were wounded as described in *Materials and Methods* and sacrificed at different time points after injury. 20 μg total cellular RNA from normal and wounded skin were analyzed by RNase protection assay for the expression of the different keratins. 50 μg tRNA was used as a negative control. 1000 cpm of the hybridization probes were loaded in the lanes labeled probe and used as size markers. The same set of RNAs were used for all protection assays shown in this figure. 1 μg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays.

Interestingly, the mRNA levels of this keratin declined significantly (54 % reduction) within 3 days after injury (Fig. 8 and 9) and remained low until day 7 after wounding (Fig. 8 and 9). At day 13 after injury when the wound was completely reepithelialized, K15 mRNA levels had returned to the basal levels.

These results suggest that the high levels of growth factors and cytokines present in the wound do indeed suppress K15 expression.

Interestingly, K14 expression was differently regulated by wounding compared to K15. Expression levels of K14 increased continuously until day 5 after wounding. Maximal levels were seen between day 5 and day 7 (Fig. 8 and 9). At this stage of the repair process, K14 mRNA levels were approximately 6-fold higher compared to non-wounded skin. At day 13 after injury, K14 expression had still not declined to the basal level.

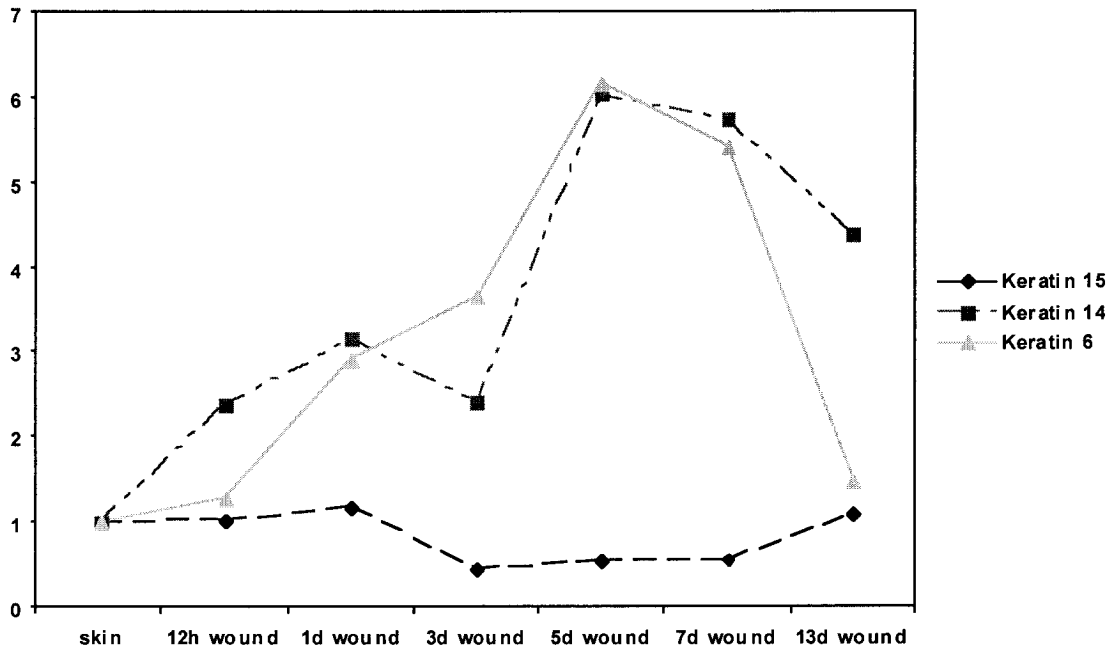

Fig. 9 The time course of keratin expression during wound healing. The degree of keratin mRNA induction or repression at different stages of the repair process as assessed by laser scanning densitometry of the autoradiograms is shown. The expression level in non-wounded skin was arbitrarily set as 1.

As a control for our *in vivo* studies we analyzed the expression of keratin 6 (K6) during wound healing. Expression of this keratin is restricted to hair follicle keratinocytes in non-wounded skin but is induced in the hyperproliferative

epidermis of wounds (Mansbridge *et al.*, 1987). Consistent with these results, we found a strong increase (6-fold induction) in K6 mRNA expression within 5 to 7 days after injury (Fig. 8 and 9). These time points correlate with the hyper-proliferative phase of the wound epithelium. At day 13 after wounding expression levels of this keratin had almost declined to the basal level. Taken together, our results demonstrate a differential regulation of keratins 15, 14 and 6 during cutaneous wound repair.

5.1.1.5 K15 expression is downregulated in the hyperproliferative epithelium of the healing wound

To localize K15 in the wound tissue we stained normal and wounded skin with antibodies against K14, K15 and K 6. A monospecific polyclonal antibody directed against the carboxyterminus of K15 (Leube *et al.*, 1991) was used for this purpose. As shown in Fig. 10A and D, K15 was seen in the outer root sheath of the hair follicles and in all basal cells of the epidermis adjacent to the wound. However, only a weak staining was observed within the hyperproliferative wound epithelium above the granulation tissue. By contrast, the K14 antibody stained all keratinocytes of the epidermis adjacent to and within the wound (Fig. 10B). K6 was not detectable in interfollicular epidermis of normal back skin (data not shown), but was seen in all areas of the hyperthickened epithelium within the wound and adjacent to the wound (Fig. 10E). For a direct comparison, we performed double-immunostaining with the following combinations: anti-keratin 15 (red)/anti-keratin 14 (green) and anti-keratin 15 (red)/anti-keratin 6 (green). Coexpression of K15 and K14 (yellow color) was seen in hair follicle keratinocytes (Fig. 10C), in the basal layer of the normal epidermis (not shown) and the slightly hyperthickened epidermis at the wound edge (Fig. 10C) but not in the strongly hyperthickened epithelium above the granulation tissue. Co-expression of keratins 15 and 6 was restricted to hair follicle keratinocytes and to the basal cells of the slightly hyperthickened epidermis adjacent to the wound where the keratinocytes are in

contact with a basement membrane (Fig. 10F). By contrast, the keratinocytes of the strongly hyperthickened epithelium above the granulation tissue expressed K6 but not K15. These data demonstrate a differential expression of keratins 14, 6 and 15 in the wound.

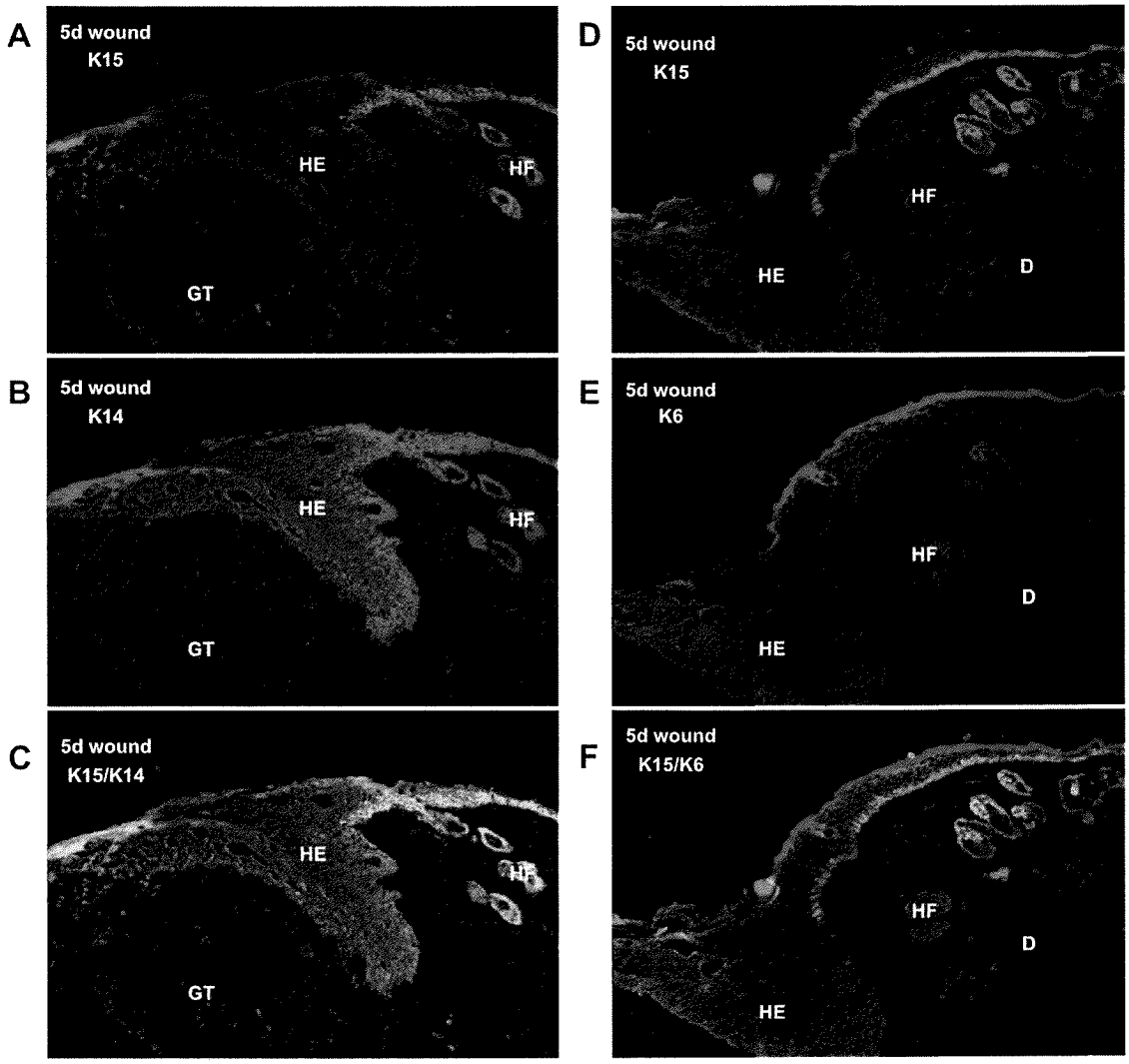

Fig. 10 Keratin 15, 14 and 6 are differentially expressed in wounded skin. Frozen sections were taken from the middle of a 5d wound and incubated with antibodies directed against K15 (A, C, D, F), K14 (B, C), and K6 (E, F). Double-immunofluorescence staining with antibodies directed against K15 (red), K14 (green) and K6 (green) are shown in (C) and (F). Areas where two types of keratins are expressed appear yellow. Magnification: 100x. HF: Hair follicle; HE: Hyperproliferative epithelium; GT: Granulation tissue, D: dermis.

We subsequently determined a possible correlation between K15 expression and cell proliferation. For this purpose we performed double staining with the anti-K15 antibody (red) and anti-Tec-3 (green). The latter detects the Ki67 protein present in proliferating cells. As shown in Fig.11, proliferating cells were predominantly seen within the basal and lower suprabasal layers of the hyperthickened wound epithelium and in the hair follicles adjacent to the wound.

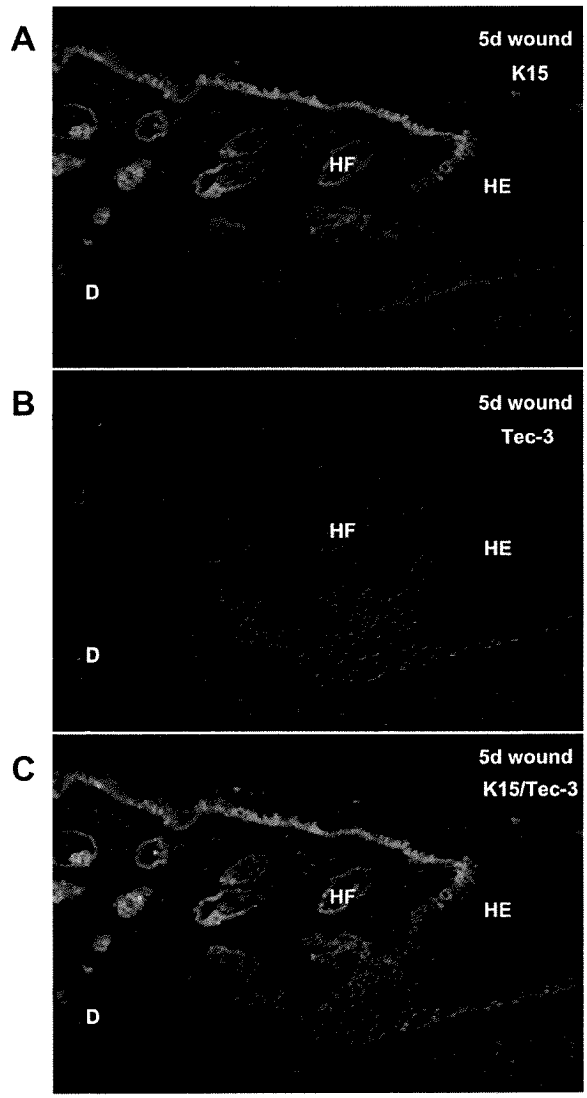

Fig. 11 Expression of K15 in comparison to Tec-3 in wounded skin. Frozen sections were taken from the middle of a 5d wound and incubated with antibodies directed against K15 (A, C) and Tec-3 (B, C). Double-immunofluorescence staining with antibodies directed against K15 (red) and Tec3 (green) is shown in (C). Magnification: 100x. HF: Hair follicle; HE: Hyperproliferative epithelium; D: Dermis.

Coexpression of Ki67 and K15 was restricted to some hair follicle keratinocytes (Fig. 11C), to basal keratinocytes of the normal epithelium (data not shown) and to the slightly hyperthickened epithelium adjacent to the wound (Fig. 11C). These data demonstrate a preferential expression of K15 in non-dividing cells, although K15 expression is not strictly incompatible with cell proliferation.

5.1.1.6 Discussion

Keratins are intermediate filament proteins which are specifically expressed in epithelia. They can be grouped into two types, the smaller and acidic type I keratins and the larger and basic/neutral type II keratins. In contrast to other types of intermediate filaments, keratins are obligatory heteropolymers, and one member of each type of keratin is required to form the heterotypic tetramers representing the intermediate filament subunits (reviewed by Fuchs and Weber, 1994). Keratins are frequently expressed in specific pairs of acidic and basic keratins in epithelial tissues which are characteristic for the differentiation pathway. In the epidermis, K14 and K5 are expressed at high levels in the basal layers and only at low levels in the suprabasal layers (for review see Fuchs, 1990). In contrast to these well-characterized and abundant keratins, K15 has been poorly characterized. This keratin is expressed in the basal layer of the adult epidermis and in other stratified epithelia, but its function is yet unknown (Moll *et al.*, 1982a, Moll *et al.*, 1982b, Leube *et al.*, 1988, Lloyd *et al.*, 1995). It has been shown to dimerize with K5 in the absence of K14 (Lloyd *et al.*, 1995, Jonkman *et al.*, 1996), although K5/K15 heterodimers do obviously not assemble into mature 10-nm intermediate filaments and higher order keratin bundles like K5/K14 dimers (Jonkman *et al.*, 1996). A characteristic expression pattern of K15 has also been demonstrated in the hair follicle of sheep, mice and humans (Whitbread *et al.*, 1998, Lyle *et al.*, 1998, Waseem *et al.*, 1999), and it has been suggested that K15 is a marker for human hair follicle stem cells and that loss of K15 is one of the earliest signs of the transition from a stem cell to a transit-amplifying cell (Lyle *et al.*, 1998). Finally, expression of K15 was shown to be downregulated in hyperproliferating epidermis

as seen in psoriasis and hypertrophic scars and in activated keratinocytes growing in skin equivalent cultures, indicating that the activated phenotype is not compatible with the K15 expression (Waseem *et al.*, 1999). However, the factors which regulate expression of K15 *in vitro* and *in vivo* have as yet not been identified.

In this study we identified the K15 gene as a novel gene regulated by the multifunctional cytokine TGF-β1. Surprisingly, we identified a novel K15 variant which lacks the coding sequence for the carboxyterminal half of the protein. In addition, the 5'-end of the mRNA including the AUG was replaced by an unknown sequence. Since we detected a protected fragment of the expected size by RNase protection assay with this cDNA probe, our DDRT-PCR fragment is not the result of a cloning artefact. Furthermore, the generation of this fragment is not due to a chromosomal rearrangement in the HaCaT cell line, since it was also obtained with RNA from primary human keratinocytes. Nevertheless, the corresponding mRNA is unlikely to encode a functional protein, since we could not detect a longer open reading frame. Therefore, it might either be derived from a pseudogene or it could be the result of alternative splicing.

The existence of an additional band which is likely to be generated by RNA lacking the unknown sequence at the 5'-end but also the nucleotides encoding the carboxyterminal half of K15 suggests the existence of a carboxy-terminally truncated K15 protein which might exert a dominant-negative effect. However, only a single band of the expected size was obtained in a Northernblot with a probe corresponding to the 5'-end of the published sequence. Therefore the additional transcript (3, Fig.3) either does not include the published 5'-end or is of similar length as the transcript which encodes the published K15 variant. The lack of smaller K15 gene products which include the published amino-terminus was further supported by results from RT-PCR experiments, since we did not amplify a smaller fragment using primers overlapping the ATG and the stop codon or the 3'-end of the DDRT-PCR fragment. These results suggest that shorter K15 proteins which include the published amino-terminus do not exist, However, we cannot exclude the existence of truncated K15 variants which include another 5'-end.

Interestingly and relevant to the goal of our study, expression of all variants was strongly suppressed by TGF-β1 and TNF-α and to a lesser extent also by the keratinocyte mitogens EGF and KGF. The downregulation by factors which differentially regulate keratinocyte proliferation, demonstrate that the suppression of K15 expression is not generally associated with the regulation of cell proliferation *in vitro*. These results provide new insight into the regulation of K15 expression in cultured keratinocytes, since growth factor- and cytokine-dependent expression of this keratin has as yet not been demonstrated. However K15 expression in HaCaT cells was shown to be dependent on the concentration of retinoic acid in the medium (Breitkreutz *et al.*, 1993). Furthermore, its expression is regulated by cell density, since it was expressed at higher levels in confluent cultures compared to cells grown at low density (Ryle *et al.*, 1989).

The cytokine-mediated suppression of K15 expression is likely to be of biological significance, since expression of the full-length published K15 was also regulated by these factors as determined by RNase protection assay and Northern blot analysis using a probe corresponding to the amino-terminus of the protein. In contrast to K15, expression of the major basal type I keratin, K14, was not regulated by EGF, KGF and TNF-α, and upregulated by TGF-β1. The latter finding is in agreement with results from Jiang *et al.* (1995) who observed transcriptional induction of K14 and K5 genes by TGF-β. These results indicate that growth factors alter the ratio between the two types of keratin.

To gain insight into the *in vivo* importance of this regulation we analyzed the expression of K15 and K14 during cutaneous wound healing where high levels of these factors are present (Werner *et al.*, 1992, Marchese *et al.*, 1995, Hübner *et al.*, 1996c, Frank *et al.*, 1996). Expression of K6 which is known to be upregulated in wounded skin was analyzed for comparison (Mansbridge *et al.*, 1987). Consistent with the *in vitro* results, we found a strong downregulation of K15 expression after skin injury. By contrast, expression of K14 and particularly of K6 was upregulated during the wound healing process. These results suggest that the regulation of these genes by growth factors that we observed *in vitro* might also be

of biological importance in the *in vivo* situation. Interestingly, the lowest levels of K15 mRNA were seen during the phase of reepithelialization which is characterized by the presence of activated keratinocytes which migrate and proliferate. K15 has been shown to be excluded from activated keratinocytes in psoriatic skin and in hypertrophic scars (Waseem *et al.*, 1999). Consistent with this result we found a strong decline in K15 protein expression in the activated keratinocytes within the strongly hyperthickened wound epithelium above the granulation tissue. However, there was no strong correlation between cell proliferation and expression of K15, since the proliferating keratinocytes at the wound edge did still express this protein. By contrast, the availability of K15 suppressing growth factors and cytokines might determine the level of K15 expression. This hypothesis is supported by the expression of K15 in basal cells of the slightly hyperthickened epidermis adjacent to the wound above the basement membrane and the underlying dermis, but not within the strongly hyperthickened epithelium above the granulation tissue. Thus the multitude of factors which are produced in the granulation tissue, including KGF, TGF-βs and TNF-α (Martin, 1997) are likely to suppress expression of K15 in the adjacent keratinocytes in a paracrine manner.

The functional consequences of the reduced expression of K15 in the hyperthickened wound epithelium are as yet unknown and a functional analysis will require the targeted overexpression of this molecule in these activated keratinocytes. Nevertheless, the strong regulation of K15 expression by various growth factors present in the healing wound suggests a novel role of these factors in the organization of the cytoskeleton in keratinocytes which might influence migration, proliferation and/or differentiation of these cells during wound repair and in hyperproliferative skin disease.

5.1.2 Induction of *leu-13* expression by cytokines *in vitro* and by cutaneous injury *in vivo*

Using the DDRT-PCR technology, we obtained a 182 bp cDNA fragment of a gene, which seemed to be upregulated after TGF-β1 treatment in HaCaT cells. The band was eluted from the gel, the fragment was reamplified, and cloned into the transcription vector pBluescript KSII(+). Sequence analysis revealed that the amplified fragment corresponds to nucleotides 465-647 of the published *leu-13* sequence (Deblandre *et al.*, 1995). Leu-13 is a 16 kDa protein, which is expressed after interferon (IFN)-α or -β treatment of different cell types including leukocytes, fibroblasts, epithelial and endothelial cells (Pumarola-Sune, *et al.*, 1986, Deblandre *et al.*, 1995). It is part of a multimeric signal transduction complex of membrane proteins, which triggers growth inhibition and cell adhesion (Chen *et al.*, 1984, Evans *et al.*, 1993, Tedder *et al.*, 1994).
Unfortunately, the upregulation of *leu-13* mRNA by TGF-β1 could not be confirmed by RNase protection assay using the DDRT-PCR fragment as a template. However, *leu-13* expression turned out to be strongly upregulated by cytokines in keratinocytes, which made it interesting for further investigations.

5.1.2.1 Expression of *leu-13* is upregulated by IFN-α and TNF-α in cultured keratinocytes

As demonstrated by the DDRT-PCR result, *leu-13* is expressed in the keratinocyte cell line HaCaT. Since *leu-13* expression can by induced in lymphocytes, epithelial cells and fibroblasts by interferons-α and-β (Deblandre *et al.*, 1995), we investigated the expression of this gene in IFN-α-treated keratinocytes. As shown in Fig. 12, *leu-13* mRNA increased significantly 2 hours after IFN-α addition.

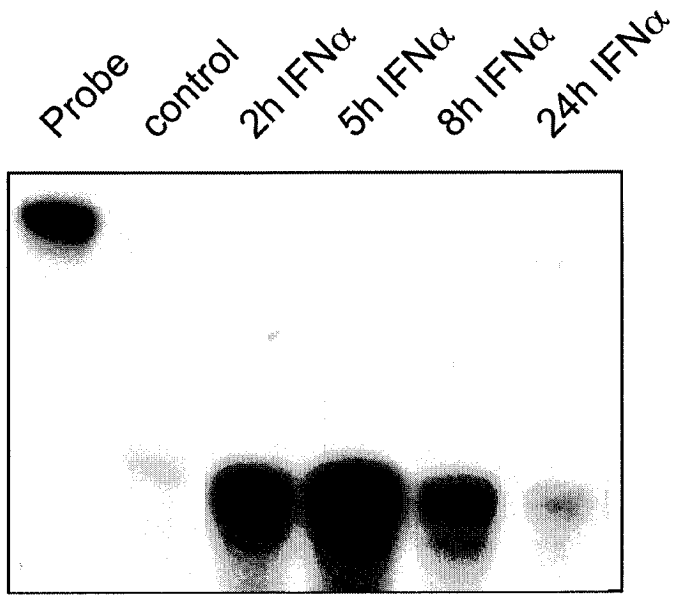

Fig. 12 *Leu-13* expression is upregulated by IFN-α in HaCaT keratinocytes. Cells were rendered quiescent by serum starvation. They were subsequently treated with IFN-α. Total RNA was harvested after 2, 5, 8, and 24h as indicated. Twenty μg total cellular RNA was analyzed by RNase protection assay for the expression of *leu-13*. 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used a size marker.

Since interferons have a growth inhibitory effect on keratinocytes (Nickoloff *et al.*, 1984, Khan *et al.*, 1993), we determined, whether other cytokines and growth factors that induce (KGF and EGF) or suppress (TNF-α, TGF-β and activin) the proliferation of keratinocytes (Coffey *et al.*, 1988a, Marchese *et al.*, 1990, Rheinwald and Green, 1977, Detmar and Orfanos, 1990, Shimizu *et al.*, 1998) can also regulate *leu-13* expression. We demonstrated that TNF-α induces *leu-13* expression in HaCaT cells (Fig. 13). *Leu-13* mRNA was upregulated 3h after TNF-α addition and remained high for at least another 24 hours. In contrast, several growth factors, including KGF, EGF, TGF-β1 and activin A, had no effect on *leu-13* expression (data not shown).

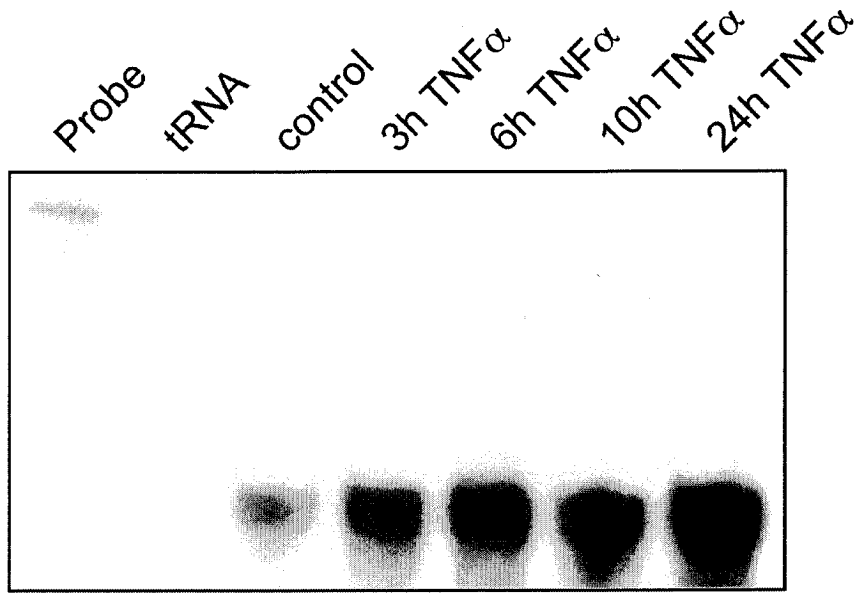

Fig. 13 *Leu-13* expression is upregulated by TNF-α in HaCaT keratinocytes. Cells were rendered quiescent by serum starvation. They were subsequently treated with TNF-α. Total RNA was harvested 3, 6, 10, and 24h after addition of TNF-α as indicated. Twenty μg total cellular RNA was analyzed by RNase protection assay for the expression of *leu-13*. 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker.

5.1.2.2 *Leu-13* expression is upregulated during the tissue repair process

TNF-α expression is strongly induced during wound healing. It is predominantly expressed by neutrophils and macrophages during the inflammatory phase of the repair process (reviewed by Clark, 1995, Hübner *et al.*, 1996b). To determine a possible regulation of *leu-13* by this cytokine *in vivo*, we analyzed the expression of *leu-13* during cutaneous wound repair. Full-thickness excisional wounds were generated on the back of mice, and the wound tissue was isolated at different time points after injury and used for RNA isolation. As shown in the RNase protection assay of Fig. 14, *leu-13* was weakly expressed in non-wounded skin. Interestingly, the mRNA levels increased strongly 1 day after wounding and remained high until day 5 after injury (Fig.14).

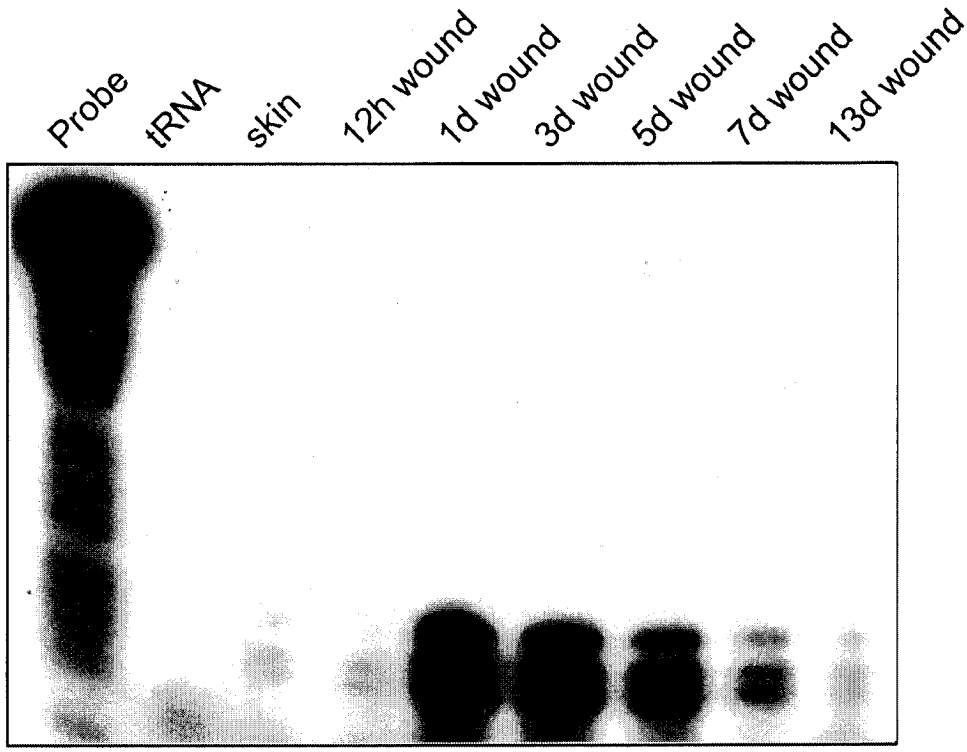

Fig. 14 Expression of *leu-13* during cutaneous wound repair in mice. Mice were wounded as described in *Materials and Methods* and sacrificed at different time points after injury. Twenty μg total cellular RNA from normal and wounded skin was analyzed by RNase protection assay for the expression of *leu-13*. Fifty μg tRNA was used as a negative control. 1000 cpm of the hybridization probe were loaded in the lane labeled probe and used as a size marker.

This result suggests that the high levels of cytokines, which are present in the wound, do indeed increase *leu-13* expression *in vivo*.

5.1.2.3 Induction of *leu-13* expression after injury is reduced in glucocorticoid-treated mice

To further analyze the possibility, that inflammatory cell-derived cytokines such as TNF-α are responsible for the induction of *leu-13* expression during wound repair, we investigated the *leu-13* mRNA levels after injury in glucocorticoid-treated mice. These mice show severe defects in tissue repair, including reduced numbers of infiltrated inflammatory cells, delayed reepithelialization and impaired granulation tissue formation. The induction of pro-inflammatory cytokines during wound healing

is dramatically reduced in comparison to normal mice. The levels of TNF-α mRNA in dexamethasone-treated wounds were shown to be 55-65 % lower than in control wounds (Hübner *et al.*, 1996c).

Dexamethasone was injected daily subcutaneously at 9 a.m. over a period of seven days before wounding and 1-5 days after wounding. RNA was isolated at different time points after wounding and analyzed by RNase protection assay for *leu-13* expression (Fig. 15).

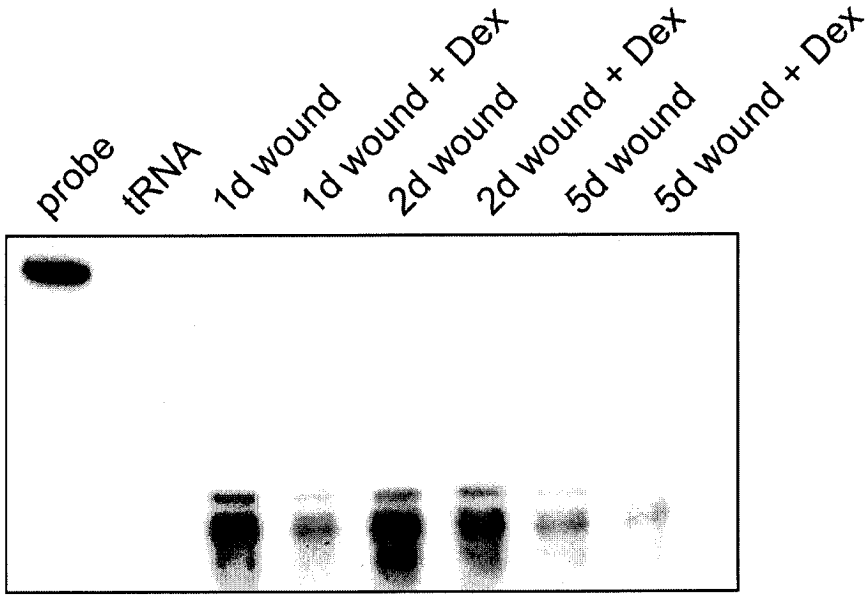

Fig. 15 Induction of *leu-13* mRNA expression is reduced during wound healing in glucocorticoid-treated mice. Balb/c mice were treated with dexamethasone (1 mg/kg) as described in *Materials and Methods*. Mice injected with phosphate-buffered saline were used as a control. Total cellular RNA was isolated 1, 2 and 5 days after wounding. 20 µg of the RNA was analyzed by RNase protection assay for *leu-13* expression. 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probe were loaded in the lane labeled probe and used as a size marker.

The induction of *leu-13* mRNA expression was significantly reduced in the dexamethasone-treated mice in comparison to control mice at day 1 after wounding. However, no significant differences were detected at later time points after injury.

The reduced expression of TNF-α and *leu-13* in the early wounds of glucocorticoid-treated mice further supports the hypothesis that *leu-13* is regulated by these

cytokines *in vivo*.

5.1.2.4 *Leu-13* mRNA is expressed in inflammatory cells of the granulation tissue

To localize *leu-13* mRNA in wounded tissue we performed *in situ* hybridization with sections from wounded skin (day 5 after wounding). *Leu-13* mRNA expression was detected in the inflammatory cells of the granulation tissue, particularly in macrophages, but not in epithelial cells (Fig. 16A-C).

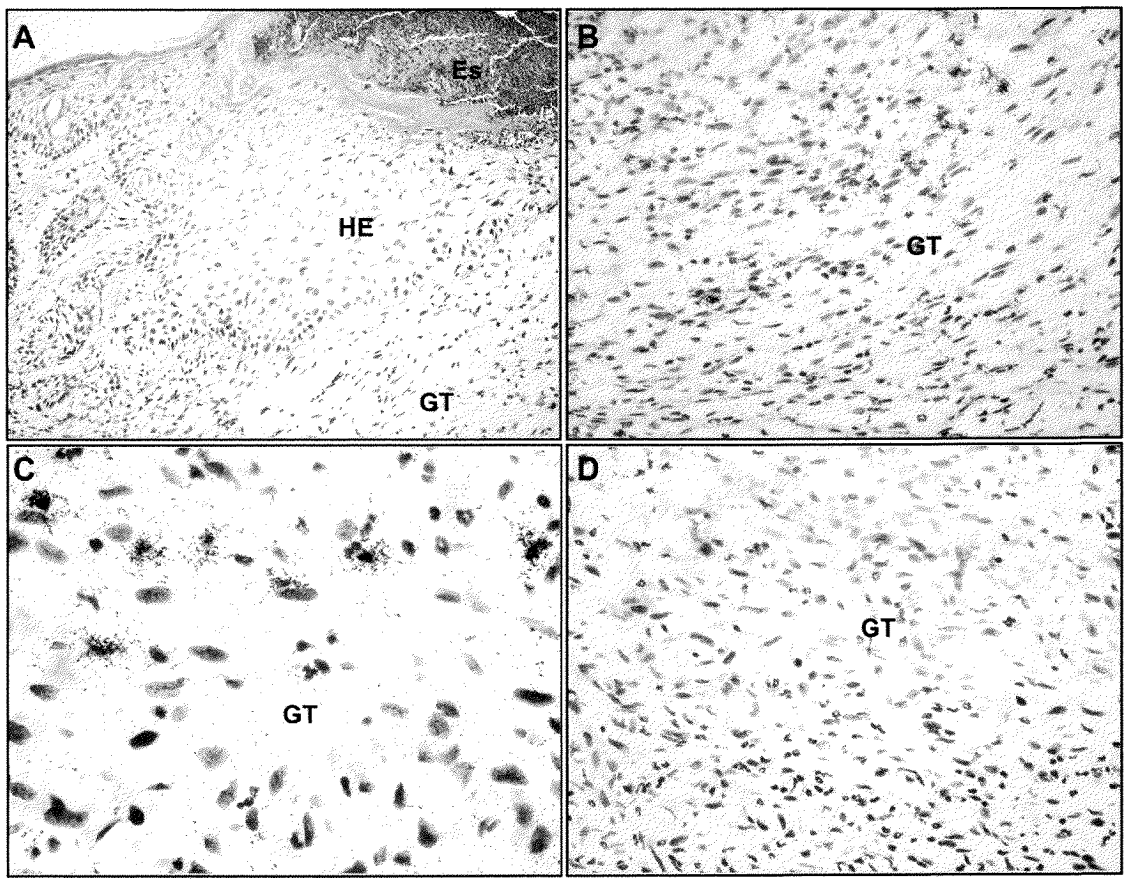

Fig. 16 *Leu-13* is expressed in inflammatory cells of the granulation tissue. Paraformaldehyde-fixed frozen sections were taken from the middle of 5d wounds and analyzed by *in situ* hybridization for the presence of *leu-13* mRNA using a [α-^{35}S]-labeled mouse *leu-13* antisense RNA. Hybridization with the corresponding sense RNA was used as a negative control (D). Note the presence of *leu-13* mRNA in inflammatory cells of the granulation tissue. Magnifications: 100x (A), 200x (B,D), 400x (C). D: Dermis, Es: Eschar, HE: Hyperproliferative epithelium and GT: Granulation tissue.

5.1.2.5 *Leu-13* mRNA expression is upregulated in inflammatory bowel disease

As described above, we demonstrated a strong increase in *leu-13* expression during the early inflammatory phase of skin repair. Therefore, we speculated that *leu-13* expression might also be induced during inflammatory processes of other organs. To address this question, we analyzed *leu-13* expression in inflammatory bowel disease (IBD), a multifactorial disease of the gut. Two forms of IBD can be distinguished: Crohn's disease (CD) and ulcerative colitis (UC). These disorders are characterized by phases of acute inflammation followed by remission. Often a total surgical resection of the affected parts is required, because of intractable disease, presence of dysplasia and/or the risk of perforation (for review see Rubin and Farber, 1994). CD mainly affects the terminal ileum. Segments of the inflamed tissue are directly adjacent to non- affected segments. In contrast, UC affects predominantly the colon. It spreads continuously, usually extending from the rectum for a variable distance proximally (for review see: Podolsky *et al.*, 1991, Gibson and Pavli, 1992, Rubin and Farber, 1994). To determine a possible role of Leu-13 in inflammatory bowel disease, we analyzed total cellular RNA extracted from surgical specimens of CD-patients for *leu-13* expression. A total of 28 specimens from 6 CD patients were investigated. In all cases between 3 and 6 specimens from differently affected areas were taken to correlate the *leu-13* expression with the degree of inflammation. Besides histological analysis, the degree of inflammation was determined by analysis of IL-1β expression. In highly inflamed areas, which show epithelial destruction and large amounts of inflammatory cells in the mucosa, a strong IL-1β expression could be detcted (Hübner *et al.*, 1997).

A strong overexpression of *leu-13* was found in highly inflamed areas of the CD patients (Table 1, Fig. 17). The strongest overexpression was 26-fold in a specimen which was characterized by particularly severe inflammation and tissue damage (Tab. 1).

Patient No.	Tested samples	Maximal degree of overexpression
1	6	3-fold
2	4	2-fold
3	3	2-fold
4	7	26-fold
5	5	2-fold
6	4	3-fold

Table 1: Overexpression of *leu-13 mRNA* in CD patients. The degree of over-expression was determined by comparison of *leu-13* expression in non-inflamed or weakly inflamed areas with the levels of leu-13 mRNA in highly-inflamed areas.

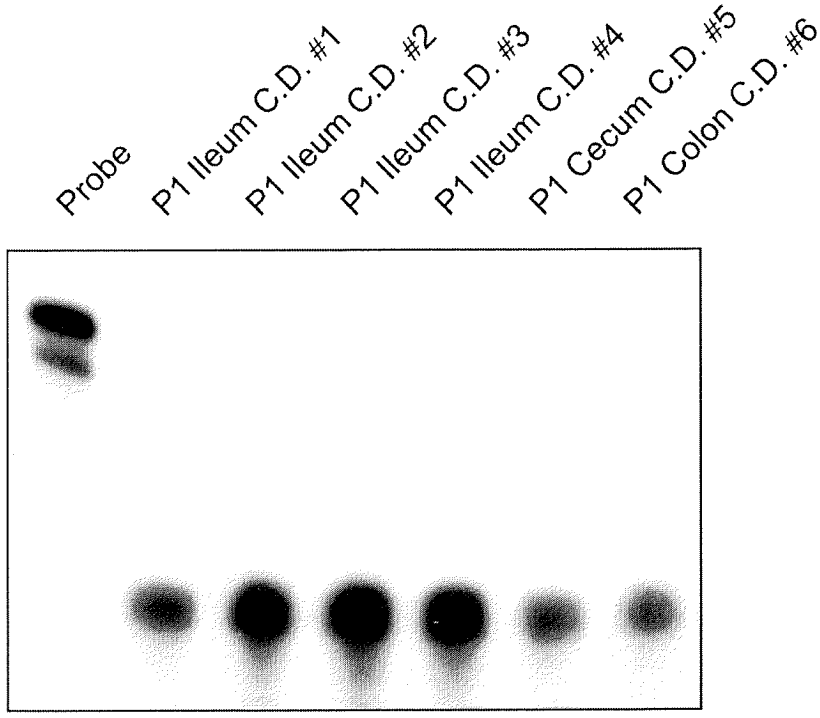

Fig. 17 Overexpression of *leu-13* in Crohn's disease. Total cellular RNA was isolated from surgical specimens of different areas of the ileum, cecum and colon from one patient. 30µg was analyzed by RNase protection assay for *leu-13* expression. 50 µg tRNA was used as a negative control (data not shown). 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker. #1 non-inflamed area; #2,#5 and #6 weakly inflamed areas and #3 and #4 highly inflamed areas.

5.1.2.6 *Leu-13* mRNA is expressed in inflammatory cells of the inflamed gut

To determine the sites of *leu-13* mRNA expression in the inflamed gut, we performed *in situ* hybridization with sections from CD patients. We selected areas with a different degree of inflammation (Fig. 18A, B).

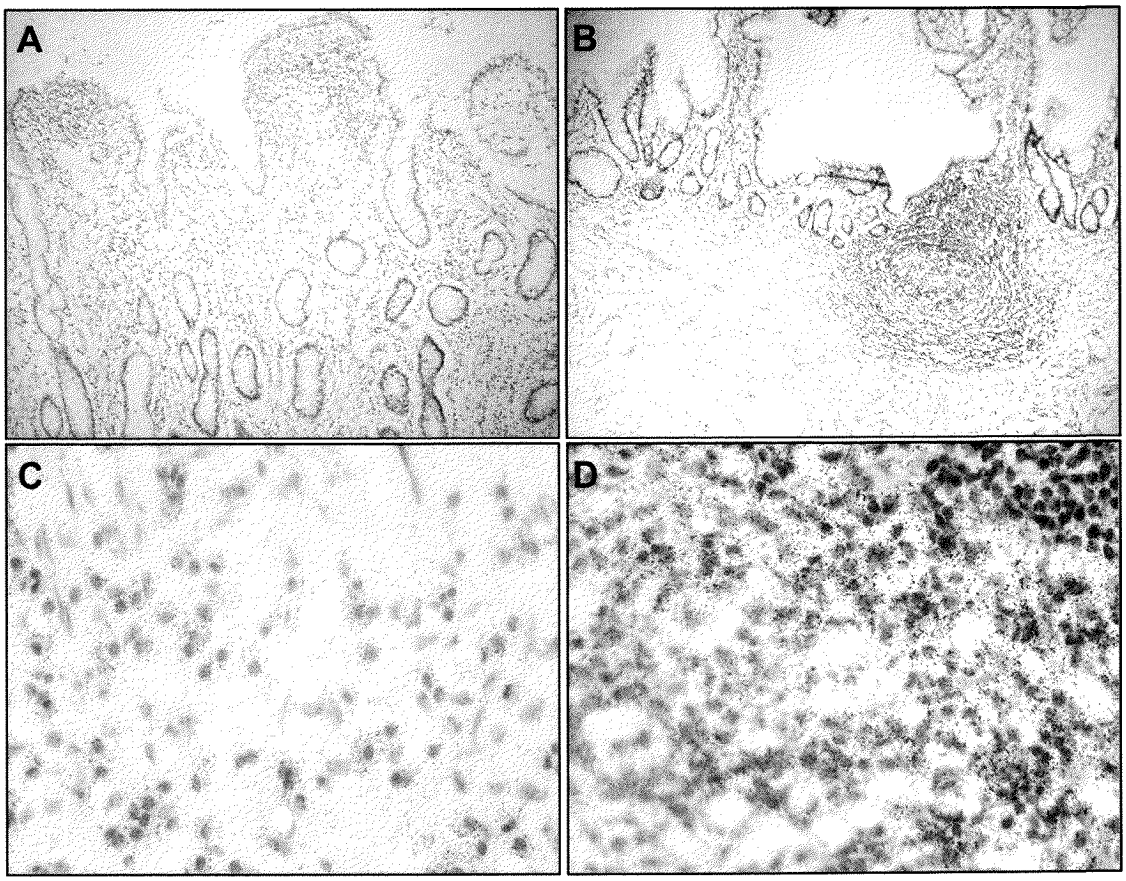

Fig. 18 *Leu-13* mRNA is expressed in inflammatory cells in Crohn's disease. Paraformaldehyde-fixed frozen sections were taken from differently inflamed areas of CD patients. Specimens A and C were taken from a weakly inflamed area, whereas specimens B and D belong to a highly inflamed area. The sections were analyzed by *in situ* hybridization for the presence of *leu-13* mRNA using a [α-^{35}S]-labeled mouse *leu-13* antisense RNA. Hybridization with the corresponding sense RNA was used as a negative control (data not shown). Note the presence of *leu-13* mRNA in inflammatory cells and in the epithelium. Magnifications: 50x (A, B), 400x (C, D).

Highly inflamed areas showed a completely destroyed epithelium and large amounts of inflammatory cells. (Fig. 18 B). High levels of *leu-13 mRNA* were detected in the inflammatory cells (Fig. 18 C, D).

5.1.2.7 Discussion

In the present study, we identified *leu-13* as a cytokine-regulated gene in keratinocytes. Leu-13 is described as a 16 kDa protein, which is expressed after IFN (-α, -β or -γ) treatment on various cell types, including lymphocytes, epithelial cells, fibroblasts and endothelial cells (Hillman *et al.*, 1987, Jaffe *et al.*, 1989, Deblandre *et al.*, 1995). However, expression of *leu-13* by keratinocytes has not been previously shown. The Leu-13 protein is part of a multimolecular signal transduction complex, which is located on the cell surface. In lymphocytes, it is associated in this complex with CD19, CD21 and CD81 (Tedder *et al.*, 1994). Leu-13 plays an important role in the regulation of proliferation and cell-cell adhesion. Thus it inhibits the growth of lymphocytes and induces homotypic lymphocyte aggregation (Hillman *et al.*, 1987, Evans *et al.*, 1993).

In this study we identified keratinocytes as a novel *leu13*-producing cell type. To determine a possible role of Leu-13 in the regulation of keratinocyte proliferation, we analyzed the regulation of *leu-13* mRNA expression by growth inhibitory factors such as IFN-α and TNF-α. We found a strong upregulation of *leu-13* expression after treatment with these cytokines, indicating a role of *leu-13* in the control of keratinocyte proliferation. Similar to lymphocytes and endothelial cells (Hillman *et al.*, 1987, Jaffe *et al.*, 1989), the inhibition of keratinocyte proliferation through regulation of *leu-13* expression seems to be controlled by members of the interferon family. But in contrast to lymphocytes (Evans *et al.*, 1993), TNF-α is an additional inducer of *leu-13* expression in keratinocytes. By contrast, growth factors, which regulate the proliferation of keratinocytes, including TGF-β, activin, EGF and KGF (Rheinwald and Green, 1977, Coffey *et al.*, 1988a, Marchese *et al.*, 1990, Shimizu *et al.*, 1998) had no effect, indicating that *leu-13* expression is not simply a proliferation marker. By contrast, it might fulfill more specific, but as yet unidentified functions in kerationcytes.

To gain insight into a possible *in vivo* function of Leu-13 in keratinocytes, we analyzed the expression of this gene during cutaneous wound repair, where high

levels of pro-inflammatory cytokines are present. Analyzing 5 day wounds by *in situ* hybridization, we found a strong expression of *leu13* mRNA in inflammatory cells of the granulation tissue, but not in keratinocytes. These results argue against an *in vivo* function of Leu-13 in keratinocytes, although the sensitivity of our *in situ* hybridization might have been too low to detect *leu-13* transcripts in these cells. Furthermore, expression of *leu-13* in keratinocytes might occur earlier during the repair process, as suggested by the earlier maximum of TNF-α expression during wound healing (Hübner *et al.*, 1996c). Thus, analysis of 1 and 3 day wounds by *in situ* hybridization will be necessary to determine a possible role of *leu-13* in keratinocytes during wound healing.

Leu-13 mRNA was found at high levels in inflammatory cells of the granulation tissue in 5-day wounds. The shape of their nuclei suggests that the *leu-13*-expressing cells are predominantly macrophages. The reason for the upregulation of *leu-13* expression in these inflammatory cells still needs to be investigated, since no regulatory mechanisms of *leu-13* expression in macrophages have been described yet. Therefore, the effects of growth factors and cytokines on *leu-13* expression in these cells should be determined.

The function of *leu-13* for macrophages is as yet unknown. It might play a role in the regulation of their proliferation or it could modulate their adhesive properties as previously shown for lymphocytes. Thus *leu-13* plays a role in the suppression of L-selectin-mediated lymphocyte binding to high endothelial venules (Frey *et al.*, 1997). Furthermore it activates lymphocyte homotypic adhesion processes (Evans *et al.*, 1993) and it stimulates the β_1 integrin-mediated adhesion of lymphocytes to extracellular matrix molecules (Frey et al., 1997). Therefore, it will be interesting to determine the effect of *leu-13* on macrophage adhesiveness.

We also investigated the *leu-13* expression in inflammatory bowel disease. Interestingly, high levels of *leu-13* mRNA were detected in severely affected areas of patients suffering from CD. Similar as in wounds, inflammatory cells were identified as the major *leu-13* expressing cells in the inflamed gut. These results

suggest that *leu-13* expression is a general feature of inflammatory processes and might be used as a marker for the degree of inflammation.

5.2 Identification of activin regulated genes by expression analysis of known differentiation markers

Since activin has been shown to play a role in the regulation of keratinocyte proliferation and differentiation, we determined the effect of activin A and its homologue TGF-β1 on the expression of known differentiation markers. For this purpose we investigated, among others, the regulation of the transcriptional regulator Mad1 by these growth factors. Mad1 is a basic region/helix-loop-helix/leucine zipper transcriptional regulator which can dimerize with the Max protein (Ayer *et al.*, 1993). Mad1/Max heterodimers antagonize the transcriptional activation exerted by a complex between Max and the Myc proto-oncoprotein (Grandori *et al.*, 2000). Mad1 is a member of a family of transcriptional regulators that also includes Mxi1/Mad2, Mad3, and Mad4 (Zervos *et al.*, 1993, Hurlin *et al.*, 1995a).

Expression of Mad1 appears closely linked to terminal differentiation of many different cell types (reviewed by Grandori *et al.*, 2000). Most importantly, a series of *in vitro* and *in vivo* expression studies and the inhibition of colony formation in a mouse keratinocyte cell line by Mad1 overexpression (Västrik *et al.*, 1995, Hurlin *et al.*, 1995a,b, Lymboussaki *et al.*, 1996, Gandarillas and Watt, 1997) suggest that Mad1 is involved in the cessation of cell proliferation associated with keratinocyte differentiation.

5.2.1 Regulation of *mad1* mRNA and protein expression in HaCaT cells by activin A and TGF-β1

To analyze the effect of activin A and TGF-β1 on *mad1* expression, we stimulated quiescent human immortalized keratinocytes (HaCaT cells) with activin A or TGF-β1 and isolated the RNA at different time points.

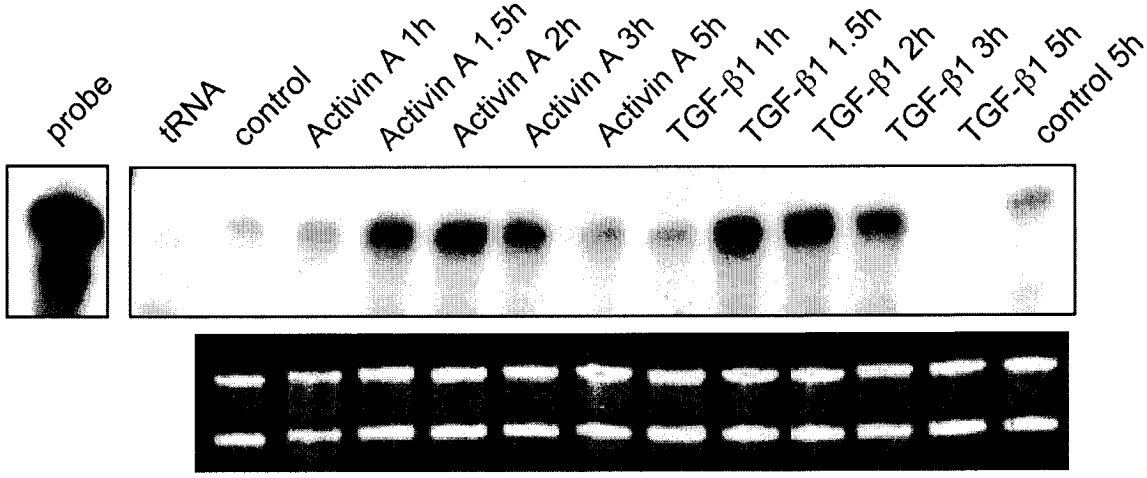

Fig. 19 Activin A and TGF-β1 induce *mad1* mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A or 1 ng/ml TGF-β1. Total cellular RNA was isolated from these cells at different time points after addition of activin A or TGF-β1 as indicated. 20 µg RNA were analyzed by RNase protection assay for the expression of *mad1* mRNA. 1 µg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide (shown below the RNase protection assay). 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probes were loaded in the lanes labeled "probe" and used as a size marker.

As shown by RNase protection assay (Fig. 19), *mad1* mRNA levels were 5-fold up-regulated 90 minutes after addition of activin A to the culture medium. An even stronger mRNA induction (7-fold) was seen upon TGFβ-1-treatment (Fig. 19). Pretreatment of the cells with the protein synthesis inhibitor cycloheximide strongly increased the basal and activin A-/TGF-β-mediated expression of *mad1*, demonstrating that induction of this gene is a direct response to the action of these growth and differentiation factors (Fig. 20).

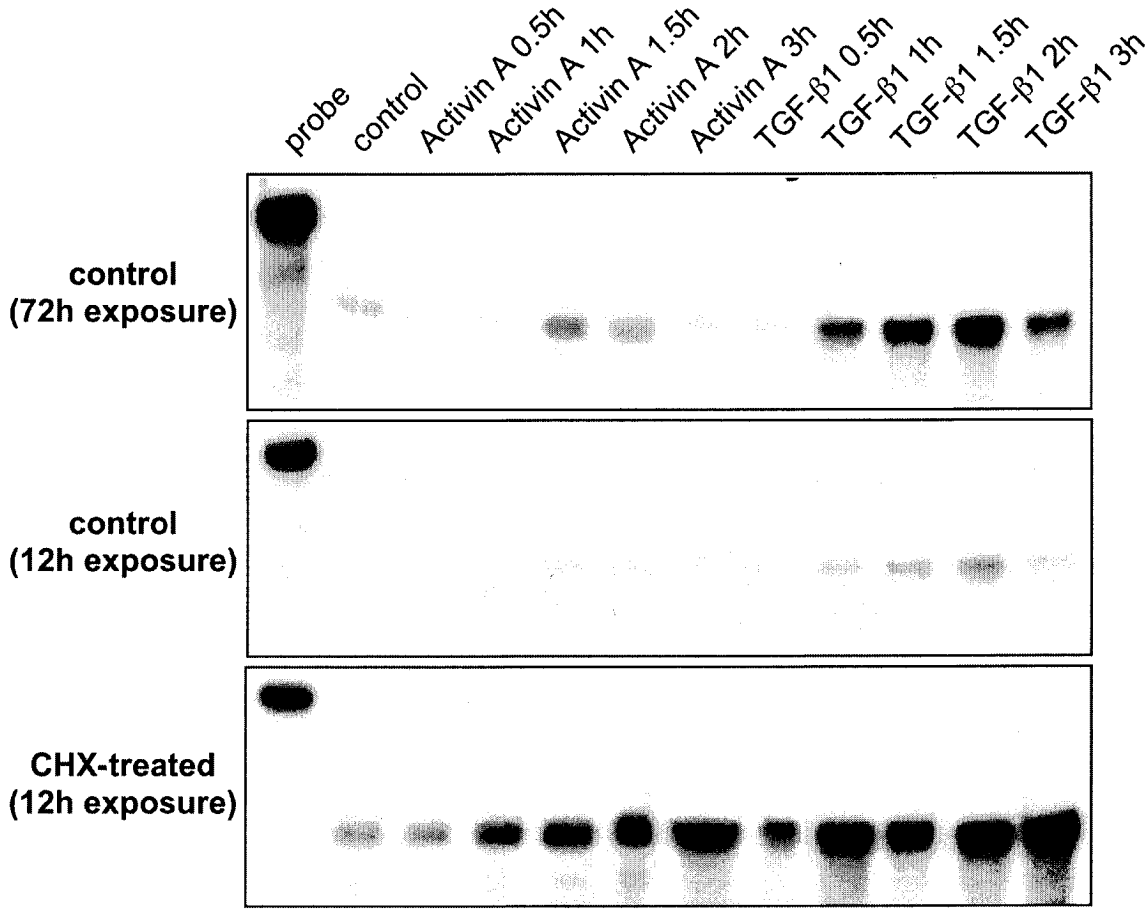

Fig. 20 Activin A and TGF-β1 directly induce *mad1* mRNA expression in HaCaT keratinocytes. To analyze if the induction of *mad1* by activin A and TGF-β1 is a direct effect, HaCaT keratinocytes were rendered quiescent by serum starvation and treated with 10 µg/ml cycloheximide dissolved in DMSO. After 1h 18 ng/ml activin A or 1 ng/ml TGF-β1 were added. Control cells were treated with the solvent DMSO. Total cellular RNA was isolated from these cells at different time points after activin A or TGF-β1 addition as indicated. Samples of 20 µg RNA were analyzed by RNase protection assay for the expression of *mad1* mRNA. 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probes were loaded in the lanes labeled "probe" and used as a size marker. Note the increased basal and activin A-/TGF-β1 induced expression levels of *mad1* in the cycloheximide-treated cells.

In an analogous experiment, we prepared total cell lysates, nuclear lysates and cytosolic fractions from activin A- and TGF-β1-treated cells and determined the level of Mad1 in the lysates by westernblot analysis. As shown in Fig. 21A, a 4-fold

up-regulation of the Mad1 protein (35 kDa) occurred after activin A treatment and a 7-fold increase was seen in the presence of TGF-β1.

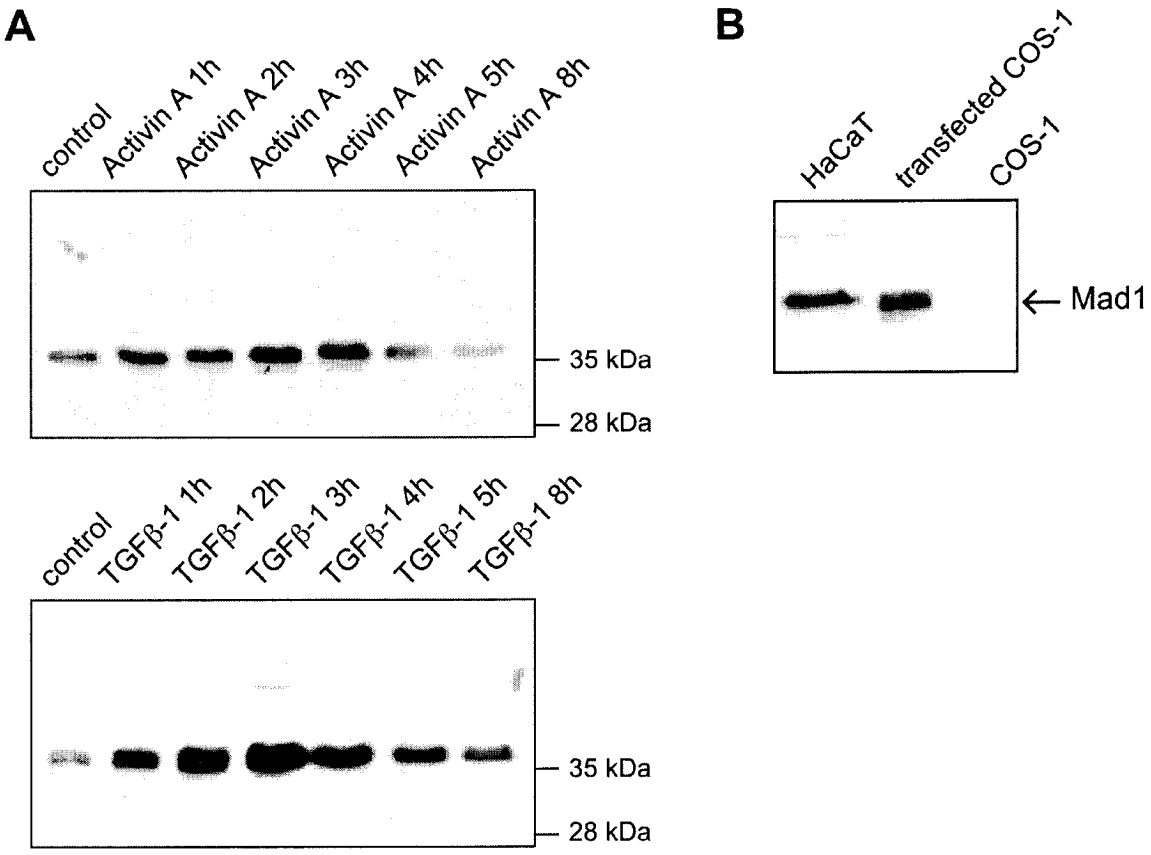

Fig. 21 Activin A and TGF-β1 induce Mad1 protein expression in HaCaT keratinocytes. (A) HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A or 1 ng/ml TGF-β1. Total cell lysates were prepared at different time points after addition of activin A or TGF-β1. 120 µg protein was analyzed by westernblotting for the presence of Mad1. Bands were visualized with the ECL detection system. (B) Total lysate from COS-1 cells that had been transfected with a *mad1* expression vector was used as a positive control.

Maximal induction was observed 3 hours after growth factor addition. After 5 to 8 hours Mad1 had returned to its basal level. The identity of the detected protein with Mad1 was confirmed by westernblot analysis of COS-1 cells that had been transiently transfected with a Mad1 expression vector. The transfected protein was detected by the Mad1 antibody and corresponded in size to the one that reacted with the antibody in HaCaT cells (Fig. 21B). No signal was observed when the blot was probed with the Mad1 antibody that had been pre-treated with the

immunization peptide, demonstrating the specificity of the Mad1 signal (data not shown). Since Mad1 can only inhibit c-Myc function when present in the nucleus, we determined the intracellular localization of the protein. As shown in Fig. 22, Mad1 was predominantly found in the nucleus. By contrast, little Mad1 was detected in the cytosolic fraction. The enrichment of Mad1 in the nucleus after activin/TGF-β1 treatment suggests that these growth and differentiation factors increase the amount of functionally active Mad1 in the cell.

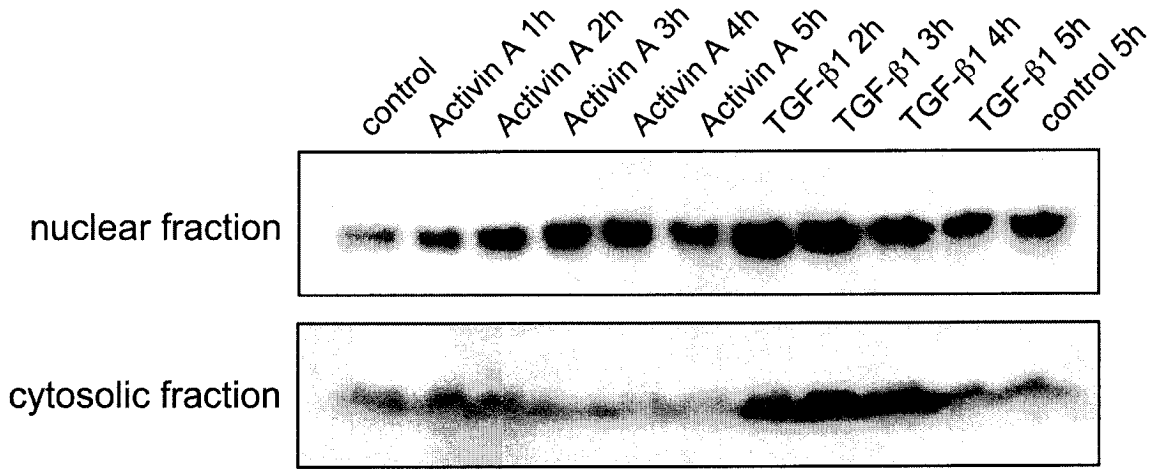

Fig. 22 Enrichment of Mad1 in the nucleus after activin A and TGF-β1 treatment in the nucleus. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A or 1 ng/ml TGF-β1. Nuclear lysates or cytosolic fractions were prepared at different time points after addition of activin A or TGF-β1. 80 μg (cytosolic fraction) or 40 μg (nuclear lysate) protein was analyzed by westernblotting for the presence of Mad1. Bands were visualized with the ECL detection system.

5.2.2 Regulation of Mad1 activity by activin A and TGF-β1 in HaCaT keratinocytes

To further determine whether the increase in *mad1* expression correlates with increased Mad1 DNA-binding activity, we next studied the Mad1 DNA-binding activity after activin A and TGF-β1 treatment. For this purpose we used a solid-phase DNA-binding assay as described by Larsson *et al.* (1997). The latter is a

combination of immunoprecipitation and DNA-binding assay. Within 3 hours after growth factor addition we observed an increased binding of Mad1 immunocomplexes to the labeled CMD-oligonucleotide which contains an optimal Myc/Max-binding site (Fig. 23).

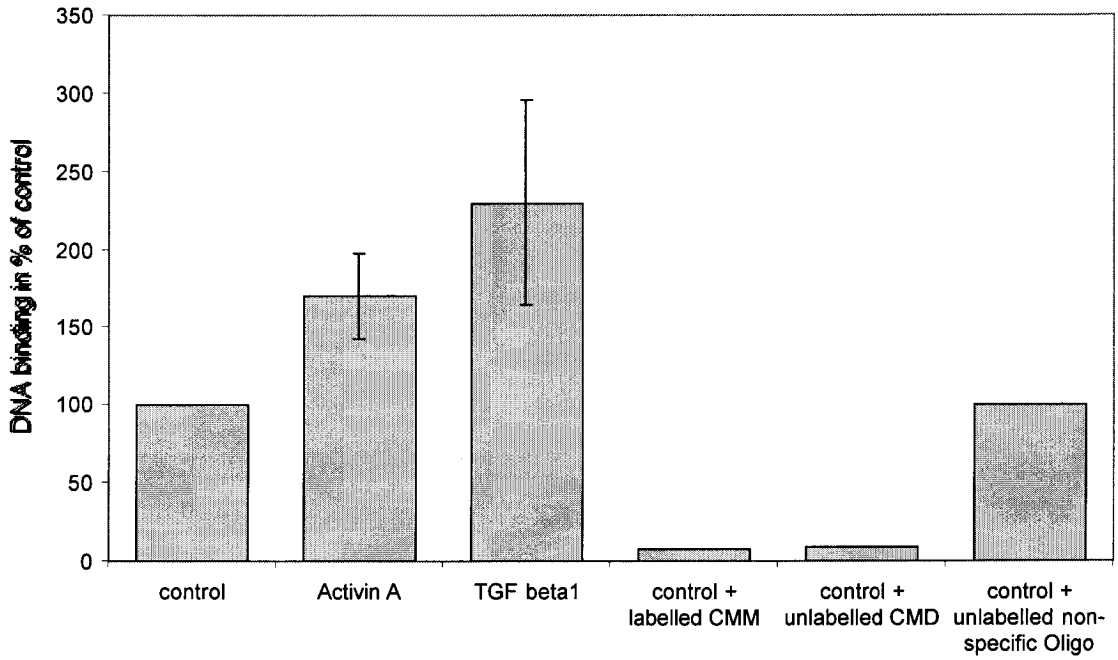

Fig. 23 Activin A and TGF-β1 induce the Mad1 DNA-binding activity. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A or 1 ng/ml TGF-β1. Frackelton-buffer lysates were prepared 3 hours after the induction. Total protein lysates from quiescent (control) and activin A- or TGF-β1-treated cells were analyzed by a solid-phase DNA-binding assay. DNA-binding was analyzed with the ^{32}P-labeled CMD-oligonucleotide (which contains an optimal Myc/Max-binding site) or with the ^{32}P-labeled CMM-oligonucleotide (which has a mutated Myc/Max-binding site). The specificity of the binding was determined by competition with a 100-fold excess of unlabeled CMD- or non-specific oligonucleotide, respectively. The DNA-binding activity is indicated as % of control. Each bar indicates the mean ±S.E.M. for duplicate (activin) or triplicate (TGF-β1) determinations.

Activin A treatment enhanced the Mad1 binding activity 1.7-fold, TGF-β1 treatment caused a 2.3-fold increase. Using the labeled CMM-oligonucleotide (which contains the mutated Myc/Max-binding site) no Mad1 binding could be detected. The Mad1 binding to the CMD-oligonucleotide was completely competed with a 100-fold molar excess of unlabeled CMD, but not by addition of an unspecific oligonucleotide (Fig. 23).

5.2.3 Induction by activin A and TGF-β1 is unique to *mad1*

In contrast to *mad1*, expression of *mad2* and *mad4* was not regulated by activin and TGF-β1 under the same conditions (data not shown). Consistent with published data (Pietenpol *et al.*, 1990), expression of *c-myc* was strongly downregulated by TGF-β1 and to a much lesser extent by activin A (data not shown). In addition to Myc and Mad1, the Max transcription factor has also been shown to dimerize with the Mnt/Rox protein (Hurlin *et al.*, 1997, Meroni *et al.*, 1997). Expression of the latter, however, was not affected by either TGF-β1 or activin A (data not shown). Therefore, Mad1 appears to be the major c-Myc antagonist in activin A -/ TGF-β1- treated HaCaT cells.

5.2.4 *Mad1* expression is regulated in a biphasic manner during cutaneous wound repair

To determine a possible regulation of *mad1* expression by activin or TGF-β *in vivo*, we analyzed the expression of this transcription factor during the healing process of full-thickness excisional mouse wounds where high levels of these factors are expressed (Hübner *et al.*, 1996a, Frank *et al.*, 1996). The time points that we chose are characteristic for the different phases of wound repair: The early inflammatory phase, which is characterized by influx and activation of neutrophils and macrophages (days 1-3); the phase of new tissue formation (days 3-7), and the phase of tissue remodelling (starting around day 7). As shown in Fig. 24, *mad1* mRNA was weakly expressed in normal skin. Consistent with results from a previous study (Lymboussaki *et al.*, 1996), *mad1* mRNA levels increased after skin injury. Interestingly, we observed a biphasic regulation with a first maximum at day 1 after wounding and a second maximum after 5 days.

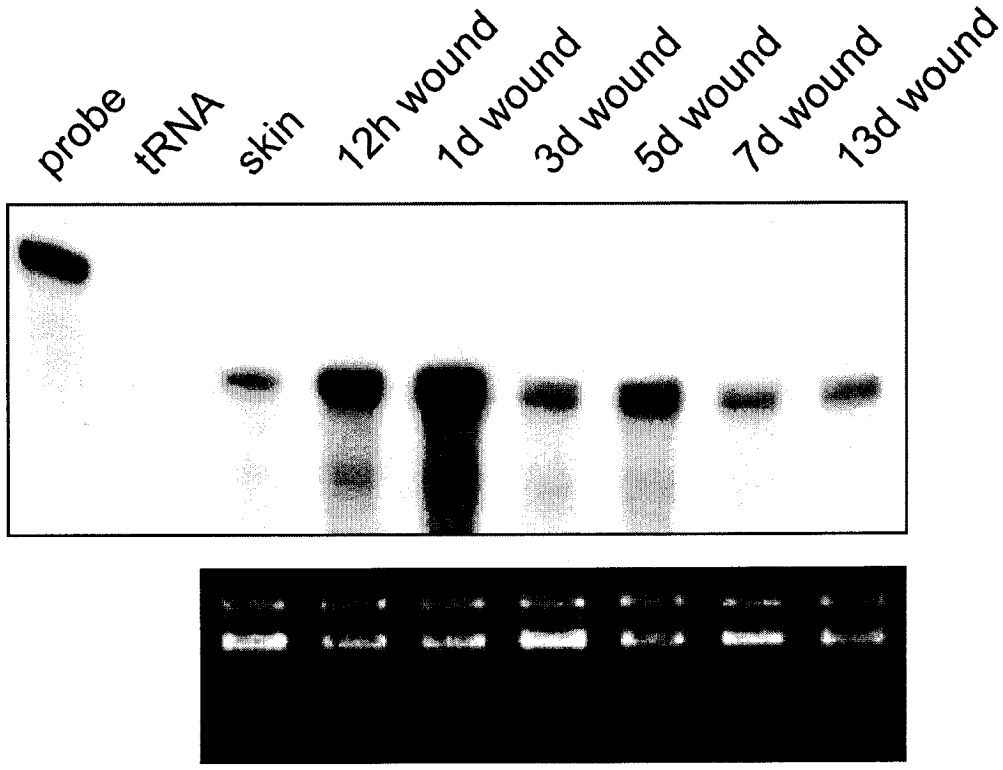

Fig. 24 Increased expression of *mad1* mRNA after skin injury. RNA was isolated from full-thickness excisional wounds of Balb/c mice at different time points after injury. 20 µg total cellular RNA from non-wounded (skin) and wounded skin (wound) were analyzed by RNase protection assay for the expression of *mad1* mRNA. 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probe was loaded in the lane labeled "probe" and used as a size marker. 1 µg of each RNA sample was loaded on a 1% agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays.

This biphasic regulation was confirmed for the protein by westernblot analysis (Fig. 25). These results were reproduced with a different set of RNAs or protein lysates from an independent wound healing experiment.

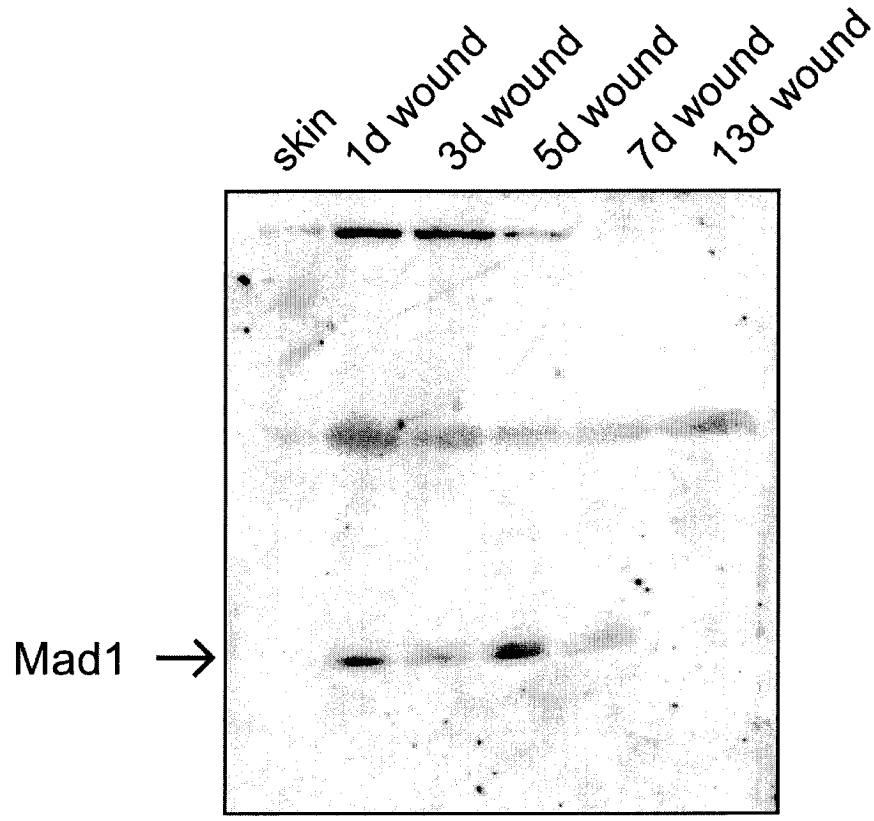

Fig. 25 Increased expression of Mad1 protein after skin injury. 120 µg total protein from normal and wounded skin was analyzed by westernblotting for the expression of Mad1.

Taken together, our findings reveal a complex regulation of *mad1* expression during *in vivo* wound repair.

5.2.5 *Mad1* is expressed in polymorphonuclear leukocytes and in suprabasal keratinocytes of the hyperproliferative epithelium

To localize *mad1* mRNA expression we performed *in situ* hybridization with sections from wounded skin (day 1 and day 5 after injury). As shown in Fig. 26A-D, *mad1* mRNA was predominantly detected within the clot (Cl) that had formed one day after injury. At higher magnification the *mad1*-expressing cells were identified as polymorphonuclear leukocytes as revealed by the characteristic shape of their nuclei (Fig. 26D).

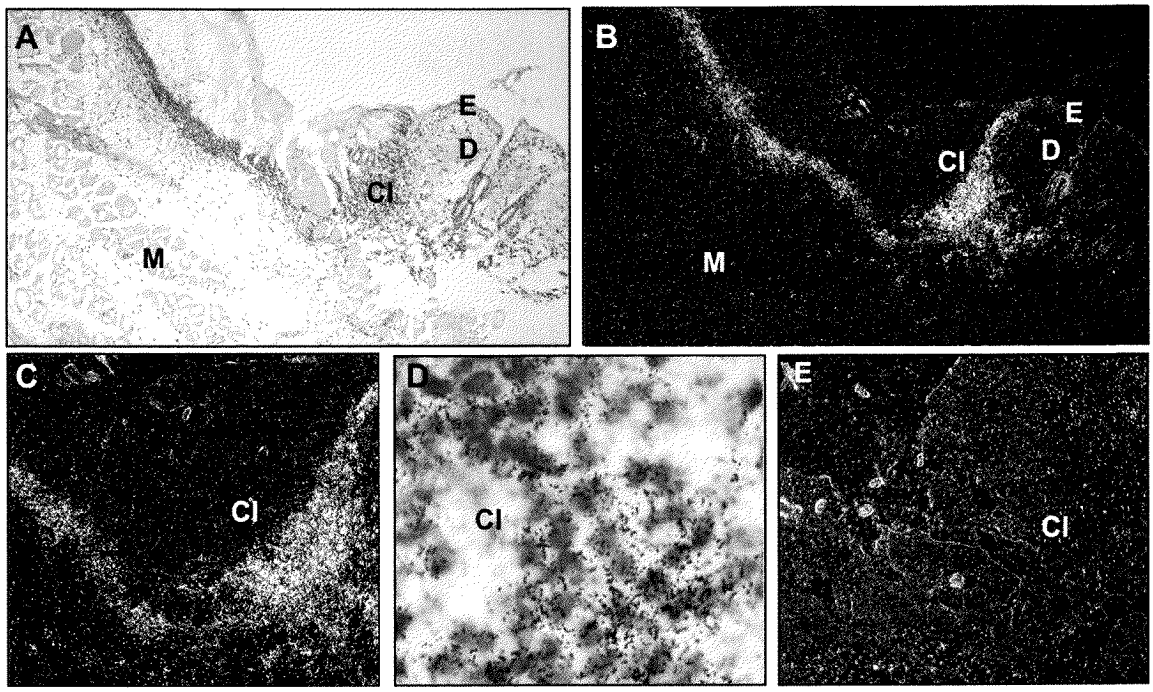

Fig. 26 *Mad1* mRNA is expressed in polymorphonuclear leukocytes in the clot of full-thickness excisional mouse wounds. Paraformaldehyde-fixed frozen sections were taken from the middle of 1d (A-E) wounds and analyzed by *in situ* hybridization for the presence of *mad1* mRNA using a [α-^{35}S]-labeled mouse *mad1* antisense RNA. Hybridization with the corresponding sense RNA was used as a negative control (E). Signals appear as white dots in the dark field surveys (B, C, E) and as black dots in the bright field surveys (A, D). Note the presence of *mad1* mRNA in polymorphonuclear leukocytes. Magnifications: 100x (A, B), 200x (C, E), 1000x (D). E: Epidermis; Cl: Clot, D: Dermis, M: muscle (*Panniculus carnosus*).

Five days after wounding *mad1* was expressed in keratinocytes of the upper suprabasal layers of the hyperproliferative epithelium (HE) which have lost their proliferative capacity. By contrast, only weak signals were observed in basal, non-differentiated keratinocytes and in the underlying granulation tissue (Fig. 27A-C). Furthermore, *mad1* mRNA was not detectable in the normal epidermis at a distance from the wound (data not shown). No specific signals were obtained with the sense riboprobe (Fig. 27D). Thus *mad1* expression is predominantly associated with differentiated cells in the wound area.

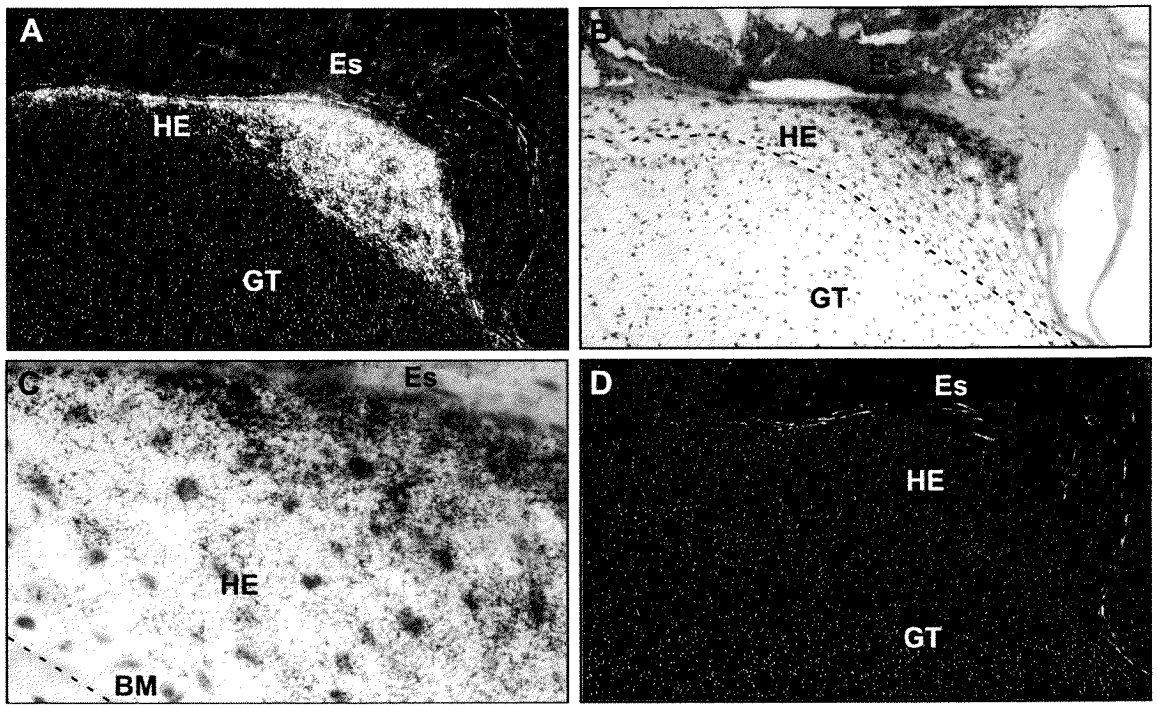

Fig. 27 *Mad1* mRNA is expressed in differentiated keratinocytes of the hyperproliferative epithelium of full-thickness excisional mouse wounds. Paraformaldehyde-fixed frozen sections were taken from the middle of 5d (A-D) wounds and analyzed by *in situ* hybridization for the presence of *mad1* mRNA using a [α-^{35}S]-labeled mouse *mad1* antisense RNA. Hybridization with the corresponding sense RNA was used as a negative control (D). Signals appear as white dots in the dark field surveys (A, D) and as black dots in the bright field surveys (B, C). Note the presence of *mad1* mRNA in suprabasal keratinocytes of the hyperproliferative epithelium. The border between the hyperproliferative epithelium and the granulation tissue is marked with a dotted line in (B) and (C). Magnifications: 200x (A, B, D), 1000x (C). HE: Hyperproliferative epithelium; GT: Granulation tissue, Es: Eschar, BM: Basement membrane.

5.2.6 Expression of the other *mad*-family members, *mxi1/mad2*, *mad3* and *mad4* is differentially regulated compared to *mad1* during cutaneous wound repair

To determine a possible role of other Mad proteins in the healing process, we investigated the regulation of *mxi1/mad2*, *mad3* and *mad4* mRNA expression after skin injury.

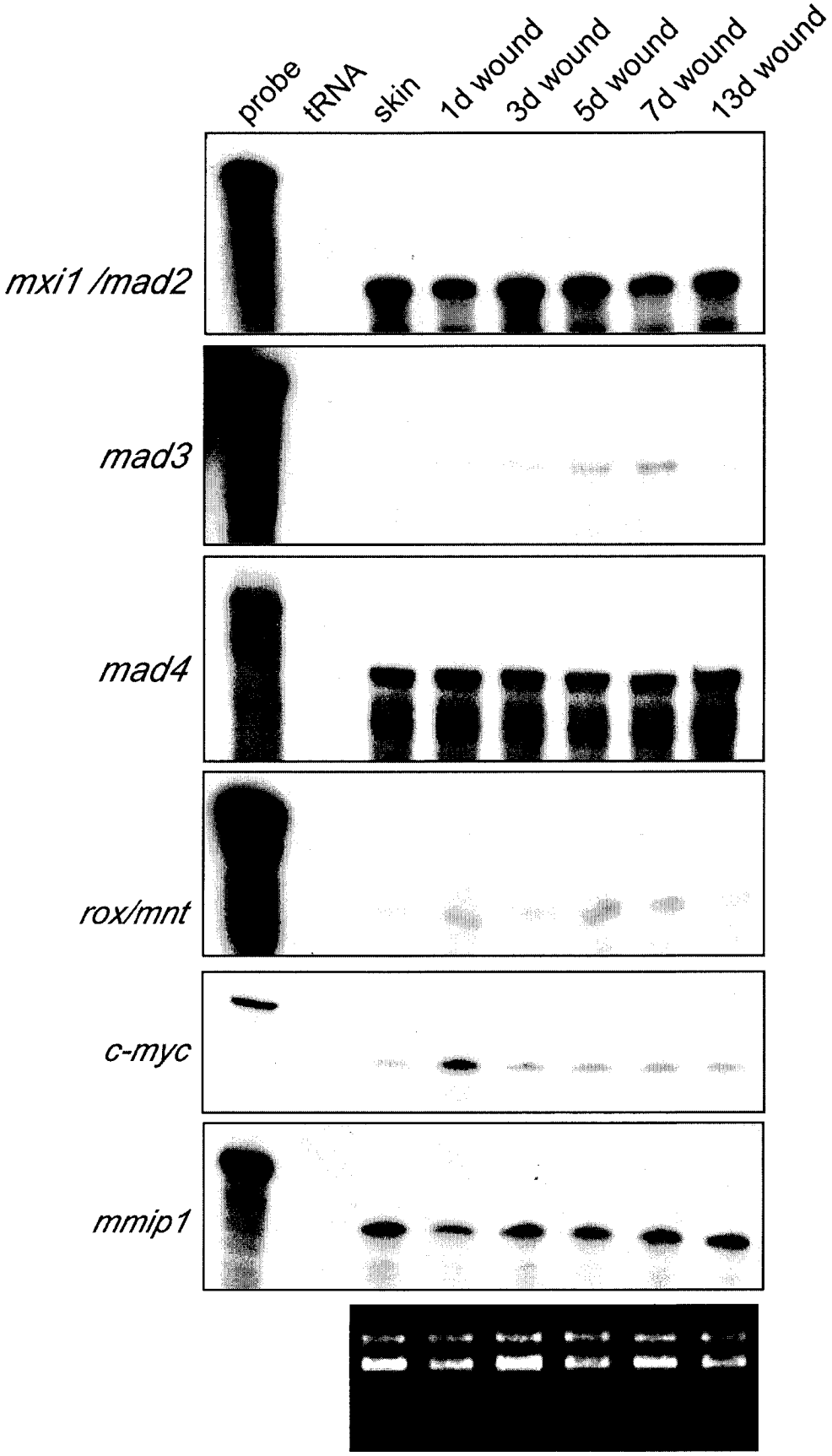
probe
tRNA
skin
1d wound
3d wound
5d wound
7d wound
13d wound
mxi1 /mad2
mad3
mad4
rox/mnt
c-myc
mmip1

Fig. 28 (page 112) **Expression of *mxi1/mad2*, *mad3*, *mad4*, *mnt/rox*, *c-myc* and *mmip1* mRNAs in normal and wounded skin.** RNA was isolated from full-thickness excisional wounds of Balb/c mice at different time points after injury. 20 µg total cellular RNA from non-wounded (skin) and wounded skin (wound) were analyzed by RNase protection assay for the expression of *mxi1/mad2*, *mad3*, *mad4*, *rox/mnt*, *c-myc* and *mmip1* mRNAs as indicated. 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probes were loaded in the lanes labeled "probe" and used as size markers. 1 µg of each RNA sample was loaded on a 1 % agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays. The same set of RNAs was used for the RNase protection assays shown in Fig. 24 and 28.

As shown in Fig. 28, *mxi1/mad2* and *mad4* were expressed at fairly high levels in both normal and wounded skin, but their expression levels did not change after injury. *Mad3* mRNA, which was hardly detectable in normal skin, was weakly up-regulated 5 to 7 days after skin injury.

In addition, the mRNA encoding *mnt/rox* was hardly detectable in normal and wounded skin. *C-myc* was expressed at low levels in normal skin, but was transiently up-regulated within 6h – 24h after wounding (Fig. 28 and data not shown). Maximal induction occurred after 6 hours.

Mad1 function has recently been shown to be antagonized by the leucine zipper protein Mmip-1 (Gupta *et al.*, 1998) and by the unrelated RING finger-containing protein Mmip-2 (Yin *et al.*, 1999). *Mmip-1* mRNA was expressed at moderate levels in normal and wounded skin, but its expression was not regulated by injury (Fig. 28). Expression of *mmip-2* mRNA could neither be detected in normal nor in wounded skin (data not shown). These results were reproduced with a different set of RNAs from an independent wound healing experiment.

5.2.7 *Mad1* expression is upregulated in psoriatic skin

The strong expression of *mad1* in the suprabasal cells of the hyperproliferative wound epithelium tempted us to speculate about a role of Mad1 in hyperproliferative skin disease. Therefore, we determined its expression in the skin of patients suffering from the inflammatory skin disease psoriasis. The latter is characterized by hyperproliferation of the keratinocytes and abnormalities in their

differentiation process. As shown in Fig. 29, *mad1* mRNA levels were indeed 4-fold higher in the affected skin of psoriatic patients (n=8, 4 patients shown) compared to healthy control patients (n=5, 2 patients shown).

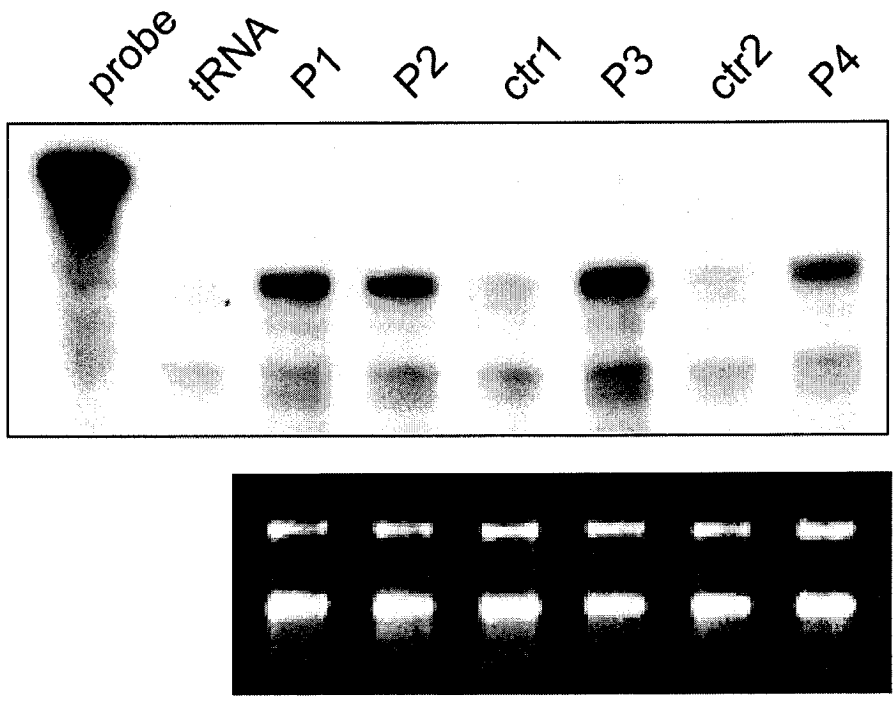

Fig. 29 Increased expression of *mad1* mRNA in psoriatic skin. 10 µg RNA isolated from skin biopsies of normal (ctr) and psoriatic skin (patient; P) was analyzed by RNase protection assay for the presence of *mad1* mRNA. 50 µg tRNA was used as a negative control. 1000 cpm of the hybridization probe was loaded in the lane labeled "probe" and used as a size marker. 1 µg of each RNA sample were loaded on a 1% agarose gel and stained with ethidium bromide. A picture of the RNA gel is shown below the RNase protection assays.

This result was confirmed by *in situ* hybridization. Thus, *mad1* mRNA was hardly detectable in normal human skin (Fig. 30C, D), but strong signals were found in the upper suprabasal layers of the hyperthickened epidermis of psoriatic patients (Fig. 30A, B). No signal was obtained with the sense probe (not shown).

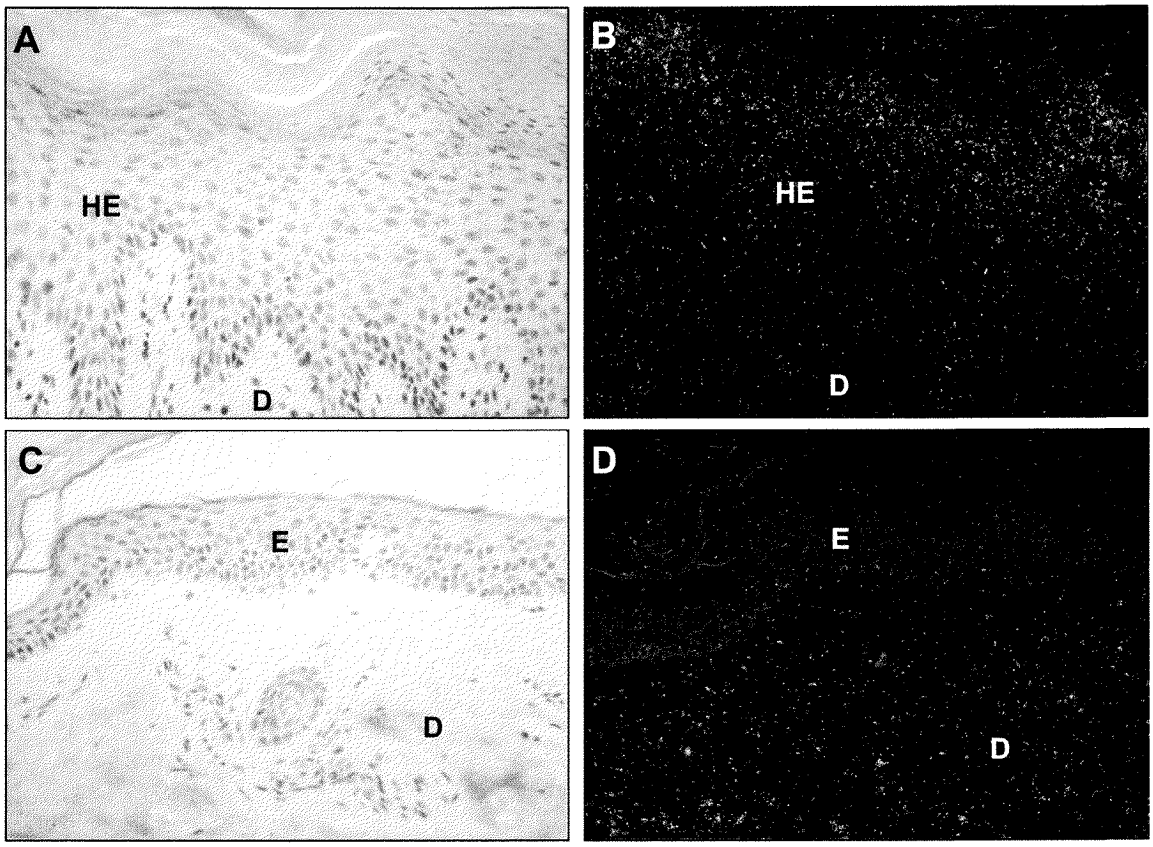

Fig. 30 *Mad1* mRNA is expressed in differentiated keratinocytes of the hyperproliferative epithelium of psoriatic skin. (A-D) *In situ* hybridization showing *mad1* expression in the suprabasal layers of psoriatic skin. Paraformaldehyde-fixed frozen sections were taken from normal (C, D) and psoriatic (A, B) human skin and analyzed by *in situ* hybridization for the presence of *mad1* mRNA using a [α-^{35}S]-labeled human *mad1* antisense RNA. Signals appear as white dots in the dark field surveys (B, D) and as black dots in the bright field surveys (A, C). Magnifications: 200x. E: Epidermis; D: Dermis, HE: Hyperproliferative epithelium.

5.2.8 Discussion

Recent studies from us and from others have revealed a novel role of the TGF-β superfamily member activin in the regulation of keratinocyte proliferation and differentiation (Shimizu *et al.*, 1998; Seishima *et al.*, 1999, Munz *et al.*, 1999). Interestingly, the effects of activin on keratinocytes seem to differ from those of TGF-β, since the latter caused a much stronger inhibition of keratinocyte proliferation *in vitro* compared to activin (Shimizu *et al.*, 1998, Seishima *et al.*,

1999). *In vivo*, the differences were even more striking, since targeted expression of TGF-β1 in the epidermis of transgenic mice caused severe inhibition of keratinocyte proliferation (Sellheyer *et al.*, 1993, Wang *et al.*, 1999). By contrast, activin overexpression in the epidermis even resulted in keratinocyte hyperproliferation and thickening of the epidermis (Munz *et al.*, 1999). This effect is probably indirect, since activin is a weak inhibitor of keratinocyte proliferation *in vitro* (Shimizu *et al.*, 1998). Thus, a dermally derived mitogen that is induced by the activin released from keratinocytes might be responsible for the stimulation of keratinocyte proliferation. Interestingly, activin overexpression in the skin was shown to affect keratinocyte differentiation (Munz *et al.*, 1999), whereas this was not the case with TGF-β1 (Sellheyer *et al.*, 1993, Wang *et al.*, 1999). Therefore, it seemed likely that the two TGF-β superfamily members regulate the expression of a different set of genes. In a search for genes which are regulated by TGF-β and/or activin we recently identified several genes which are exclusively regulated by TGF-β1, including calpactin I (Munz *et al.*, 1997) and keratin 15 (Werner *et al.*, 2000). By contrast, genes that are regulated by activin in keratinocytes have as yet not been identified.

In the present study we have identified the gene encoding the Mad1 transcriptional regulator as the first activin target gene in epidermal keratinocytes. Interestingly, the effect was direct, demonstrating that *mad1* is a primary response gene in keratinocytes. Mad1 is one of four members of the Mad family that have all been shown to antagonize the function of the Myc proteins by recruiting a transcriptional repressor complex that contains histone deacetylase activity (Sommer *et al.*, 1997, Laherty *et al.*, 1997, Alland *et al.*, 1997). Expression of all Mad proteins correlates in general with growth inhibition and terminal differentiation of many cell types, including keratinocytes of the epidermis (reviewed by Grandori *et al.*, 2000). Thus, Mad family members are predominantly expressed in the suprabasal, differentiated layers of embryonic and adult skin (Hurlin *et al.*, 1995a, b; Västrik *et al.*, 1995; Lymboussaki *et al.*, 1996). Furthermore, induction of keratinocyte differentiation *in vitro* is accompanied by an increase in *mad1* and *mxi1/mad2* expression

(Gandarillas and Watt, 1995). Finally, transfection of a mouse keratinocyte cell line with *mad1* strongly inhibited colony formation of these cells (Västrik *et al.*, 1995). Therefore, it seems likely that in addition to the suppression of *c-myc* expression by TGF-β (Pietenpol *et al.*, 1990), the increased expression of *mad1* in response to TGF-β1 and activin is at least partially responsible for the growth inhibitory effect of these factors for keratinocytes. Thereby the stronger induction of *mad1* expression by TGF-β1 correlates with the more potent growth inhibitory effect of the latter in comparison to activin. This inhibitory effect of Mad1 on keratinocyte proliferation is likely to be a prerequisite for subsequent differentiation of these cells. However, *mad1* expression is probably not sufficient for the induction of the differentiation process, since the potent *mad1* inducer TGF-β has little effect on keratinocyte differentiation (see above) and since overexpression of *mad1* in keratinocytes was obviously not sufficient to induce the differentiation program (described in Grandori *et al.*, 2000 as unpublished data by P Gallant & RN Eisenman).

To gain insight into a possible role of activin and TGF-β1 in the regulation of *mad1* expression *in vivo*, we analyzed its expression during cutaneous wound repair where high levels of these factors are present (Frank *et al.*, 1996, Hübner *et al.*, 1996, reviewed by Martin, 1997, O`Kane and Ferguson, 1997). Consistent with previous data (Lymboussaki *et al.*, 1996), the mRNA expression of *mad1* was upregulated after skin injury. In the present study we show that the increased mRNA also leads to elevated levels of Mad1 protein. The upregulation was shown to be unique for *mad1*, since the other *mad* family members were expressed at equal levels in normal and wounded skin. Most interestingly, we identified two peaks of *mad1* expression. The first peak occurred between 12 and 24 hours after injury, a period that is characterized by a strong inflammatory response. The second peak was found between day 5 and day 7 after wounding and coincides with the strongly enhanced formation of new tissue. *In situ* hybridization revealed that suprabasal, redifferentiating keratinocytes of the epidermis are responsible for the second peak (Lymboussaki *et al.*, 1996, this study).

In addition, polymorphonuclear leukocytes were identified as a novel site of *mad1* expression in wounds that explains the early peak in the levels of *mad1* mRNA and protein. These cells are attracted to the site of injury and subsequently become activated to fulfill their task of bacterial destruction. After this period they become entrapped within the wound clot or they become senescent and are phagocytosed by macrophages (reviewed by Clark, 1996). The high levels of Mad1 in these cells indicate a role of this transcription factor in neutrophil activation as supported by results obtained in *in vitro* differentiation models (Larsson *et al.*, 1994, Sommer *et al.*, 1998). Alternatively, Mad1 might inhibit apoptosis of these cells at this stage of the repair process. This hypothesis is supported by the recently demonstrated anti-apoptotic activity of Mad1 in other cells (Gehring *et al.*, 2000). Most importantly, granulocytic precursor cells of mice lacking the *mad1* gene were characterized by decreased survival under cytokine-limiting conditions (Foley *et al.*, 1998), whereas *mad1* overexpression decreased the proliferative capacity of these cells and increased their survival when cytokine leves were reduced (Queva *et al.*, 1999).

Interestingly, the temporal and spatial expression of *mad1* correlates nicely with the expression pattern of TGF-β1 and activin. Thus, TGF-β1 is released in large amounts from degranulating platelets after wounding (Assoian *et al.*, 1993) and might thus be responsible for the early increase in *mad1* expression. Furthermore, both activin and TGF-β are highly expressed during the proliferative phase of wound repair, and particularly high levels of the corresponding mRNAs and proteins were detected in the *mad1* positive suprabasal keratinocytes of the hyperproliferative wound epithelium at the same stage of repair in the same wound healing model (Frank *et al.*, 1996, Hübner *et al.*, 1996). Therefore, it seems likely that activin and TGF-β are also involved in the regulation of *mad1* expression *in vivo*. This hypothesis is further supported by preliminary findings from our laboratory that revealed a higher *mad1* expression in the skin of mice that overexpress activin in the epidermis.

The strong expression of *mad1* in polymorphonuclear leukocytes as well as in suprabasal keratinocytes suggests a possible role of this transcription factor in the pathogenesis of skin diseases that are characterized by inflammation and/or epidermal hyperproliferation. This hypothesis is supported by the particularly strong expression of *mad1* in the granular layer of chemically- or virally-induced mouse skin carcinomas as well as in human squamous and basal cell carcinomas that retain differentiation capacity. In these skin tumors the levels of *mad1* mRNA increased with the thickness of the differentiated cell layers (Hurlin *et al.*, 1995b, Lymboussaki *et al.*, 1996). In this study we demonstrate that *mad1* overexpression is not restricted to malignant tumors but is also observed in the skin of patients suffering from the benign inflammatory skin disease psoriasis that is characterized by epidermal hyperproliferation. Due to the known inhibitory effect of Mad1 on keratinocyte proliferation, it seems possible that the presence of this protein in hyperthickened epidermis of wounds, psoriatic lesions and skin tumors limits the hyperproliferation of keratinocytes under these conditions. Alternatively, Mad1 could also inhibit keratinocyte death by terminal differentiation, which has been shown to share features with programmed cell death (reviewed by Gandarillas, 2000). This hypothesis is supported by the potent anti-apoptotic effect of Mad1 for many other cell types (Gehring *et al.*, 2000). The analysis of the wound healing process in mice lacking different *mad* genes (Foley *et al.*, 1998, Schreiber-Agus *et al.*, 1998, Queva *et al.*, 2001) as well as skin carcinogenesis studies in these animals will help to further define the role of Mad1 in keratinocyte proliferation, differentiation and apoptosis *in vivo*.

5.3 Identification of activin A regulated genes using a cDNA array

To identify activin A regulated genes we hybridized a commercially available cDNA array (*Atlas human cDNA expression array I,* Clontech) with radioactively labeled cDNA from quiescent and activin A treated HaCaT cells (Fig. 31).

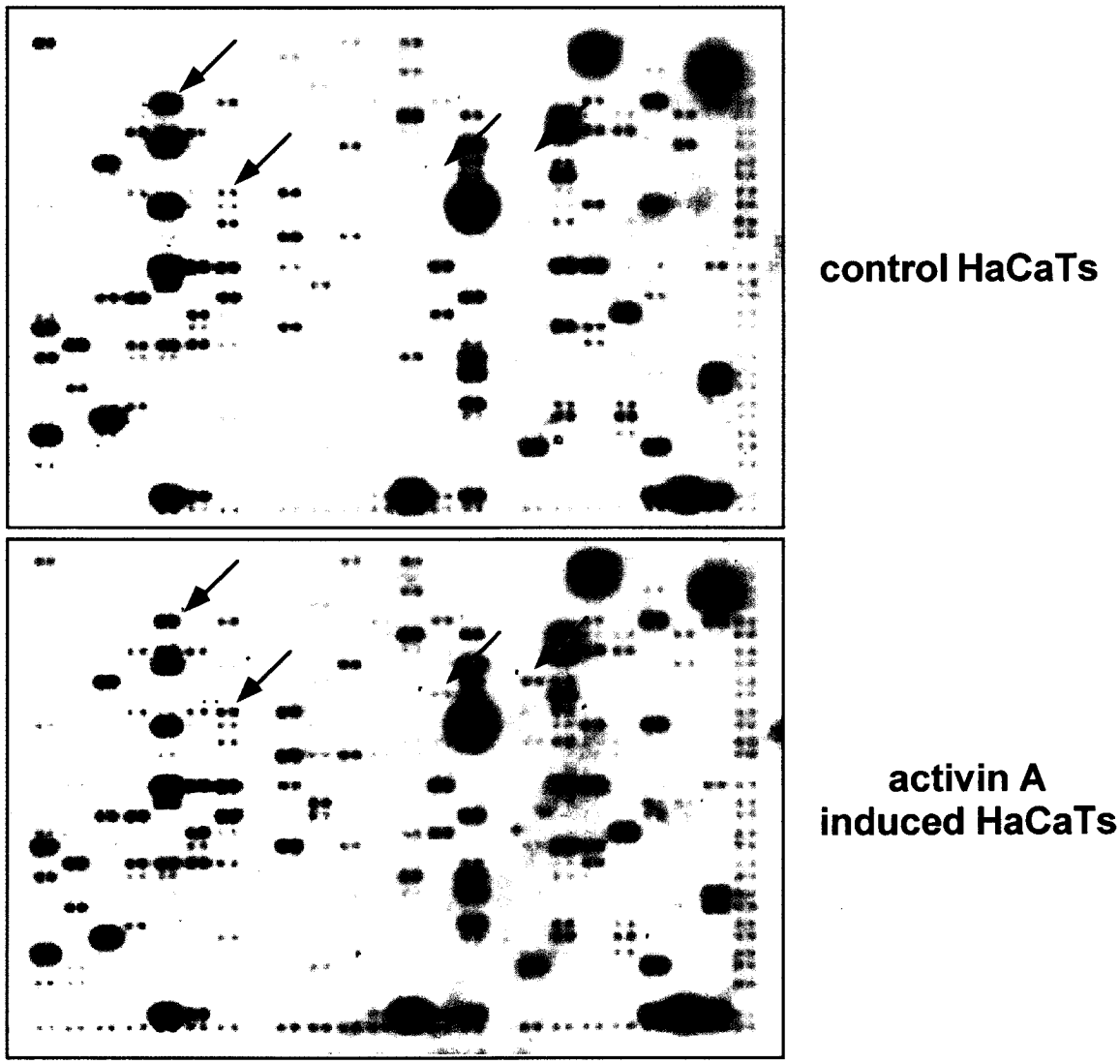

Fig. 31 Hybridization of a cDNA array with radioactively labeled cDNA from quiescent and activin A treated HaCaT cells. The hybridization was performed as described by the manufacturer. ^{32}P labeled cDNA probes were generated by reverse transcription of 1µg polyA$^+$ mRNA from quiescent (control) and activin A treated (for 5h and 8h) HaCaT cells. Genes upregulated by activin A are marked with green arrows, genes downregulated by activin A with red arrows. Names of the identified genes can be found in table 2.

The 588 cDNA fragments, which are spotted on this array, can be subdivided into 6 groups of proteins: oncoproteins, stress response proteins, proteins involved in apoptosis, proteins involved in signal transduction, transcription factors and proteins involved in cell-cell communication. Using the Atlas array, 4 regulated genes were identified, which are listed in table 2. Most of them play a role in the proliferation and/or differentiation of keratinocytes (Cook *et al.*, 1995, Nakanshi *et al.*, 1997, Alani *et al.*, 1999, Nickoloff *et al.*, 2000, Langlands *et al.*, 2000).
The low number of identified activin A regulated genes supports the hypothesis, that there are few activin regulated genes in HaCaT cells. This is consistent with the DDRT-PCR results, where no activin regulated gene could be identified (5.1).

Name or protein/gene	Verification
Induced genes	
Rach1	not tested
Interferon regulatory factor	false positive
Adenosine A1 receptor	not tested
Repressed genes	
DNA-binding protein inhibitor Id-1	verified

Table 2: Activin A regulated genes, identified by hybridization of a cDNA array.

5.3.1 Regulation of Id-family members by activin A and TGF-β1

Using the cDNA array technology we identified the gene encoding Id-1 as an activin A regulated gene in HaCaT keratinocytes. Id-1 belongs to the family of Id-proteins. They are negative regulators of the basic helix-loop-helix (bHLH) transcription factors. Id-proteins have a basic helix-loop-helix protein binding domain, but not a DNA binding domain. Through formation of heterodimers with bHLH transcription factors, they prevent the binding of these proteins to DNA (Benezra *et al.*, 1990, Sun *et al.*, 1991). Id-proteins play a role in the inhibition of

cell differentiation and in the promotion of cell growth (Ellmeier *et al.*, 1992, Zhu *et al.*, 1995).

To verify the activin A regulation of Id-1 mRNA a human cDNA fragment was amplified by RT-PCR of HaCaT cDNA and cloned into the transcription vector pBluescript KSII(+). This fragment was used as a template for RNase protection assay with RNAs from quiescent and activin A stimulated keratinocytes. As shown by RNase protection assay Id-1 expression was indeed downregulated by activin A after 5h and 8h (Fig. 32).

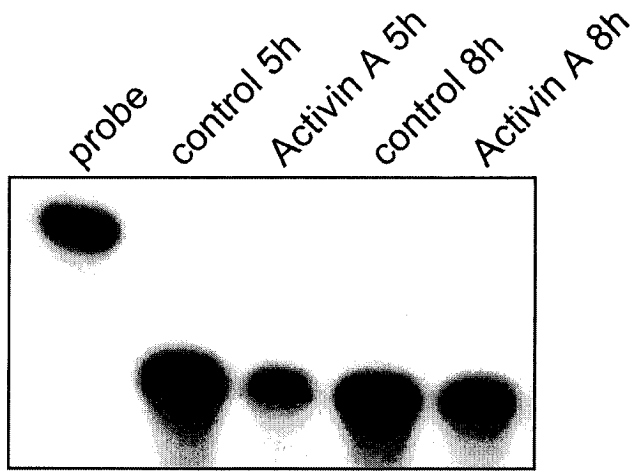

Fig. 32 Activin A reduces *Id-1* mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A. Total cellular RNA was isolated from these cells at different time points after addition of activin A as indicated. 20 µg RNA was analyzed by RNase protection assay for the expression of *Id-1* mRNA. 50 µg tRNA was used as a negative control (data not shown). 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker.

We also investigated the effect of the activin A homologue TGF-β1 on *Id-1* expression in keratinocytes. After 5 hours of TGF-β1 treatment the *Id-1* mRNA levels had strongly declined (Fig. 33). This is consistent with results from a recent study (Chen *et al.*, 2001).

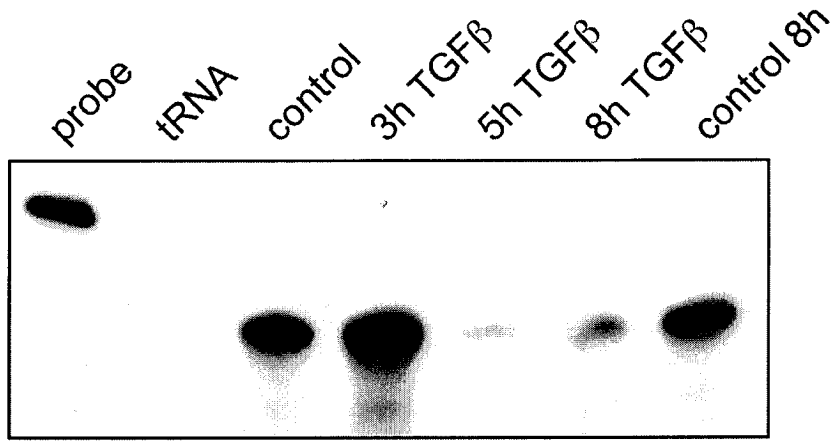

Fig. 33 TGF-β1 reduces *Id-1* mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 1ng/ml TGF-β1. Total cellular RNA was isolated from these cells at different time points after addition of TGF-β1 as indicated. 20 µg RNA was analyzed by RNase protection assay for the expression of *Id-1* mRNA. 50 µg tRNA was used as a negative control (data not shown). 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker.

Besides Id-1, two other members of the Id-family are expressed in keratinocytes: Id-2 and Id-3. We investigated the regulation of these two family members by activin A and TGF-β1 in HaCaT keratinocytes.

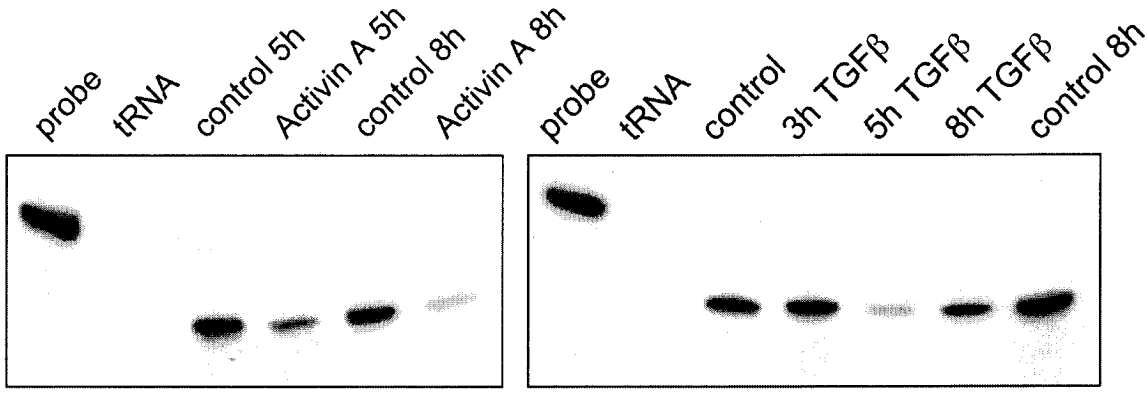

Fig. 34 Activin A reduces *Id-2* mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A. Total cellular RNA was isolated from these cells at different time points after addition of activin A as indicated. 20 µg RNA was analyzed by RNase protection assay for the expression of *Id-2* mRNA. 50 µg tRNA was used as a negative control (data not shown). 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker.

Id-2 (Fig. 34) and *Id-3* (Fig. 35) mRNA levels declined upon addition of activin A or TGF-β1 to HaCaT cells.

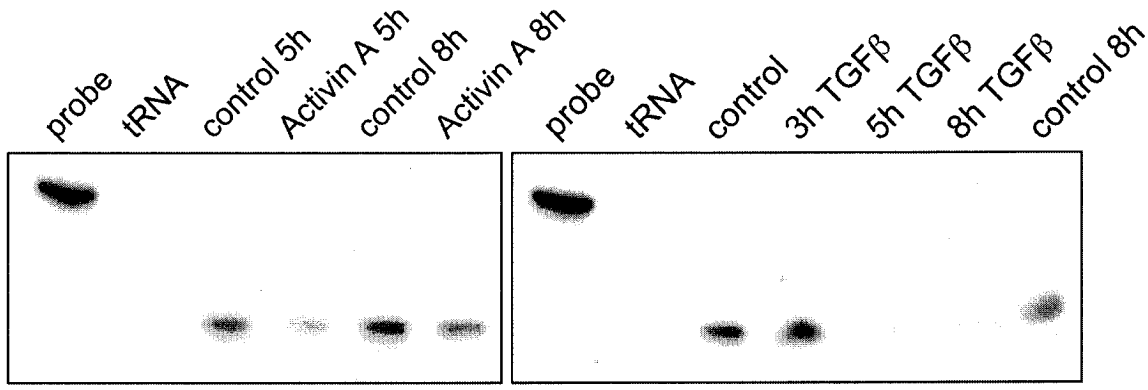

Fig. 35 Activin A reduces *Id-3* mRNA expression in HaCaT keratinocytes. HaCaT keratinocytes were rendered quiescent by serum starvation and subsequently treated with 18 ng/ml activin A. Total cellular RNA was isolated from these cells at different time points after addition of activin A as indicated. 20 µg RNA was analyzed by RNase protection assay for the expression of *Id-3* mRNA. 50 µg tRNA was used as a negative control (data not shown). 1000 cpm of the hybridization probe were loaded in the lane labeled "probe" and used as a size marker.

5.3.2 Discussion

Id-proteins are bHLH proteins, which are important for the inhibition of cell differentiation and for the promotion of cell proliferation (Ellmeier *et al.*, 1992, Zhu *et al.*, 1995). In this study, we could demonstrate that the expression of the Id-proteins is downregulated by activin A and TGF-β1 in keratinocytes. It was shown, that activin A induces the differentiation (Shimizu *et al.*, 1998, Seishima *et al.*, 1999, Munz *et al.*, 1999) and TGF-β1 inhibits the proliferation of keratinocytes (Coffey *et al*, 1988a). Therefore, the downregulation of Id-proteins might at least partially underlie the growth inhibitory/differentiation promoting effects of TGF-β and activin A.

Recently, Id-2 was identified as a c-Myc target gene (Lasorella *et al.*, 2000). Since *c-myc* expression is negatively regulated by activin A (this work) and TGF-β1

(Coffey *et al.*, 1988b), it seems likely that the regulation of Id-proteins by activin A and TGF-β is mediated via c-Myc.

6 Final discussion

Injury to the skin initiates various complex processes, including reepithelialization of the wounded area as well as formation and reeorganization of the granulation tissue. These different processes are controlled by a multitude of growth factors and cytokines, which are secreted by platelets or expressed by different types of activated cells, including keratinocytes, fibroblasts and macrophages (reviewed by Clark, 1996 and Martin, 1997).

Activin and TGF-β, both growth factors of the TGF-β superfamily, are important modulators of the tissue repair process. Both of them have been shown to accelerate wound healing in animal models. Whereas several functions of TGF-β during the repair process have been demonstrated, including promotion of extracellular matrix synthesis and regulation of cell migration, little is as yet known about the role of activin in wound repair, and its mechanisms of action during the repair process have not yet been characterized.

Activin and TGF-β can act on various cell types. The major goal of my thesis project was to determine the effects of activin and TGF-β on keratinocytes. Published *in vitro* studies have shown that activin induces differentiation of these cells but has little effect on proliferation, whereas TGF-β strongly inhibits proliferation of keratinocytes (Coffey *et al.*, 1988a, Shimizu *et al.*, 1998, Seishima *et al.*, 1999). Therefore, we wanted to elucidate the molecular mechanisms underlying these different effects. For this purpose we attempted to identify and characterize genes, which are regulated by these two factors in keratinocytes, with particular emphasis on those genes that are differentially regulated by both factors.

We used three different strategies to analyze the gene expression after activin A and TGF-β1 treatment. First, we looked for differential gene expression using the differential display RT-PCR technology and by hybridization of an Atlas cDNA array. Both technologies showed advantages and disadvantages. The hybridization

of a commercially available cDNA array is a very rapid and easy method. However, the number of genes that can be analyzed is limited, and novel genes cannot be identified with this technology. Furthermore, weakly expressed genes cannot be analyzed. By contrast, the differential display RT-PCR technology is a large-scale method and allows the identification of all regulated genes, including weakly expressed and novel genes. However, a lot of false positive genes are usually obtained with this technology.

Since the only known effect of activin on keratinocytes is the induction of differentiation, we also searched for activin regulated genes by analyzing the expression of various known differentiation markers.

We had no problem in identifying TGF-β regulated genes, but only very few activin regulated genes could be identified using all three methods. This indicates that the number of activin A regulated genes in keratinocytes is low in comparison to TGF-β regulated genes. Therefore, it seems likely that activin exerts very specific effects on these cells. Alternatively, activin might act in concert with other growth factors and cytokines as demonstrated by the synergistic effects of TGF-β1 and activin on collagen synthesis by kidney fibroblasts (Sugiyama *et al.*, 1998). Thus it might be worthwhile to determine whether activin modifies the transcriptional responses elicited by other growth factors.

In our screens, we identified the *keratin 14* and *15* genes as TGF-β1 regulated genes and *mad1*, *c-myc* and the *id* genes as TGF-β1 and activin A regulated genes. However, no gene could be identified, which was only regulated by activin A. Mad-1, c-Myc and the Id-proteins have all been shown to regulate proliferation and differentiation of keratinocytes (Västrik *et al.*, 1995, Chin *et al.*, 1995, Hurlin *et al.*, 1995a, b, Alani *et al.*, 1999, Nickoloff *et al.*, 2000, Langlands *et al.*, 2000). Thus, these genes are likely to play a role in the growth inhibitory effect of TGF-β as well as in the differentiation-inducing effect of activin. However, they do not explain the observed differences in the biological effects exerted by TGF-β on the one hand and by activin on the other hand. Thus, it seems likely that additional

proteins are regulated by these factors which mediate the specific responses. Alternatively, the intensity or the time-course of expression of the activin/TGF-β target genes might be important for the biological response. Indeed, the intensity of regulation of *mad1* and the *id* genes was different in response to activin and TGF-β. Inducible overexpression of these genes might help to clarify whether the intensity of overexpression affects proliferation and/or differentiation.

Whereas the as yet identified activin regulated genes are all involved in the regulation of keratinocyte proliferation/differentiation, TGF-β has been shown to regulate a series of other genes, e.g. those encoding the keratins 14 and 15 (this study), calpactin I (Munz *et al.*, 1997), peroxiredoxin VI (Frank *et al.*, 1997), enzymes involved in nucleotide biosynthesis (Gassmann et al., 1999), and also the structural proteins caveolin-1 and caveolin-2 (Gassmann and Werner, 2000). Therefore, TGF-β seems to affect a wide variety of different processes in keratinocytes, whereas the major function of activin is probably the regulation of keratinocyte differentiation. The identification and characterization of additional activin regulated genes will help to further elucidate its mechanisms of action in keratinocytes.

To investigate a potential role of activin and TGF-β in the regulation of the identified genes *in vivo*, we analyzed the expression of *mad1* and the *keratins* during the wound healing process, where high levels of activin and TGF-β are present. Indeed, regulation of these genes during the repair process could be correlated with the expression pattern of activin and TGF-β. Therefore, the chosen system appears suitable to identify genes, which are regulated by these growth factors during the tissue repair process.

The results of this work provide new insights into the mechanisms of activin and TGF-β action in keratinocytes *in vitro*. Furthermore, our wound healing studies suggest that the genes identified in our screen are also mediators of activin and

TGF-β action *in vivo*. To support this hypothesis, it will be necessary to analyze the expression of these genes in activin A and follistatin overexpressing transgenic mice, which are available in our laboratory (Munz *et al.*, 1999, Wankell *et al.*, submitted). First experiments were performed to analyze the *mad1* expression in activin overexpressing mice and they confirmed the results obtained for the *mad1* regulation by activin *in vitro*.

Finally, inhibition of the identified growth factor regulated genes *in vitro* and *in vivo* in keratinocytes, for example by using an antisense technology or a dominant negative approach, will allow us to determine their specific roles in the regulation of keratinocyte proliferation and differentiation by activin and TGF-β.

7 Literature

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8 List of abbreviations

APS	ammonium persulfate
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Ci	Curie
cm	centimeter
cpm	counts per minute
dATP	desoxyadenosine triphosphate
dCTP	desoxycytosine triphosphate
ddH_2O	bidistilled water
DDRT	differential display reverse transcription
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's modified eagle's medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
DNase	desoxyribonuclease
dNTP	desoxyribonucleoside triphosphate
DTT	Dithiothreitol
ECL	enhanced chemoluminescene
ECM	extracellular matrix
E.coli	Escherichia coli
EDTA	ethylendiamine tetraacetic acid
EGF	Epidermal growth factor

FCS	fetal calf serum
g	gram
GSCN	Guanidine thiocyanate
IgG	immunoglobulin G
IFN	Interferon
IL	Interleukin
IPTG	Isopropylthiogalactoside
kDa	kilodalton
KGF	Keratinocyte growth factor
l	liter
LB-medium	Luria-Bertani medium
M	molar
mA	milliampere
mg	milligram
mM	millimolar
min	minute
ml	milliliter
µg	microgram
µl	microliter
µM	micromolar
mRNA	messenger ribonucleic acid
nm	nanometer
OD	optical density
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIPES	Piperazine-*N*-*N*'-bis-[2-ethenesulfonic acid]
PMSF	Phenylmethylsulfonylfluoride
RNA	ribonucleic acid
RNase	ribonuclease
RPM	revolutions per minute

RT	room temperature
SDS	sodiumdodecylsulfate
TEMED	*N,N,N',N'*-Tetramethylendiamine
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
Tris	Tris[hydroxymethyl]aminomethane
tRNA	transfer ribonucleic acid
Tween 20	Polyoxyethylenesorbitanmonolauryl acid
U	unit
UV	ultraviolet light
UTP	Uracil triphospahte
VEGF	Vascular endothelial growth factor
V	volt
v/v	part of volume/volume
w/v	part of weight/volume
X-Gal	5-Bromo-6-chloro-3-indolyl-β-D-galactoside

9 Curriculum Vitae

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