

**NF1 TUMOR SUPPRESSOR IN
EPIDERMAL DIFFERENTIATION
AND GROWTH - IMPLICATIONS
FOR WOUND
EPITHELIALIZATION AND
PSORIASIS**

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OULU 2003



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PSORIASIS**

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium of the Department of Anatomy and Cell Biology, on October 3rd, 2003, at 12 noon

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2003

Abstract

Neurofibromatosis type 1 (NF1) is a dominantly inherited neurocutaneous disorder caused by mutations in the NF1 gene. Common clinical manifestations associated with NF1 are neurofibromas, café-au-lait macules (CALM), axillary freckling and Lisch nodules of the iris. Other important manifestations are vasculopathy, a variety of osseous lesions, including short stature, scoliosis and pseudoarthrosis, optic gliomas and an increased risk for certain malignancies. The best characterized function of the NF1 gene is to act as a downregulator of Ras proto-oncogene signalling by accelerating the switch of active Ras-GTP into inactive Ras-GDP. The NF1 gene is considered a tumor suppressor since some malignancies may display a loss of heterozygosity or homozygotic inactivation of the gene.

The present study investigated the behaviour and function of the NF1 gene during keratinocyte differentiation, wound healing and psoriasis using human epidermis and epidermal keratinocytes as a model. The NF1 protein was shown to associate with the intermediate filament network during keratinocyte differentiation both *in vitro* and *in vivo*, and it is thus suggested to play a role in the cytoskeletal re-organization or in the formation of cell adhesions. NF1 gene expression was also studied in psoriasis, in which keratinocytes are hyperproliferative and cell differentiation is altered. NF1 gene expression was downregulated in psoriatic keratinocytes both *in vivo* and *in vitro*, suggesting that the NF1 gene might have role in downregulating keratinocyte proliferation. During epidermal wound healing, NF1 gene expression was increased. However, the process of wound healing showed no apparent differences between NF1 patients and controls. Furthermore, an increased number of cells immunoreactive for active Ras-MAPK was demonstrated in vascular tissues of NF1 patients, but not in epidermal keratinocytes or dermal fibroblasts. The finding suggests that the NF1 protein functions as a Ras-GAP in some, but not all tissues.

Keywords: cell adhesion, cell differentiation, cytoskeleton, keratinocytes, neurofibromatosis 1, neurofibromin 1, psoriasis, ras, skin, wound healing

To my family

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Oulu, August 2003

Jussi Koivunen

Abbreviations

Ab	antibody
cAMP	cyclic adenosine monophosphate
CK/ K	cytokeratin
CSK buffer	cytoskeletal extraction buffer
kDa	kilodalton
EGF	epidermal growth factor
ERK/ p44/42MAPK	extracellular regulated kinase
FTI	farnesyl transferase inhibitor
GAP	GTPase-activating protein
GDP	guanosine 5`-diphosphate
GEF	guanine nucleotide exchange factor
GRD	GAP-related domain
GTP	guanosine 5`-triphosphate
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated kinase, ERK kinase
MPNST	malignant peripheral nerve sheath tumor
mRNA	messenger RNA
NF1	type 1 neurofibromatosis
PDGF	platelet-derived growth factor
PI3-kinase	phosphatidylinositol 3-kinase
PKA	protein kinase A
PLC	phospholipase C
RaIGDS	Ras-related guanine nucleotide dissociation stimulator
TEWL	transepidermal water loss
PKC	protein kinase C
TGF α	transforming growth factor α

TGF β
TNF α

transforming growth factor β
tumor necrosis factor α

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

- I Koivunen J, Ylä-Outinen H, Korkiamäki T, Karvonen S-L, Pöyhönen M, Laato M, Karvonen J, Peltonen S & Peltonen J (2000) New function for NF1 tumor suppressor. *J Invest Dermatol* 114: 473-479
- II Koivunen J, Kuorilehto T, Kaisto T, Peltonen S & Peltonen J (2002) Ultrastructural localization of NF1 tumor suppressor protein in human skin. *Arch Dermatol Res* 293: 646-649.
- III Karvonen S-L, Koivunen J, Nissinen M, Ylä-Outinen H, Björkstrand A-S & Peltonen J (2003) NF1 tumor suppressor gene expression is deficient in psoriatic skin *in vivo* and *in vitro*: A potential link to increased Ras activity. *Br J Dermatol*, in press
- IV Koivunen J, Karvonen S-L, Ylä-Outinen H, Aaltonen V, Oikarinen A & Peltonen J NF1 tumor suppressor in epidermal wound healing. Submitted.

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1 Introduction

Neurofibromatosis type 1 (NF1) is a dominantly inherited disease with an incidence of 1/3500. It is caused by mutations in the NF1 gene located in chromosome 17. Common clinical manifestations associated with NF1 are neurofibromas, café-au-lait macules, axillary freckling and Lisch nodules, pigmented spots of the iris. Other important manifestations are vasculopathy, a variety of osseous lesions, including short stature, scoliosis and pseudoarthrosis, optic gliomas and an increased risk for certain malignancies, such as malignant peripheral nerve sheath tumors (MPNST) and leukemias. NF1 patients also display learning disabilities. The best characterized function of the NF1 gene is to act as a downregulator of Ras proto-oncogene signaling by accelerating the switch of active Ras-GTP into inactive Ras-GDP. The NF1 gene is considered a tumor suppressor since some cancers, such as MPNST, leukemias and melanomas, can display loss of heterozygosity (LOH) or homozygotic inactivation of the gene.

The present study was designed to elucidate the role of the NF1 gene in keratinocyte differentiation and activation and its relationship with the clinical manifestations associated with NF1. Epidermal differentiation, wound healing and psoriasis were used to study the behavior and function of the NF1 gene. Epidermal keratinocytes serve as an excellent model for studying various cellular processes, such as differentiation and activation, that occur during wound healing and certain pathological conditions, both *in vitro* and *in vivo*. It is likely that a tumor suppressor is not constantly expressed in keratinocytes and may behave specifically during the differentiation or activation of these cells. Furthermore, the specific function of the NF1 gene is not known, and it may have other functions apart from acting as a GAP protein

2 Review of the literature

2.1 Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1) is a dominantly inherited disease caused by mutations in the NF1 gene. The incidence of the disease is estimated to be about 1/3500 with no ethnic or gender-related variability (Fuller *et al.* 1989, Huson *et al.* 1989, Poyhonen *et al.* 2000). CALMs, neurofibromas, axillary/inguinal freckling and Lisch nodules, pigmented spots of the iris, are the most common clinical manifestations associated with the disease. Other important manifestations are vasculopathy involving multiple vessels with lesions varying from stenotic to aneurysmatic, a variety of osseous lesions, including short stature, scoliosis and pseudoarthrosis, optic gliomas and an increased risk for certain malignancies such as MPNSTs and leukemias. NF1 patients also display learning disabilities (Friedman & Riccardi 1999). The diagnostic criteria for NF1 are shown in Table 1 (Gutmann *et al.* 1997). The NF1 gene is considered as a tumor suppressor because either LOH or homozygous inactivation of the gene has been demonstrated in a number of tissues or cells, including neurofibromas (Sawada *et al.* 1996, Serra *et al.* 1997, Kluwe *et al.* 1999), MPNST (Glover *et al.* 1991, Legius *et al.* 1993), myelogenous leukaemias (Side *et al.* 1997) or melanomas (Andersen *et al.* 1993b).

The most common skin manifestations seen in NF1 are CALMs, neurofibromas and axillary/inguinal freckling (Friedman & Riccardi 1999). Dermal neurofibromas are benign tumors rising from small nerves. They are usually elevated upwards from skin and are slightly reddish in color. Neurofibromas are largely composed of Schwann cells, fibroblasts, perineural cells and extensive collagenous extracellular matrix (Harkin 1986, Peltonen *et al.* 1988). CALMs are pigmented patches of the epidermis 10mm or more in diameter. The histological features of CALMs show basilar hyperpigmentation with occasional melanocytic hyperplasia. CALMs are characterized by the presence of melanin macroglobules within melanocytes and keratinocytes. (Jimbow *et al.* 1973, Martuza *et al.* 1985) Freckling of skin occurs mainly in non-sun-exposed areas, such as the axillary or inguinal region (Friedman & Riccardi 1999).

Genetic and epigenetic factors have been proposed to play a role in the skin manifestations associated with the disease. LOH has been demonstrated in benign

neurofibromas (Sawada *et al.* 1996, Serra *et al.* 1997). Physical factors, such as trauma or elevated skin temperature, have also been proposed to play a part in the formation of neurofibromas, CALMs and freckling (Friedman & Riccardi 1999, Karvonen *et al.* 2000, Kaufmann *et al.* 2001). Furthermore, monozygous twins with NF1 have been shown to carry different phenotypes of the disease, suggesting the role of epigenetic factors in the development of clinical manifestations (Akesson *et al.* 1983).

Table 1. Diagnostic criteria for NF1 (Gutmann et al. 1997).

NF1 patients should fulfill 2 or more of the following criteria
1. Six or more café-au-lait macules <ul style="list-style-type: none"> - $\geq 1.5\text{cm}$ in diameter in postpubertal individuals - $\geq 0.5\text{cm}$ in prepubertal individuals
2. Two or more neurofibromas of any type or one plexiform neurofibroma
3. Multiple freckles in the axillary area or groin
4. Optic glioma
5. Two or more Lisch nodules of the iris
6. A distinct osseous lesion, such as <ul style="list-style-type: none"> - sphenoid dysplasia - thinning of the long bone cortex with or without pseudoarthrosis
7. A first-degree relative who meets the above criteria for NF1

2.2 NF1 gene

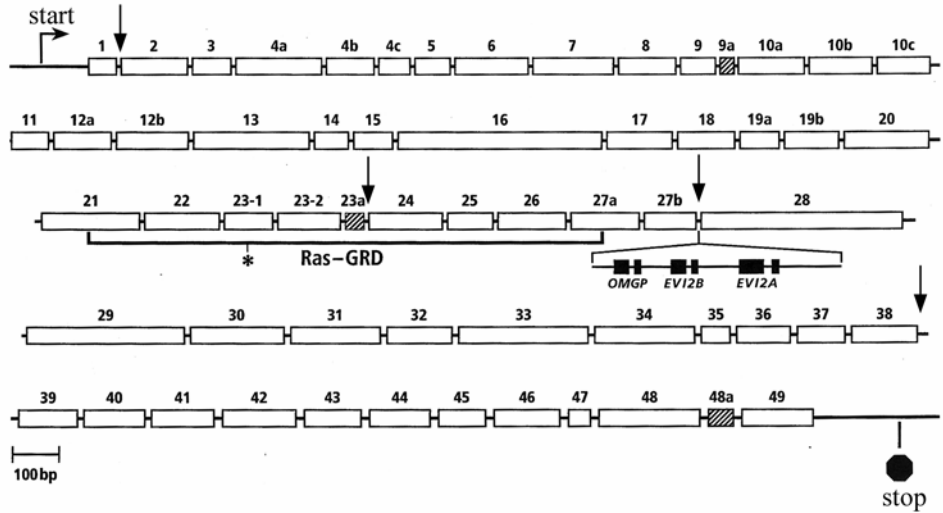
The NF1 gene is located in the long arm of chromosome 17 (17q11.2). (Cawthon *et al.* 1990, Viskochil *et al.* 1990). It is a very large gene spanning over 350 kb of genomic DNA (Marchuk *et al.* 1991) with 60 exons organized into four clusters, which are separated by four large introns (Fig. 1) (Li *et al.* 1995). The NF1 gene is ubiquitously expressed, resulting in 11-13 kb NF1 mRNA with many alternatively spliced variants (Fig. 1) (Gutmann *et al.* 1995, Gutmann *et al.* 1999, Vandenbroucke *et al.* 2002a). The NF1 gene is highly conserved between species. There is ~98% homology of the protein product between mouse and human, and ~60% homology between drosophila and human (Bernards *et al.* 1993, The *et al.* 1997).

The NF1 gene contains a domain (exons 21-27) related to Ras-GTPase-activating proteins (Ras-GAPs) (Fig. 1) (Ballester *et al.* 1990). The Ras-GAP proteins increase the intrinsic GTPase activity of proto-oncogene Ras. The NF1 gene has even longer homology to the yeast inhibitory regulator proteins (IRA), which are also involved in regulating the Ras-signaling pathway (Ballester *et al.* 1990). There are no other areas significantly homologous to other genes. One of the introns in the NF1 gene contains three embedded genes (OMGP, EVI2A and EVI2B), which are transcribed in an orientation opposite to NF1 gene (Fig. 1) (Viskochil *et al.* 1991). The functions of these three genes are unknown. The mutation rate of the NF1 gene is one of the highest known in the human genome, $3.1\text{-}6.5 \times 10^5$ (Vogel & Motulsky 1997). Thus, ~50% of all NF1

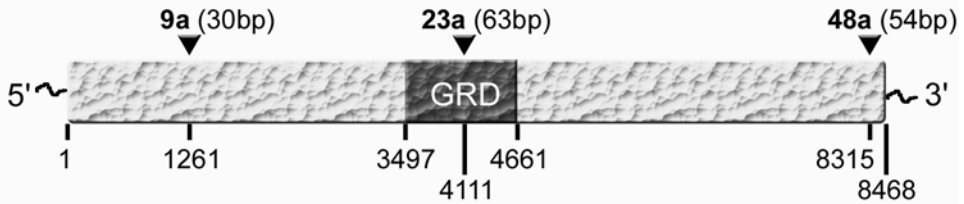
patients lack a family history of the disease (Huson & Hughes 1994). It has been speculated that the high mutation rate is caused by the large size of the gene and the complexity of its processing (Upadhyaya *et al.* 1994). Recent advances in detecting mutations of the NF1 gene have made it possible to identify >95% of the mutations (Messiaen *et al.* 2000). There are no general hot spot areas for mutations in the NF1 gene, but the exons 10 and 37 have been shown to have the highest mutation rate and count for ~30% of the mutations (Messiaen *et al.* 2000).

The NF1 gene is ubiquitously expressed, as one would expect based on the diverse clinical manifestations of the disorder (DeClue *et al.* 1991, Gutmann *et al.* 1991, Daston & Ratner 1992). However, the highest level of NF1 gene expression is seen in neural tissues (Daston *et al.* 1992). There are three major alternatively spliced exons of the gene, but also many other splice variants with low expression levels (Fig. 1) (Gutmann *et al.* 1995, Gutmann *et al.* 1999, Vandenbroucke *et al.* 2002b). The most commonly expressed alternative isoform includes exon 23a (63bp), which is located in the GAP region. The isoform containing exon 23a has been shown to have decreased Ras-GAP activity (Andersen *et al.* 1993a). Two other common alternatively spliced exons are 9a (30bp) and 48a (54bp). The isoform containing exon 48a is highly expressed in muscle tissues (Gutmann *et al.* 1995), and expression of the 9a-containing isoform is seen during embryonic development of the brain (Gutmann *et al.* 1999). The functions of these two latter isoforms are not known. The 3' untranslated region of NF1 mRNA shares strong homology between human and mouse, suggesting that it is important for mRNA stability (Bernards *et al.* 1993). A few proteins have been identified that bind to the NF1 3' untranslated region, including the human RNA-binding protein (HuR), which is known to bind mRNAs of proto-oncogenes, cytokines and transcription factors (Haeussler *et al.* 2000). Furthermore, NF1 mRNA has recently been identified to be targeted towards the cell-cell adhesion zone (Yla-Outinen *et al.* 2002).

NF1 gene



NF1 mRNA



NF1 protein

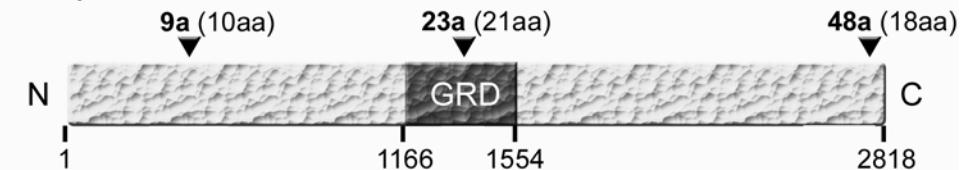


Fig. 1. Schematic presentation of the NF1 gene, mRNA and protein. This illustration of the NF1 gene presents all the exons of the NF1 gene, and the four large introns are marked with arrows. The OMGP, EVI2A and EVI2B genes in intron 27 are marked in the picture. The diagram is modified from (Friedman & Riccardi 1999). The illustrations of NF1 mRNA and protein present the alternatively spliced exons (9a, 23a and 48a) and the GAP-related domain (Gutmann & Collins 1993, Skuse & Cappione 1997). aa, amino acid; bp, base pair; GRD, GAP-related domain.

2.3 NF1 protein

The NF1 protein (neurofibromin) is relatively large, containing 2818 amino acids with a calculated molecular mass of 327kDa (Fig. 1) (Marchuk *et al.* 1991). However, the molecular mass identified in electrophoretic analysis is 220-280kDa (DeClue *et al.* 1991, Gutmann *et al.* 1991, Daston *et al.* 1992, Hirvonen *et al.* 1998). This is most likely due to the protein folding during migration through denaturing polyacrylamide gels because there is no evidence of glycosylation or processing of the full-length protein (Gutmann & Collins 1993). The NF1 protein has a GAP-related (GRD) domain, which is about 300-400 amino acids long, in the middle of the protein product (Gutmann & Collins 1993).

The NF1 protein is expressed in a variety of cell types in adults and during embryonic development (Daston & Ratner 1992, Daston *et al.* 1992, Huynh *et al.* 1994). The expression is most abundant in the nervous system (Daston *et al.* 1992). Subcellular localization studies have shown the NF1 protein to occur in the particulate and soluble fractions of the cytoplasm (DeClue *et al.* 1991, Golubic *et al.* 1992, Hattori *et al.* 1992). The NF1 protein has been shown to associate with microtubules, actin filaments, mitochondria, smooth endoplasmic reticulum, the nuclear/perinuclear area or heparan sulphate proteoglycans (Gregory *et al.* 1993, Nordlund *et al.* 1993, Roudebush *et al.* 1997, Hsueh *et al.* 2001, Li *et al.* 2001). The NF1 protein has also been shown to be constitutively phosphorylated at the cysteine/serine-rich domain of the N-terminus and the C-terminal region (Izawa *et al.* 1996). The half-life of the NF1 protein is regulated by a post-translational mechanism, which is likely to depend on protein phosphorylation. Its half-life is increased in melanocytes by the addition of growth factors, such as the basic fibroblast growth factor (bFGF) or phorbol myristate acetate (PMA) (Griesser *et al.* 1997, Kaufmann *et al.* 1999b). A recent study has shown that the NF1 protein is rapidly degraded in response to various growth factors, and the protein levels are re-elevated shortly afterwards in NIH3T3 fibroblasts, primary IMR90 cells and RT4 Schwannoma cells (Cichowski *et al.* 2003).

The best-known function of the NF1 protein is to act as a Ras-GAP. The Ras-GAP proteins stimulate the intrinsic Ras-GTPase to hydrolyze Ras attached GTP to GDP and inactivate the Ras by doing this (Fig. 2). The Ras-GAP function of the NF1 protein was first suggested after gene cloning and the identification of a region homologous to other Ras-GAP proteins (Ballester *et al.* 1990, Xu *et al.* 1990b). p120GAP and GAPIII are the closest relatives, sharing ~30% identity with the GAP region of the NF1 protein. The NF1 protein has been shown to interact with Ras-GTP and to stimulate its GTPase activity (Martin *et al.* 1990, Xu *et al.* 1990a, Bollag & McCormick 1991). The affinity of the NF1 protein to Ras-GTP is higher than that of p120GAP, but the stimulation of the intrinsic GTPase activity of Ras is less marked compared to p120GAP (Martin *et al.* 1990, Bollag & McCormick 1991). The NF1 protein also binds to oncogenic Ras, but is not able to stimulate its intrinsic GTPase activity (Xu *et al.* 1990a, Bollag & McCormick 1991). Furthermore, certain lipids (e.g., arachidonic acid, phosphatidic acid, stearic acid, oleic acid, phosphatidylinositol-4,5-bisphosphate, some n-6 and n-3 polyunsaturated fatty acids) decrease the GAP activity of the NF1 protein (Bollag & McCormick 1991, Golubic *et al.* 1991).

Elevated Ras-GTP levels have been demonstrated in some cells and tissues obtained from NF1 patients. Elevated Ras-GTP levels have been demonstrated in Schwann cells, primary leukemias, malignant schwannomas, neurogenic sarcomas and dermal neurofibromas (Basu *et al.* 1992, Yan *et al.* 1995, Bollag *et al.* 1996, Guha *et al.* 1996, Sherman *et al.* 2000). However, low levels of NF1 protein have not been shown to lead to increased Ras-GTP levels in various cell types, including melanomas, neuroblastomas, melanocytes and fibroblasts (Boddrich *et al.* 1995, Griesser *et al.* 1995, Sherman *et al.* 2000). Transfection studies have shown that overexpression of NF1 protein may lead to suppression of cell growth, transformed phenotype and decreased tumorigenicity without any change in Ras-GTP levels, suggesting that the NF1 gene can act independently of GAP activity (Johnson *et al.* 1994, Li & White 1996). Furthermore, in *Drosophila*, the NF1 protein seems to be a regulator of the cAMP-PKA-dependent signaling pathway instead of the Ras pathway (Guo *et al.* 1997, The *et al.* 1997, Tong *et al.* 2002). Defective calcium signaling has also been demonstrated in keratinocytes cultured from NF1 patients (Korkiamaki *et al.* 2002).

Taken together, the NF1 protein seems to be involved in the regulation of the Ras-MAPK pathway. However, the GAP region of the NF1 protein is only a fraction of the length of the total protein product. Consequently, it is likely that the NF1 protein has functions other than acting as a Ras-GAP. These other functions could involve regulation of the cAMP/PKA pathway or calcium-related signaling. The complexity of the regulation of the NF1 protein is shown by studies demonstrating various subcellular localizations for it.

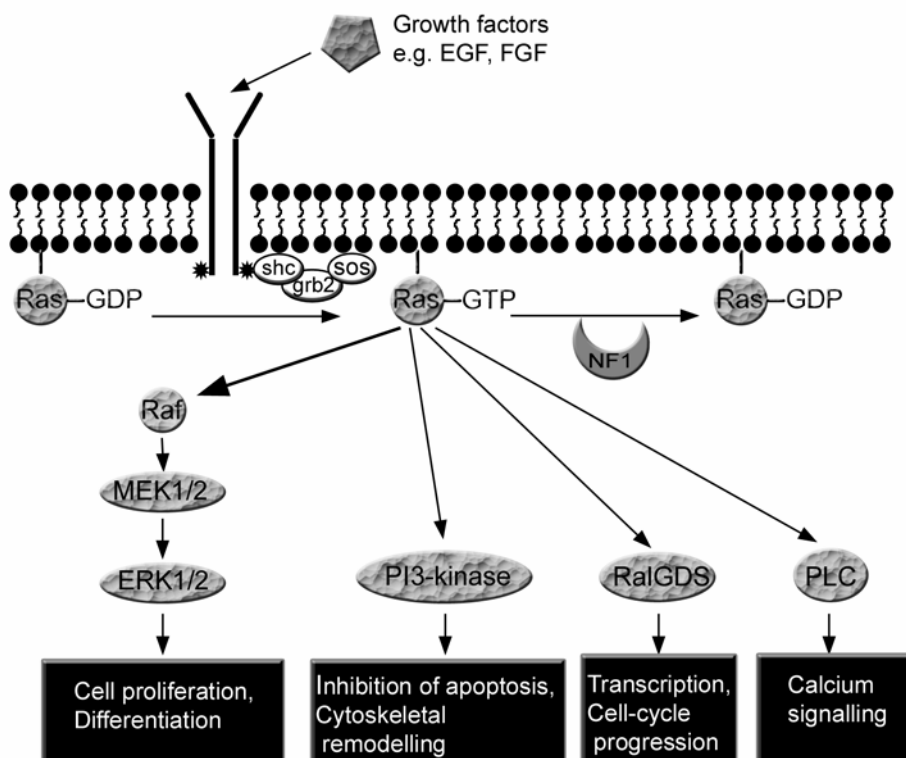


Fig. 2. The Ras-MAPK-signaling pathway. Growth signals (ligand coupled to receptor) activate the guanine nucleotide exchange factor sos, which is bound to the receptor by the shc-grb2 complex. Activation of sos enables GTP binding to Ras proteins. The NF1 protein acts as a negative regulator of Ras. The GRD of the NF1 protein accelerates the switch of active Ras-GTP into inactive Ras-GDP. The signaling pathways downstream of Ras-GTP include Raf-MEK-ERK kinase, phosphatidylinositol-3-kinase (PI3-kinase), RalGDS and phospholipase C (PLC) cascades.

2.4 Ras-MAPK signaling pathway

Ras proteins belong to the superfamily of monomeric GTPases, which are involved in receptor-mediated signal transduction pathways (Fig. 2). Other members of the family are Rho GTPase, a remodeller of the actin cytoskeleton, and Rab GTPase, a regulator of intracellular trafficking. Ras proteins are located on the inner surface of the plasma membrane and attached to the membrane by a farnesyl residue. Ras proteins transmit extracellular signals that promote the growth, proliferation, differentiation and survival of cells. The signaling cascade starts from the plasma membrane where the growth factor (e.g. epidermal growth factor (EGF), FGF) binds to its enzyme-linked receptor causing

receptor dimerization. This leads to phosphorylation of the intracellular parts of the receptors, which activates guanine exchange factors (GEF), such as sos. GEFs are attached to the receptor by the adaptor proteins shc and grb-2. GEFs promote the exchange of GDP attached to inactive Ras to GTP, which leads to activated Ras. Ras-GTP is constantly inactivated by Ras-GAP proteins (e.g., NF1 protein, p120GAP), which promote the intrinsic GTPase of Ras. This leads to the hydrolyzation of active Ras-GTP into inactive Ras-GDP (Alberts 2002). The major downstream target of Ras-GTP is mitogen-activated protein kinase (MAPK), but it is also known to activate other targets, e.g. phosphatidylinositol 3-kinase (PI3-kinase), Ras-related guanine nucleotide dissociation stimulator (RalGDS) and phospholipase C ϵ (PLC ϵ) (Rodriguez-Viciano *et al.* 1994, Spaargaren & Bischoff 1994, Kelley *et al.* 2001). Activation of MAPK occurs through specific phosphorylation of both a threonine and a tyrosine separated by a single amino acid. The first component of MAPK is called Raf, which is activated on the plasma membrane by Ras-GTP. Raf phosphorylates mitogen-activated kinase 1/2 (MEK1/2 kinase), which activates the extracellular regulated kinase 1/2 (ERK1/2 kinase or p44/42 MAPK) by phosphorylation. ERK1/2 kinase phosphorylates a variety of downstream targets, which results in changes in gene expression and the catalytic activities of enzymes (Alberts 2002). There are two other signaling pathways related to MAPK. These are called p38 MAPK and c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and they are mainly involved in transmitting inflammatory signals.

Of the members of the Ras-MAPK signaling pathway, Ras and Raf have been identified as proto-oncogenes. Gain-of-function mutations (activating mutations) of these genes drive a cell towards cancer. Proto-oncogenes with activating mutations are called oncogenes. Oncogenic Ras has been identified in every fourth cancer. The most common cancers with oncogenic Ras are pancreatic (90%), thyroid (60%) and colorectal (45%) cancers (Bos 1989). Furthermore, 35% of cancers show increased MAPK activity (Hoshino *et al.* 1999).

The Ras-MAPK-signaling pathway has been linked to be responsible for the malignant phenotype, including increased proliferation, defects in apoptosis, invasiveness and ability to induce neovascularization. Consequently, different therapies towards inhibiting the pathway are under development. The receptors responsible for Ras activation have proven to be effective targets for treating human cancers and are now used in clinical practice in treatment of breast cancer (Slamon *et al.* 2001). Therapies inhibiting Ras activation itself have also aroused a lot of interest. Farnesyl transferase inhibitors (FTI) block the farnesylation of Ras proteins, which is needed for membrane attachment and activation of Ras (Sebti & Hamilton 2000). However, these drugs have not been effective in human cancer treatment, even though they were promising in animal models (Kohl *et al.* 1995, End *et al.* 2001, Rose *et al.* 2001). The major downstream target of Ras, MAPK, has also been taken as a subject for drug design. MAPK inhibitors have been shown to be effective in cancer therapy in mouse models (Sebolt-Leopold *et al.* 1999). Inhibition of the Ras-MAPK-signaling pathway has been suggested as potential therapy for NF1. In mouse models, FTIs have been shown to reverse some abnormal features seen in NF1-deficient Schwann cells (Kim *et al.* 1997), but not in fibroblasts (Atit *et al.* 1999). Furthermore, FTIs can eliminate the learning difficulties seen in NF1 heterozygous mice (Costa *et al.* 2002). FTIs have also recently been tested in NF1 patients (Weiss *et al.* 1999).

2.5 Animal models for NF1

After cloning of the NF1 gene, many animal models have been developed to elucidate the molecular background of the disease. The first mouse models showed homozygous (-/-) mutations to be lethal due to heart malformations on embryonic day 13.5 (Brannan *et al.* 1994, Jacks *et al.* 1994, Henkemeyer *et al.* 1995). Other pathological changes seen in homozygous mice included a delay of muscle, renal and hepatic development and hyperplasia of sympathetic ganglia (Brannan *et al.* 1994). Heterozygous (+/-) mice were viable and showed no clinical signs for NF1, i.e. neurofibromas or CALMs (Brannan *et al.* 1994, Jacks *et al.* 1994). However, these mice were predisposed to the formation of tumors with advancing age. The mice had increased numbers of pheochromocytomas and leukaemias, which both are associated with NF1. (Jacks *et al.* 1994) In *Drosophila*, individuals homozygous (-/-) for the NF1 gene show 25% smaller body size but no other obvious developmental defects (The *et al.* 1997).

Animals with mutations of the NF1 gene have later been used to explore the pathogenetic mechanism of the disease. The unexpected absence of neurofibromas in mice heterozygous for the NF1 gene and the possible role of LOH in tumor formation have encouraged investigators to generate mice with some cells homozygous and some cells heterozygous for the NF1 gene. Chimeric mice, which have both homozygous and heterozygous cells, developed plexiform neurofibromas but no dermal neurofibromas (Cichowski *et al.* 1999). The development of plexiform neurofibromas was also seen in conditional knockout mice, which were homozygous for the NF1 gene in Schwann cells and heterozygous for it in other cells. However, these mice did not show neurofibromas after removal of the heterozygous background, suggesting that the formation of these tumors occurs in co-operation between many tissues. Skin pigmentation, carcinogenesis and wound healing have also been addressed in NF1 knockout mice. Heterozygous mice showed defects in the wound healing process. The wounds displayed fibroblast hyperplasia and increased collagen accumulation, and *in vitro* studies detected abnormal responses of fibroblasts to various growth factors. (Atit *et al.* 1999). Increased skin pigmentation and tumorigenesis were also seen in mice heterozygous for the NF1 gene after chemical carcinogen application. (Atit *et al.* 2000). Learning disabilities are commonly associated with NF1. Interestingly, both NF1 knockout mice and *Drosophila* showed learning and memory defects (Guo *et al.* 1997, Silva *et al.* 1997). Furthermore, removal of the alternatively spliced exon 23a on mice causes learning disabilities, but no other developmental defects or tumor predisposition (Costa *et al.* 2001). A recent study has addressed the role of the NF1 gene in endothelial cells. Mice homozygous for the NF1 gene in endothelial cells showed the same heart abnormalities as seen in homozygous mouse embryos. This finding highlights the importance of the NF1 gene in endothelial cells and indicates that endothelial cells, rather than neural crest cells or heart muscle cells, are responsible for the embryonic vascular abnormalities seen in NF1 homozygous mice (Gitler *et al.* 2003).

Animal models for NF1 have revealed the important cell signaling routes that involve the gene. The NF1 gene has been shown to have a critical function in regulating the Ras-MAPK-signaling route in various tissues. Mouse models have shown the importance of Ras-GAP function in cardiovascular morphogenesis (Gitler *et al.* 2003) and in learning

(Costa *et al.* 2002). In *Drosophila*, the NF1 gene is involved in cAMP-PKA signal transmission, but not in the Ras-MAPK pathway, and the NF1 knockout phenotype can be reversed by activation of the cAMP-PKA pathway (Guo *et al.* 1997, The *et al.* 1997, Guo *et al.* 2000, Tong *et al.* 2002).

2.6 Cytoskeleton and cell adhesions

The cytoskeleton consists of dynamic filamentous structures. There are three major types of filaments: actin filaments, intermediate filaments and microtubules. The cytoskeleton is not only a structural element, but also contributes to many other cellular processes, such as transportation, mitosis, secretion and formation of cell extensions. Cell adhesions serve as anchoring points between epithelial cells to ensure appropriate integrity and tensile strength of epithelial sheets. There are various types of cell adhesions, namely desmosomes, hemidesmosomes, adherens junctions, tight junctions, gap junctions and focal adhesions. The adhesive structures are connected to intermediate filaments (desmosomes and hemidesmosomes) or actin filaments (adherens junctions, tight junctions and focal adhesions) (Fig. 3). Association with the cytoskeleton is necessary for stable cell-cell and cell-matrix adhesion and for the integration of cell-cell contacts with changes in morphology that are characteristic of epithelial cells.

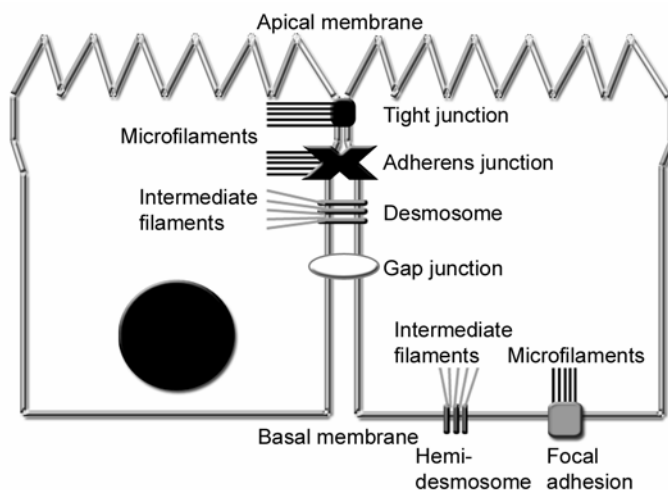


Fig. 3. A schematic presentation of cell adhesions and their association with the cytoskeleton in epithelium.

2.6.1 Cytoskeleton

Actin filaments are composed of two strands of polymerized actin protein. The filament diameter is 5-9nm. Actin filaments are dispersed around the cell, but are mostly concentrated just underneath the plasma membrane. Actin filaments have a special role in regulating the cell surface shape. Cell surface structures, such as lamellopodia, filopodia and microvilli, are supported by actin filaments. The formation of these structures is regulated by Rho-GTP-binding proteins, which belong to the Ras superfamily. The ability of cells to move is largely based on actin filaments and their association with myosin motor proteins (Alberts 2002).

Intermediate filaments have a diameter of 10nm. There are four types of intermediate filament proteins: nuclear lamins, vimentin-like proteins (vimentin, desmin and glial fibrillary acidic protein), keratins and neurofilament proteins. Intermediate filaments are composed of protein monomers (48nm length), which have a central helical domain and globular domains at both ends. The monomers are attached to each other in a particular order to form a filament. Specifically, two monomers first form a dimer and two dimers then link to each other and form a tetramer. Eight tetramers join together to form an intermediate filament with 10nm diameter. Keratin filaments are formed from heterodimers, in which a type I keratin protein (acidic) is joined together with a type II keratin protein (basic). All other types of intermediate filaments are constituted from homodimers with identical monomer proteins (Fuchs & Cleveland 1998, Alberts 2002). The most important function of intermediate filaments is to increase the mechanical strength of cells and tissues. Intermediate filaments are able to resist mechanical forces more effectively than microtubules or actin filaments (Janmey *et al.* 1991). Mutations in intermediate filament genes are linked to a variety of human diseases with defects in tissue or cell durability. Specifically, keratin mutations are linked to skin blistering and hypertrophy, diseases in which keratinocytes are not able to resist mechanical stress (Fuchs & Cleveland 1998).

Microtubules are formed of tubulin subunits. One subunit itself is composed of two closely related α - and β -tubulin proteins. The subunits attach to each other to form a protofilament, and 13 protofilaments join together to form a tubular filament with 25nm diameter. Microtubules rise from a microtubule-organizing center or centrosome. They are highly dynamic structures. Microtubules are involved in the positioning of membrane-enclosed organelles and direct intracellular transport (Alberts 2002).

2.6.2 Cell adhesions

Adherens junctions are constituted by transmembrane cadherin proteins and catenin-linker proteins that attach the junctions to the actin cytoskeleton. The extracellular parts of cadherin proteins from adjacent cells interact with each other in a calcium-dependent manner to form a junction (Nose *et al.* 1988). The cytosolic parts of cadherins connect to β -catenin, which attaches to α -catenin, a linker protein between the actin cytoskeleton and adherens junction (Fig. 4) (Gumbiner & McCreary 1993). There are three major types

of cadherin proteins found in adherens junctions: E; epithelial, P; placental and N; neuronal cadherins (Yap *et al.* 1997). Adherens junctions have been shown to have a wide variety of functions apart from just acting as simple adhesional structures. E-cadherin has been found to be a key determinant of cell recognition with diverse effects on cell behavior. Adherens junctions have been presented as adhesion-activated signaling receptors (Yap & Kovacs 2003). Specifically, adherens junctions are known to activate Rho-GTPases and PI3-kinase in an adhesion-dependent manner (Kovacs *et al.* 2002). Furthermore, adherens junctions have been linked to the Ras-MAPK cascade because a loss of adherens junctions by α -catenin knockout in mouse skin has been shown to cause sustained Ras-MAPK activation with strong hyperproliferation (Vasioukhin *et al.* 2001a).

Desmosomes function as adhesive intercellular junctions and as linkers of intermediate filaments in epithelia and cardiac muscle. Desmosomes are composed of transmembrane desmosomal cadherins (desmocollins 1-3, desmogleins 1-3) and a linker protein called desmoplakin. Desmoplakin attaches the cytoplasmic parts of cadherins to the intermediate filament network (Fig. 4). Furthermore, plakoglobin and plakophilin proteins have been demonstrated in the cytoplasmic part of the desmosome. Similarly to adherens junctions, desmosomal cadherins form junctions in a calcium-dependent manner (Garrod 1993, Garrod *et al.* 1996, Garrod *et al.* 2002). Desmosomes are thought to be especially important in forming mechanically stable cell junctions. This has been demonstrated in autoimmune diseases, with antibodies against desmosomal cadherins, and in diseases with mutations in desmosomal proteins, which both show the mechanical fragility of tissues (Armstrong *et al.* 1999, Rickman *et al.* 1999, Anhalt & Diaz 2001). However, recent studies have reported that desmosomes have a more versatile role in cell behavior. They have been shown to be involved in cell positioning and morphogenesis (Runswick *et al.* 2001) as well as in cell invasion (Tselepis *et al.* 1998).

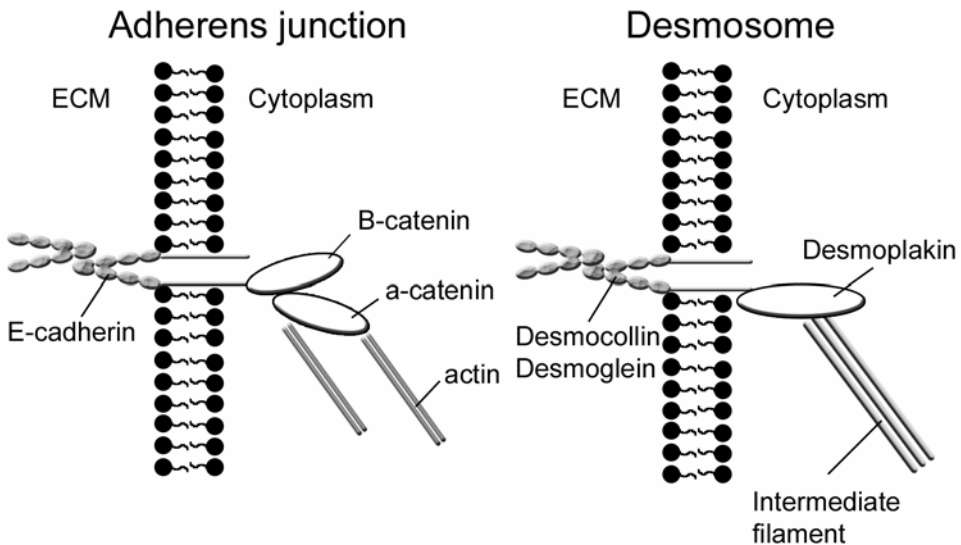


Fig. 4. Schematic presentation of molecular interactions within adherens and desmosomal junctions.

Gap junctions act as intercellular channels and function in signal transduction between neighboring cells (Richard 2000). They are permissive for small molecules (molecular weight <1kDa), such as nutrients, metabolites, ions and small signaling transducers (Kumar & Gilula 1996). Each gap junction is composed of a connexon joined with another connexon of an adjacent cell. A connexon is composed of six connexin proteins assembled in a channel-like fashion (Goodenough *et al.* 1996).

Tight junctions have a role in forming an impermeable barrier and dividing the plasma membrane of epithelial cells in the basolateral and apical domains. Claudin and occluding proteins are the transmembrane parts of the junction, and the cytoplasmic part is linked to the microfilament network (Tsukita & Furuse 1999, Tsukita *et al.* 2001).

Hemidesmosomes and focal adhesions represent the two major cell-matrix adhesions found in epithelial cells. Hemidesmosomes connect the basal cells to the basement membrane and are linked to the intermediate filament network. $\alpha 6\beta 4$ -integrin and PB180 act as transmembrane proteins and are linked to the cytoplasmic plaque composed of plectin and BP230. Plectin and BP230 attach the junction to the intermediate filament network (Garrod 1993, Borradori & Sonnenberg 1996). Focal adhesions are assembled from integrin transmembrane proteins, which are linked to the cytoplasmic plaque and further to the actin cytoskeleton (Petit & Thiery 2000, Geiger *et al.* 2001). Hemidesmosomes and focal adhesions have been linked to various cellular processes, such as cell attachment, proliferation, differentiation and death (Borradori & Sonnenberg 1996, Geiger *et al.* 2001).

2.6.2.1 Cell-cell adhesion assembly

The formation of adherens junctions and desmosomes requires extracellular calcium. Keratinocyte cultures serve as a model for adhesion assembly. Keratinocytes can be induced to form strong cell-cell adhesions by raising the calcium concentration of the culture medium from <0.1mM to >1.2mM (Hennings *et al.* 1980). This causes rapid (~15min) re-organization of actin and intermediate filament networks and re-localization of adherens junctional and desmosomal proteins towards cell-cell borders (Green *et al.* 1987, O'Keefe *et al.* 1987). The assembly of adherens junctions has been demonstrated to occur prior to the formation of desmosomes (Green *et al.* 1987), and adherens junctions are needed for desmosome arrangement (Wheelock & Jensen 1992, Lewis *et al.* 1994, Vasioukhin *et al.* 2000, Vasioukhin *et al.* 2001b).

The formation of stable cell-cell adhesions by calcium elevation was previously thought to develop from a conformational change in cadherins in response to calcium (Nose *et al.* 1988, Shapiro *et al.* 1995). However, recent studies have shed light on the molecular mechanism of cell-cell adhesions. It is thought that junctional assembly starts with the generation of filopodias, which penetrate and embed into adjacent cells. Adherens junctional proteins are clustered at the tip of the filopodia and generate a two-row adhesion zipper (Vasioukhin *et al.* 2000). Desmosomes clamp the opposing cell surfaces together and stabilize the junction (Vasioukhin *et al.* 2000, Vasioukhin *et al.* 2001b). Finally, directed actin polymerization pushes the two-row adhesion zipper into a

single row (Fig. 5). The adhesion zipper formation can be inhibited almost completely by disrupting the actin filaments by cytochalasin D (Vasioukhin *et al.* 2000). Taken together, the cell-cell adhesion development can be divided into active and passive categories. The active part utilizes actin polymerization, bringing the opposing membranes together, and the passive part consists of the establishment of cadherin-cadherin adhesions by conformational changes.

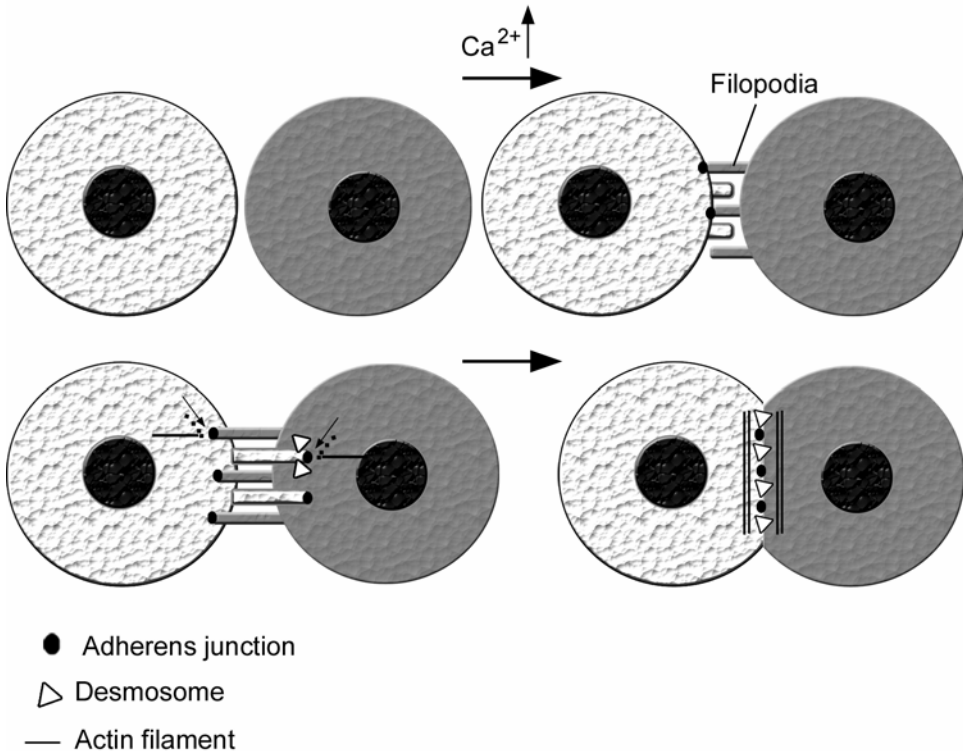


Fig. 5. Formation of cell-cell adhesion between epithelial cells after elevation of the extracellular calcium level (Vasioukhin *et al.* 2000).

2.7 Skin

The skin is one of the largest organs, accounting for 16% of body weight and having a surface area of $\sim 2\text{m}^2$. It has various functions: it is a protective layer against injuries and the invasion of microorganisms; it prevents dehydration; it is a sensory organ; and it participates in the thermoregulation by functioning as an insulator and producing sweat to remove excess heat. The skin consists of three layers: epidermis, dermis and hypodermis (Fig. 6). The epidermis is a surface epithelial layer. The dermis is a connective tissue layer underlying the epidermis. The hypodermis or subcutis is a loose connective tissue

layer underneath the dermis. The skin also contains appendages, such as hair, nails and glands (Young *et al.* 2000, Fawcett *et al.* 2002).

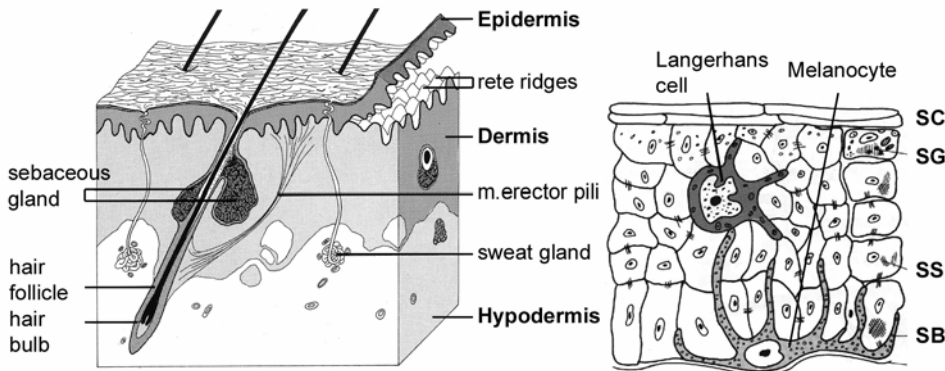


Fig. 6. Structure of human skin with its appendages and epidermis. The three layers of skin are epidermis, dermis and hypodermis. The epidermis consists of four layers: stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG) and stratum corneum (SC). The epidermis also contains some melanocytes and Langerhans cells. The figure has been modified from (Havu *et al.* 1998, Young *et al.* 2000).

2.7.1 Epidermis

The epidermis is composed of keratinized stratified squamous epithelium. It is a cellular layer with only scant extracellular matrix. Keratinocyte is the major cell type in the epidermis. Other cells found in the epidermis are melanocytes, Langerhans cells and Merkel cells (Young *et al.* 2000, Fawcett *et al.* 2002).

The epidermis comprises four layers (Fig. 6). The stratum basale, which rests on basal membrane, is the innermost cell layer. Basal cells are cuboidal and attach to each other with numerous desmosomes and to the basal membrane with hemidesmosomes. The dividing keratinocytes are located in the basal layer. The stratum spinosum is formed by differentiating keratinocytes moving upwards. It has four to six rows of cells with strong cytoplasmic keratin bundles and numerous desmosomes. The stratum granulosum consists of three to five layers of cells. The cells in this layer have marked keratohyalin granules, which stain intensely with basic dyes. There are also more cytoplasmic keratin bundles. The stratum corneum is the outermost layer composed of highly keratinized dead cells. The thickness of the layer depends on the mechanical stress directed against the skin. The stratum corneum is most abundant in such areas as palms and feet soles (Young *et al.* 2000, Fawcett *et al.* 2002).

In addition to keratinocytes, the epidermis also contains melanocytes, Langerhans cells and Merkel cells. Melanocytes are located in the basal layer and protect the skin from solar irradiation by producing melanin pigment and distributing it to the keratinocytes (Fawcett *et al.* 2002). A single melanocyte is responsible for distributing

melanin to ~36 keratinocytes with its long dendritic processes (Jimbow 1995). Langerhans cells represent 2-4% of epidermal cells and are mainly located suprabasally (Chen *et al.* 1985). They participate in immunological reactions by intaking an antigen, migrating to a lymph node and presenting the antigen to a lymphocyte (Streilein & Grammer 1989, Kripke *et al.* 1990). Merkel cells are located in the basal layer of the epidermis, and they are thought to act as mechanoreceptors (Fawcett *et al.* 2002).

2.7.1.1 Keratinocyte differentiation and activation

Basal keratinocytes have two alternative pathways to end up in (Fig. 7). They can differentiate through the spinosus, granular and cornified layers (Fig. . Differentiation be affected by calcium, retinoic acid, vitamin D3 and protein kinase C (PKC) activators (Hennings *et al.* 1980, Fuchs & Green 1981, Hosomi *et al.* 1983, Parkinson *et al.* 1984, Eckert *et al.* 1997). Basal keratinocytes express keratins 5 (K5 or CK5) and 14 (K14), while K1 and K10 are expressed by differentiating keratinocytes (Darmon & Blumenberg 1993). The other alternative pathway for keratinocytes is to become activated by a hyperproliferative and migrating phenotype. Activation occurs after epidermal injury and in certain pathological conditions, such as psoriasis (Nickoloff & Turka 1993, Tomic-Canic *et al.* 1998). A keratinocyte activation cycle has recently been proposed to exist (Freedberg *et al.* 2001). The activation cycle begins with interleukin-1 (IL-1) release from keratinocytes. Activation changes the keratin pattern from K5/14 to K6/16 (Komine *et al.* 2001) The activated phenotype is maintained by tumor necrosis factor α (TNF α) and transforming growth factor α (TGF α) (Jiang *et al.* 1993, Nickoloff & Turka 1993, Ma *et al.* 1997). In the later stages of wound healing, keratinocytes become contractile by an interferon- γ (IFN γ) signal and start to express keratin 17 (K17) (Jiang *et al.* 1994, Komine *et al.* 1996). Once the injury has been healed, a transforming growth factor β (TGF β) signal from dermal fibroblasts makes the keratinocyte phenotype basal and the cells start to express K5/14 (Jiang *et al.* 1995).

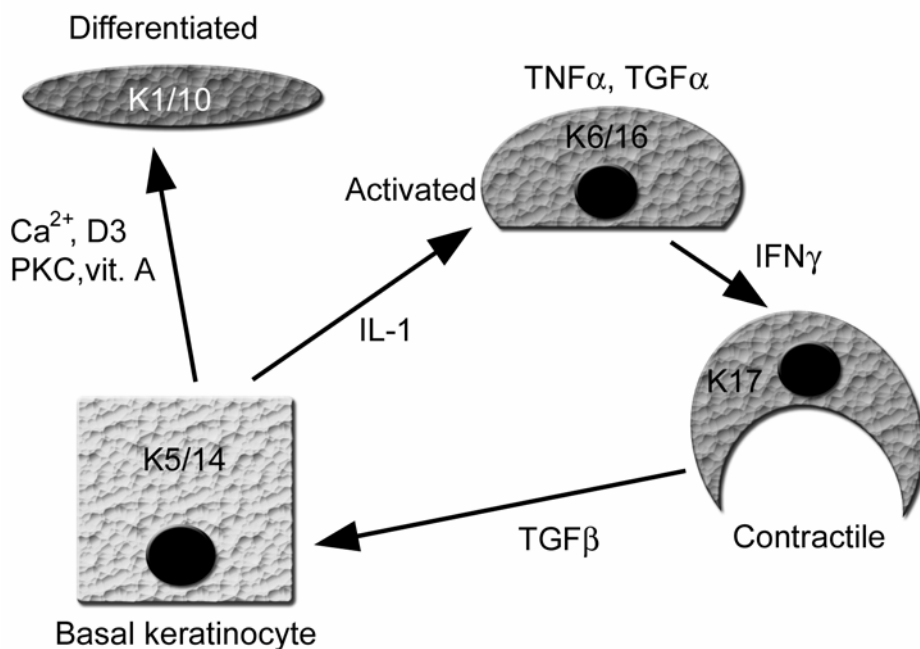


Fig. 7. Keratinocyte differentiation and activation. Basal keratinocytes can either differentiate or become activated. Differentiation is associated with keratin 1 and 10 expression (K1/K10) and can be promoted by Ca^{2+} , vitamin D (D3), protein kinase C (PKC) and vitamin A (vit. A). Activation occurs in a cyclic manner. Interleukin-1 (IL-1) promotes activation and is associated with keratin 6 and 16 (K6/16) expression. Tumor necrosis factor α ($\text{TNF}\alpha$) and transforming growth factor α ($\text{TGF}\alpha$) maintain the activated phenotype. Interferon- γ ($\text{IFN}\gamma$) helps the cells to become contractive with keratin 17 (K17) expression, and the basal phenotype is restored by transforming growth factor beta ($\text{TGF}\beta$).

2.7.2 Dermis and hypodermis

The dermis provides a supporting base for the epidermis and incorporates the abundant vascular system necessary for the metabolic support of the epidermis and for thermoregulation. The dermis mainly consists of extracellular matrix, which is mostly composed of collagens, elastin and proteoglycans, and fibroblasts. In addition to fibroblasts, other cell types include macrophages, lymphocytes and mast cells. The dermis is also characterized by abundant blood and lymphatic vessel networks. The dermis is divided into the superficial papillary and deep reticular layers. The papillary layer contains widely distributed fibroblasts between type III collagen bundles and dense capillary networks. The thick reticular layer is composed of strong and tightly packed type I collagen fibres (Young *et al.* 2000, Fawcett *et al.* 2002).

The hypodermis or subcutis is composed of loosely packed collagen fibres oriented mainly parallel to the skin surface. Adipose cells are also seen in this layer, especially in the abdomen and buttocks. The hypodermis serves as a protective layer against mechanical stress due to its mobility (Fawcett *et al.* 2002).

2.7.3 Wound healing of skin

Due to the many important functions of the skin, wound healing has to be fast and effective. Wound healing can be divided into three overlapping phases: inflammation, granulation and remodeling. The inflammatory phase begins with leakage of blood constituents from disrupted vessels. Platelets released from vessels aggregate and trigger blood coagulation and form a clot that stops the bleeding. The clot also serves as a temporary shield and forms a matrix for wound healing to take place. Later, in the inflammatory phase, neutrophils and macrophages invade into the wound area. Leukocytes act to remove bacteria and foreign bodies from the wound area. Neutrophils are the first to invade the wound area and are soon followed by macrophages. The granulation phase follows the inflammatory phase. It is characterized by cell proliferation and migration and the production of extracellular matrix. Various growth factors, such as platelet-derived growth factor (PDGF), TNF α , TGF α and TGF β , have been suggested to be operative during the granulation phase. Fibroblasts proliferate and migrate to the wound area and are responsible for the production of loose connective tissue and wound contraction. Neovascularization of the wound area occurs through growth of blood vessels from areas adjacent to the wound. Re-epithelialization of the wound is also considered to be part of the granulation phase because it acts to generate new tissue to replace the lost tissue. The last phase of wound healing is remodeling, which occurs after closure of the wound. Remodeling is characterized by replacement of the fibronectin-rich granulation matrix by collagenous matrix with increased tensile strength. TGF β has been shown to be the most important growth factor during the remodeling phase (Clark 1996, Martin 1997).

The re-epithelialization of wounds includes keratinocyte proliferation, migration and differentiation. The re-epithelializing keratinocytes mainly rise from the wound edge, but if the hair follicles or sweat glands remain intact, part of the re-epithelialization occurs through these skin appendages. Within hours after injury, keratinocytes are stimulated to migrate and cover the wound bed. Epithelial cell movement has been suggested to be driven by various factors, such as growth factors and chemotactic factors, and loss of cell-cell contacts. The migrating cells undergo metamorphosis, which is marked by retraction of tonofilaments, dissolution of desmosomes and hemidesmosomes and formation of peripheral actin filaments. Keratinocytes are able to migrate on the basement membrane or, if the basement membrane is destroyed, on the provisional matrix. After 1-2 days of wounding, keratinocytes adjacent to the original wound edge begin to proliferate. These cells form the hypertrophic zone and serve as a keratinocyte reserve. Epidermal growth factor (EGF) family members, such as EGF itself and TGF α , and keratinocyte growth factor (KGF) have been proposed to be the most important

growth factors affecting the re-epithelialization of skin (Clark 1996, Coulombe 1997, Martin 1997).

2.7.4 Psoriasis

Psoriasis is a chronic inflammatory disease of the skin. Its prevalence is 2-3% in Western populations. The disease is typically characterized by well-demarcated scaling plaques, but the clinical manifestations may display marked variation. There are four major pathological alterations associated with the disease: inflammation, hyperproliferation of the epidermis, altered maturation of the epidermis and vascular abnormalities (Arndt 1996).

The genetic aetiology of psoriasis remains obscure, but several putative candidate genes have been identified, such as human leukocyte antigen-C (HLA-C) and S100A7 (Trembath *et al.* 1997, Allen *et al.* 1999, Semprini *et al.* 1999, Asumalahti *et al.* 2000). The major pathomechanical feature involves activated CD4+ and CD8+ T-lymphocytes, which release keratinocyte-activating proinflammatory cytokines (Bata-Csorgo *et al.* 1995). However, it remains an open question whether psoriasis is primarily a disorder of the epidermal keratinocyte or, alternatively, whether the aberrant keratinocyte differentiation is a consequence of an influx of pathogenic immunocytes. Both of these possibilities may be important factors in the hyper-responsiveness of pre-psoriatic skin reported by several authors (Nickoloff 1999, Travers *et al.* 1999).

Psoriatic keratinocytes display altered gene expression profiles compared to unaffected cells. Th1 type cytokines, such as IFN γ , are thought to play a major role behind expressional changes (Uyemura *et al.* 1993, Prinz *et al.* 1994). Lesional keratinocytes show increased expression of the keratins 6, 16 and 17 (Leigh *et al.* 1995, McKay & Leigh 1995). Enhanced Ras expression and activation have also been reported in psoriatic lesions (Kobayashi *et al.* 1988, Lin *et al.* 1999). Furthermore, the NF1 protein is downregulated in lesions and its expression is restored by the healing process (Peltonen *et al.* 1995).

3 Aims of the present study

The NF1 gene was cloned in 1990. The function of the gene still remains unclear, as the Ras-GAP function is its only well-known function. However, it is likely that the NF1 gene also has other functions demonstrated by the various subcellular locations of the NF1 protein and by the large size of the gene compared to other GAP genes.

The human epidermis and epidermal keratinocytes serve as an excellent model for studying cell behavior both *in vivo* and *in vitro*. They have been used successfully to study, for example, cell differentiation and activation, a phenotype occurring during the healing of an epithelial wound. The human epidermis and epidermal keratinocytes are known to express the NF1 tumor suppressor gene, which may have a function in the tissue and the cells.

The purpose of the present study was to elucidate the role of the NF1 tumor suppressor gene in epidermal differentiation and growth. The specific aims were as follows:

1. To elucidate the role of the NF1 tumor suppressor in the cellular differentiation of keratinocytes *in vitro* and *in vivo*.
2. To study the expression the NF1 tumor suppressor in psoriatic keratinocytes, which display hyperproliferation and altered cellular differentiation.
3. To investigate the role of the NF1 tumor suppressor in normal wound epithelialization.

4 Materials and Methods

4.1 Materials

The materials used are shown summarized below in the tables 2, 3 and 4, and they have been described in detail in the original publications (I-IV).

Table 2. The patients, tissues and cell lines used are listed below. All the patient material and tissue samples were collected with appropriate approvals from the ethical committees of the respective universities and university hospitals.

Patients, tissue samples and cell cultures	Used in
Patients	
Healthy adults (22 individuals)	I, II, III, IV
NF1 patients (9 individuals)	I, IV
Psoriatic patients (5 individuals)	III
Tissue samples	
Human adult skin (from 22 individuals)	I, II, III, IV
Skin of NF1 patients (from 9 individuals)	I, IV
Lesional and non-lesional skin of psoriatic patients (from 5 individuals)	III
Cell cultures	
Human adult keratinocytes	I, II, III
Human adult fibroblasts	II
Keratinocytes cultured from NF1 patients	I
Keratinocytes cultured from psoriasis patients, lesional and non-lesional	III

Table 3. The analytical tools used for protein studies

Tool	Description	Source/Reference	Used in
Primary antibodies			
NF1GRP(D)	Rabbit polyclonal antibody against aa 2798-2818 of NF1 protein	Santa Cruz Biotech, (Gutmann <i>et al.</i> 1991)	I,II,III,IV
NF1ab67	Rabbit polyclonal antibody against 19 aa of NF1 protein representing alternatively spliced exon 23a	Our laboratory, (Hermonen <i>et al.</i> 1995)	I
NF1as159	Rabbit polyclonal antibody against 18 aa of NF1 protein representing alternatively spliced exon 48a	Our laboratory	I
Cytokeratin 14	Mouse Mab	Sigma, clone CK B1	I
α -Tubulin	Mouse Mab	Sigma, clone DM 1A	I
Desmoplakin I/II,	Mouse Mab	Boehringer Mannheim, clone Dp 2.15	I
β 4-Integrin	Mouse Mab	Life Technologies, clone 3 E1	I
Phospho-p44/42	Rabbit polyclonal antibody	Cell Signaling Technologies	IV
S-100	Rabbit polyclonal antibody	Dako	IV
Smooth muscle actin (SMA)	Mouse Mab	Dako, clone 1A4	IV
Miscellaneous			
Phalloidin	Tritc conjugated	Molecular Probes	I
Preimmune sera P67	From P67 immunized Rabbit	Our laboratory, (Hermonen <i>et al.</i> 1995)	I
Preimmune sera P159	From P159 immunized Rabbit	Our laboratory	I
Polypeptide 46	aa 1097-1126 of NF1 protein	Our laboratory, (Hermonen <i>et al.</i> 1995)	I,III
Polypeptide 67	19 aa of NF1 protein representing alternatively spliced exon 23a	Our laboratory, (Hermonen <i>et al.</i> 1995)	I
Polypeptide 159	18 aa of NF1 protein representing alternatively spliced exon 48a	Our laboratory	I
NF1GRP(D) peptide	Peptide used to generate NF1GRP(D) antibody, aa 2798-2818 of NF1 protein	Santa Cruz Biotech, (Gutmann <i>et al.</i> 1991)	I,III

Abbreviations: monoclonal antibody (Mab).

Table 4. The analytical tools used for RNA studies.

Nucleic acid probe targets	Description	Used for	Reference	Used in
NF1	0.5 and 1.2kb, cDNA	Northern blot	(Wallace <i>et al.</i> 1990)	III
NF1	650 bp, cRNA	<i>In situ</i> hybridization (anti-(Yla-Outinen <i>et al.</i> sense, sense)	2002)	III,IV
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	1.2 kb, cDNA	Northern blot	(Fort <i>et al.</i> 1985, Yla-Outinen <i>et al.</i> 2002)	III

4.2 Methods

A summary of the methods used in the thesis is shown below (Table 5), and they have been described in detail in the original publications (I-IV).

Table 5. The methods used in the original communications (I-IV).

Methods	Used in
Cell cultures	
Establishing and culturing keratinocytes	I, II, III
Establishing and culturing fibroblasts	II
Cytochemical staining	
Indirect immunofluorescence labeling	I, II,II
Avidin-biotin immunolabeling	III,IV
Immunogold labeling for electron microscopy	I,II
Protein analysis	
Western blotting	I,II,III
Cytoskeletal extractions	I
RNA analysis	
Isolation of RNA	III
Northern blot	III
<i>In situ</i> hybridization	III,IV
Microscopic techniques	
Light microscopy	III, IV
Conventional fluorescence microscopy	I,II,III
Confocal laser scanning microscopy	I
Digital image analysis	I, II, III, IV
Non-invasive measurements of skin properties	
Skin thickness measurement with an ultrasound scanner	IV
Skin elasticity measurement with a vacuum device	IV
Skin blood flow with a Doppler ultrasound scanner	IV
Transepidermal water loss (TEWL) measurement	IV
Miscellaneous	
Hematoxyline-eosin staining	III,IV
Statistical analysis	I, II, III

5 Results

5.1 NF1 protein expression during keratinocyte differentiation (I)

5.1.1 Alteration in the subcellular location of NF1 protein after induction of cellular differentiation

NF1 protein expression was studied using fluorescence microscopy in cultured keratinocytes induced to differentiate by elevating the extracellular calcium level to 1.8mM with an antibody to the far C-terminus of the NF1 protein (NF1GRP(D)). When cultured at a low calcium concentration (<0.1 mM), undifferentiated keratinocytes appeared to express NF1 protein at an equally moderate level. The immunoreaction for NF1 protein was found diffusely in cytoplasm and, in many cells, in association with fibrillary structures perinuclearly and in the cell periphery (I, Fig. 1A; Fig. 8A). Induction of cellular differentiation by elevating extracellular calcium caused a marked alteration in the subcellular location of NF1 protein. At any time point during the first 8 hours after the differentiation signal, a small subpopulation of cells was intensely immunoreactive for NF1 protein, while the majority of cells displayed only a weak immunoreaction. High microscopic magnification showed that the elevated external calcium concentration induced accumulation of NF1 protein into finger-like projections extending into the cell periphery (I, Fig. 1B; Fig. 8B). Two neighboring cells often displayed similar organization of NF1 protein, and the NF1-positive fingers of adjacent cells met, leaving only a narrow NF1-negative gap between the cells (I, Fig. 1B). After a period of intense labeling, immunoreactivity vanished, leaving the cells practically negative for NF1 protein. Re-appearance of NF1 immunoreactivity in a population of cells positioned on top of the original monolayer was noted at about 8-48 h after the calcium switch (I, Fig. 1C).

Control immunoreactions were carried out with antibodies directed against different parts of the NF1 protein. An antiserum raised against a peptide representing an alternatively spliced exon 48a (NF1as159) demonstrated immunosignals comparable to

the antibody NF1GRP(D) (I, Fig. 3A), but an antibody raised against a peptide representing an alternatively spliced exon 23a (NF1ab67) did not label any fibrillary structures. Preincubation of all NF1-specific antibodies with the peptides used for immunization completely abolished the immunosignals (I, Fig. 1D).

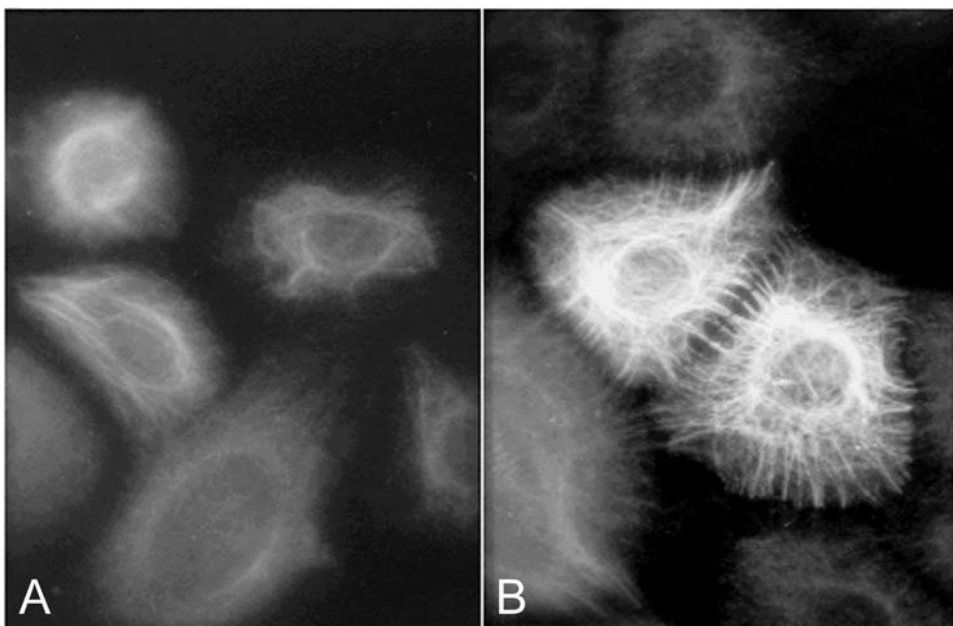


Fig. 8. Alteration in the subcellular location of NF1 protein after the induction of cellular differentiation. NF1 protein undergoes marked translocation to the filamentous network after incubation of keratinocytes with high-calcium medium (B) compared to low-calcium medium (A).

5.1.2 NF1 protein associates with an intermediate filament network during cellular differentiation

When keratinocytes were induced to differentiate, NF1 protein appeared to be associated with fibrillary structures that were most likely part of the cytoskeletal network. To identify the cytoskeletal component associated with the NF1 protein, double immunolabelings for this protein and various cytoskeletal components, i.e., tubulin, actin and cytokeratin 14 (CK14), were carried out. The results revealed a high degree of colocalization for NF1 protein and CK14 in cells intensely positive for NF1 protein (I, Fig. 2).

In order to investigate the potential interactions between NF1 protein and the keratin network, keratinocyte cultures were treated with cytoskeletal extraction buffer (CSK buffer) prior to fixation. CSK buffer removes all other cellular components apart from the

intermediate filaments and proteins associated with these filaments (Fey *et al.* 1984a, Fey *et al.* 1984b). Immunolabeling with the NF1GRP(D) antibody of cells treated with CSK buffer demonstrated an intense finger-like immunosignal in association with cytokeratin filaments in a subpopulation of cells (I, Fig. 3C). Two different isoform-specific NF1 antibodies were used to further confirm the findings showing the association of NF1 protein with bundles of intermediate filaments. As expected, antiserum NF1as159 decorated bundles of cytokeratin in differentiating keratinocytes (I, Fig. 4A). Interestingly, the antibody NF1ab67 labeled cytokeratin bundles, but the immunoreaction only became detectable after CSK buffer treatment of the cells (I, Fig. 4B). CSK preparations were also used for western transfer analysis. Western transfer analysis with the NF1GRP(D) antibody demonstrated that NF1 protein (250 kDa band) was preferably found in the insoluble fraction (represents intermediate filaments and intermediate filament-associated proteins) of CSK preparations of keratinocytes maintained at a low or high calcium concentration (I, Fig. 3D). Analogous to immunofluorescence studies, NF1 protein expression decreased in the CSK insoluble fraction of lysates generated from cultures treated for 24h at a high calcium concentration compared to low calcium or high calcium for 4h. Pre-incubation of the antibody with a corresponding peptide completely abolished the 250kDa band representing the NF1 protein (I, Fig. 3D). This indicates the specificity of the antibody towards the peptide it was originally raised for and its specificity for the NF1 protein.

5.1.3 Association of NF1 protein with the intermediate filament network is temporally parallel to the formation of desmosomes and hemidesmosomes

The subpopulation of keratinocytes that displayed an intense NF1 protein immunosignal on the intermediate filament cytoskeleton were further characterized and identified. Desmosomes and hemidesmosomes are linked to the intermediate filament network. Furthermore, previous studies have shown that elevation of the extracellular calcium level causes rapid formation of cell adhesions, including desmosomal junctions (Hennings *et al.* 1980). High extracellular calcium concentrations have been shown to initiate the transport of desmoplakin from intracellular stores along cytokeratin-containing intermediate filaments to the sites of new desmosomes (Jones & Goldman 1985, Pasdar & Nelson 1988b, Pasdar & Nelson 1988a). Consequently, double immunolabelings for NF1 protein and selected components of desmosomes and hemidesmosomes, i.e. desmoplakin and β 4 integrin, were carried out following elevation of the external calcium concentration of the cell culture medium. The results revealed a transient and temporally parallel localization of both NF1 protein and desmoplakin to the intermediate filament cytoskeleton (I, Fig. 5A, B). Specifically, analyses of cells representing distinct stages of the formation of junctions in differentiating keratinocytes collectively suggested that the order of events is as follows: localization of desmoplakin on the intermediate filament cytoskeleton becomes evident after NF1 protein has adhered

to the bundles of intermediate filaments; NF1 protein decorates cytokeratin filaments only to a point where most desmoplakin is concentrated to the cell-cell contact sites. Furthermore, NF1 protein also displays a close association with hemidesmosomes by double immunolabeling with antibody to $\beta 4$ integrin after the switch to a high calcium concentration (I, Fig. 5C).

5.1.4 Phenotype of keratinocytes cultured from NF1 patients

Keratinocyte cultures were established from nine voluntary NF1 patients to generate a model for NF1 gene deficiency. Cultures were used to investigate the potential relevance of the association of NF1 protein with intermediate filaments. The results revealed that keratinocytes obtained from NF1 patients displayed a markedly altered morphology and an altered response to the elevated calcium concentration compared to controls. Specifically, neurofibromatosis keratinocytes were larger in size, as estimated by the area occupied by the cells on the culture substratum, and displayed larger cell size variation in monolayer cultures compared to control keratinocytes (I, Fig. 7A, B). The differences between NF1 keratinocytes and control keratinocytes were most pronounced when the cells were cultured at a high calcium concentration (I, Table I). Furthermore, differentiating NF1 keratinocytes were characterized by a lower number of NF1 protein-positive fingers representing NF1 protein adhering to bundles of cytokeratin (I, Fig. 7C).

5.2 Ultrastructural localization of NF1 protein in human skin (I,II)

NF1 protein expression was further studied in human skin to extrapolate the *in vitro* findings to an *in vivo* situation. Immunofluorescence analysis of frozen sections revealed the most intense labeling for NF1 protein in the basal layer of the epidermis (II, Fig. 1A). Dermal fibroblasts and the upper layers of the epidermis also showed a positive but less intense immunosignal for NF1 protein compared to basal keratinocytes (II, Fig. 1A). High-power microscopy of basal keratinocytes demonstrated that NF1 protein is located at cellular projections directed towards neighboring cells and the dermal-epidermal junction (II, Fig. 1B).

Immunolectron microscopy was used for ultrastructural analysis of the NF1 protein in human skin. Immunogold particles representing the signal for NF1 protein were detected in basal keratinocytes and in dermal fibroblasts, but not in the upper layers of the epidermis. Both keratinocytes and fibroblasts displayed immunogold particles, mainly in the electron-dense areas of the cytoplasm containing bundles of intermediate filaments (II, Fig. 2A, B). A detailed analysis revealed that immunogold particles in basal keratinocytes intensively decorated the cytokeratin bundles, especially their lateral aspects (I, Fig. 6A, B; II, Fig. 2A). Furthermore, immunogold particles were seen in close proximity to desmosomes and hemidesmosomes. In fibroblasts, the labeling was more diffuse compared to that in keratinocytes, and no detectable cytoskeletal bundles were

seen (II, Fig. 2B). In both cell types, only a fraction of gold particles were detected in close proximity to the plasma membrane, and no signal was seen in the nuclei.

The distance between immunogold particles representing NF1 protein seemed to be consistently similar (II, Fig. 2C, D). Consequently, the distance between the two closest gold particles was measured and analyzed numerically. The results showed similar values for both keratinocytes and fibroblasts, the average distance between the closest immunogold particles being 48.1nm in keratinocytes and 47.6nm in fibroblasts (II, Fig. 3A).

5.3 NF1 gene expression in psoriatic skin and in keratinocytes cultured from psoriatic patients (III)

5.3.1 NF1 gene expression in psoriatic lesions

Skin biopsies were obtained from five psoriatic patients. The excisions extended from healthy to lesional skin. The tissues were formalin fixed and embedded in paraffin. NF1 protein expression was studied using avidin-biotin immunolabeling with NF1GRP(D) antibody. NF1 protein expression was detected in the whole epidermis, and the most intense signal was seen basally (III, Fig. 1A, B). In apparently healthy psoriatic skin, the signal was markedly more intense than in psoriatic lesions (III, Fig. 1).

NF1 mRNA expression was investigated using *in situ* hybridization with a NF1-specific anti-sense probe. NF1 mRNA was detected throughout the epidermis in both lesional and non-lesional psoriatic skin (III, Fig. 2). In healthy-looking skin, the most intense hybridization signal was located in the upper stratum spinosum (III, Fig. 2A). The expression of NF1 mRNA weakened significantly towards the lesion (III, Fig. 2B). In lesional skin, the level of NF1 mRNA was apparently the same in the upper and the lower epidermis (III, Fig. 2C, D). However, some individual cells showed a hybridization signal indicating the presence of NF1 mRNA, whereas some cells were totally devoid of signal.

5.3.2 NF1 gene expression in keratinocytes cultured from psoriatic patients

A total of four lines of lesional and three lines of non-lesional psoriatic keratinocytes and three lines of healthy control keratinocytes were used to study the NF1 gene in cultured cells. Northern transfer analysis of RNA isolated from cultured keratinocytes revealed bands of ~11 and 13 kb specific for NF1 mRNA (III, Fig. 4). The results showed that the steady-state levels of NF1 mRNA were markedly lower in both lesional and non-lesional

keratinocytes compared to healthy control cells. Healthy control keratinocytes displayed 150-500% higher levels of NF1 mRNA compared to psoriatic keratinocytes (III, Fig. 4). NF1 protein was studied with western transfer analysis generated from keratinocyte lysates. Psoriatic keratinocytes expressed lower levels of NF1 protein compared to controls (III, Fig. 3). However, the difference in protein expression was not equally extensive as that seen with NF1 mRNA.

In situ hybridization and immunocytochemistry were used to study the subcellular localization of NF1 mRNA and protein in cultured, differentiating, psoriatic keratinocytes. Keratinocyte differentiation was initiated by elevation of extracellular calcium. Granular staining representative of NF1 mRNA was distributed evenly in the cytoplasm of undifferentiated keratinocytes (III, Fig. 6). At 1 to 2 hours after raising the extracellular calcium concentration, the hybridization signal redistributed towards the cell periphery. This redistribution was more distinct in normal cells compared to psoriatic keratinocytes. At 4 hours, the NF1 mRNA signal was barely detectable in normal cells and had almost totally disappeared in psoriatic keratinocytes (III, Fig. 6). Immunofluorescence labeling of normal keratinocytes revealed NF1 protein in association with cytoskeletal filaments, some cells being strongly immunoreactive for NF1 protein (III, Fig. 5). In contrast, psoriatic keratinocytes displayed a less intense signal for NF1 protein, and the intercellular differences in labeling intensities were less pronounced (III, Fig. 5).

5.4 NF1 and epidermal wound healing (IV)

5.4.1 NF1 gene expression in healing wounds

NF1 expression and function in epidermal wound healing was studied using the suction blister method. The suction blister method is a well-characterized technique to study epidermal wound healing (Kiistala 1968). In this method, detachment of the epidermis takes place within the lamina lucida layer of the basement membrane. Re-epithelialization of a 6 mm suction blister takes place in ~ 10 days, but increased pigmentation on the healing lesion may prevail for a few months. NF1 gene expression was first studied in formalin fixed, paraffin embedded histological sections of healing, four-day-old epidermal wounds. Immunolabelings for NF1 protein in normal skin demonstrated the most intense signal in basal keratinocytes and less intense labeling in the upper epidermis and dermis. Keratinocytes in the re-epithelialization zone displayed increased NF1 protein expression compared to adjacent intact epidermis (IV, Fig. 1A). Increased expression was seen in both the hypertrophic and the migrating zones. The highest levels of expression were seen in the cells located close to the edge of the re-epithelializing front, and a less intense immunoreaction was seen in the hypertrophic zone of the epidermis. NF1 protein expression was also increased in dermal fibroblasts under the blister. Similarly, NF1 mRNA levels increased both in re-epithelializing

keratinocytes and in dermal fibroblasts (IV, Fig. 1C). The negative controls in which 1) the primary antibody was omitted or 2) hybridization was carried out with a sense probe showed only uniform background labeling (IV, Fig. 1B, D).

5.4.2 Wound healing in NF1 patients

Five NF1 patients and six age- and sex-matched controls were selected to study the possible alterations of epidermal wound healing associated with NF1. The NF1 patients were first clinically evaluated for skin abnormalities. All five patients had neurofibromas, and one had a plexiform neurofibroma (IV, Table I). CALMs were found in four patients, and all patients had axillary freckling. Since it has been speculated that skin wounds could have a role in neurofibroma and CALM formation, all previous scars of the NF1 patients were evaluated clinically for abnormalities of the skin. Most scars looked normal, but two patients had neurofibromas rising directly from old dermal scars (IV, Table I). Furthermore, non-invasive measurements of skin thickness, elasticity and blood flow were performed on the patients and the controls. The measurements showed no significant differences between these two groups (IV, Fig. 2).

The suction blister method was used to study the efficiency of epidermal wound healing in NF1 patients. The wound healing efficiency was analyzed clinically and histologically and with transepidermal water loss (TEWL) measurements four days after blister induction. Clinical evaluation of four-day-old blisters revealed no difference in wound healing between NF1 patients and healthy controls. The TEWL method measures the skin water barrier function and can be used to study wound healing efficiency (Sawchuk *et al.* 1986, Silverman *et al.* 1989). TEWL measurements showed similar values for both groups prior to, at and four days after blister induction, suggesting that epidermal wound healing is normal in NF1 patients (IV, Fig. 3A). Histological sections of the healing blisters displayed no obvious differences in the epidermis, dermis or inflammatory cell infiltration, or in the fibrin clot in NF1 patients vs. healthy controls. Furthermore, the re-epithelialization distance was measured from these sections. The analysis did not reveal marked differences between the NF1 patients and healthy controls (IV, Fig. 3B).

The blister and biopsy area of the skin was clinically re-evaluated three years afterwards to assess any long-term alterations in the process. The skin in the blister area was indistinctive from the surrounding skin in all the subjects (IV, Fig. 4). All the biopsy sites had also healed completely with normal-looking scars and without any pathological changes (IV, Fig. 4).

5.4.3 Cell proliferation and active MAPK in the skin of NF1 patients after wounding

Paraffin-embedded histological sections of four-day-old wounds were analyzed for cell proliferation and active MAPK. Proliferative cells were analyzed with the KI-67 antibody, which is known to recognize cells in other stages of the cell cycle than G0. The intact epidermis adjacent to the blister showed proliferative cells in the basal and one suprabasal cell layer in the NF1 patients and the controls (IV, Fig. 5A). In the hypertrophic zone of re-epithelializing keratinocytes, proliferative cells were seen in more superficial layers of the epidermis, but there was no difference between the two groups (IV, Fig. 5A). Dermal fibroblasts were found to be negative for KI-67 labeling (IV, Fig. 5A). Furthermore, the NF1 patients displayed an increased number of proliferative cells around the arterioles (IV, Fig. 5A).

The wounds were further analyzed for the presence of active MAPK with an antibody directed against the active p44/42 MAPK, which is a major downstream target of the Ras-signaling pathway. In the epidermis, only very few keratinocytes were positive for active p44/42 MAPK with no difference between the NF1 patients and their healthy controls (IV, Fig. 5B). Dermal fibroblasts were found to be negative for active p44/42 MAPK. Furthermore, analogous to KI-67 staining, active p44/42 MAPK labeling showed an increased number of active cells in the periarteriolar area in the NF1 patients (IV, Fig. 5B).

The KI-67 and active MAPK-positive cells were further characterized and identified. All tissue samples were labeled with antibodies directed against S100 and smooth muscle cell actin to identify Schwann cells and smooth muscle cells, which were the most likely candidate cell types. There were no S100-positive cells around the arterioles, suggesting that the positive cells were not Schwann cells (IV, Fig. 5C). Smooth muscle cell actin-positive cells were located roughly in the same area as the positive cells, and the NF1 patients displayed an increased number of smooth muscle cell actin-positive cells around the arterioles (IV, Fig. 5C). Consequently, it is likely that the positive cells were smooth muscle cells.

6 Discussion

6.1 Methodological aspects

The aim of the present work was to study the behavior and function of the NF1 gene. Human epidermis and cultured epidermal keratinocytes were selected as a model due to the versatility of this tissue and these cells. The following points were taken into consideration when human epidermis and epidermal keratinocytes were selected as a research target: 1) specific NF1 expression in the tissue (Malhotra & Ratner 1994, Hermonen *et al.* 1995), 2) differences in the skin phenotype between human disease and mouse models (Brannan *et al.* 1994, Jacks *et al.* 1994), 3) good availability of healthy and disease-affected material, and 4) possibility to study specific cellular events, such as differentiation and activation, *in vivo* and *in vitro*. The NF1 gene is considered to be a tumor suppressor. The expression levels and the subcellular distribution of a tumor suppressor may undergo major changes in cellular events, such as differentiation or cell division instead of acting as a housekeeper. For example, tumor suppressor p53 expression gradually decreases along with cellular differentiation, and the localization of the p53 protein changes through the different phases of the cell cycle (Shaulsky *et al.* 1990, Weinberg *et al.* 1995). Previous studies have shown that NF1 is specifically expressed during cellular differentiation (Gutmann *et al.* 1993, Hermonen *et al.* 1995, Li *et al.* 2001).

The present work utilized human epidermis and cultured epidermal keratinocytes as a model for studying the tumor suppressor function NF1 gene. In the normal epidermis, basal keratinocytes represent undifferentiated cells and the possibility to have two alternative fates. They may start to differentiate or they can become activated. Differentiation is marked by the formation of strong cell adhesions, the appearance of strong cytoplasmic keratin filaments, the movement of cells to the upper layers of the epidermis, and finally, apoptotic cell death. Keratinocyte activation occurs in response to epidermal injury, but the cells can also be activated in diseases such as psoriasis (Tomic-Canic *et al.* 1998, Freedberg *et al.* 2001). Cultured keratinocytes can be used as a model for the same events occurring in the epidermis. Keratinocyte differentiation following a rise of extracellular calcium is a well-characterized model for studying cellular

differentiation *in vitro* (Hennings *et al.* 1980, Eckert *et al.* 1997). The differentiation is thought to occur in a similar manner as in normal epidermis. An improved version of this method involves air-liquid interface cultivation (Kivinen *et al.* 1999), although this cultivation technique is not very practical in the kind of short cultures used in the present study. Keratinocyte activation is not equally easy to study in cultures as differentiation because undifferentiated cells are thought to have both basal and activated phenotypes with expression of both “basal” and “activated” keratins. However, there is evidence that both basal and activated phenotypes can be promoted separately by culturing cells in the presence of specific growth factors (Jiang *et al.* 1993, Jiang *et al.* 1995, Komine *et al.* 1996, Komine *et al.* 2000, Komine *et al.* 2001). Keratinocyte activation can be studied *in vivo* by experimental wound models, such as suction blisters. In the suction blister model, the epidermis is detached from the basal membrane, and the healing occurs through the movement of activated keratinocytes to re-epithelialize the wound. In conclusion, human epidermis and keratinocytes serve as an elegant model for studying tumor suppressor gene functions, such as the NF1 gene, both *in vivo* and *in vitro*.

6.2 Discussion of the results

6.2.1 NF1 tumor suppressor in epidermal differentiation

The results obtained with cultured keratinocytes demonstrated a specific change in the subcellular localization of NF1 protein during cell differentiation. Specifically, NF1 protein showed co-localization with cytokeratin 5/14 filaments shortly after the induction of keratinocyte differentiation. This co-localization was transient, and the cells later became negative for NF1 protein. The association of NF1 protein with cytokeratin filaments was temporally parallel to the re-organization of the cytoskeleton and the formation of cell-cell adhesions (Hennings *et al.* 1980, O’Keefe *et al.* 1987, Eckert *et al.* 1997). Cytokeratin-positive filaments are known to undergo specific re-organization after the calcium switch, and desmoplakin is simultaneously transported from the perinuclear area to the cell surface along cytokeratin filaments (Jones & Goldman 1985, Pasdar & Nelson 1988b, Pasdar & Nelson 1988a). Co-localization of NF1 protein and intermediate filaments reappeared after the cells had migrated to the second layer on top of the original monolayer. The cells in the second layer also showed cytoskeletal re-organization and formation of new cell-cell adhesions.

CSK buffer treatment was used to study the affinity of the adhesion between NF1 protein and intermediate filaments. NF1 protein seemed to form a high-affinity bond with cytokeratin filaments since the adhesion remained after CSK treatment. NF1 protein formed a timely and transient high-affinity bond with cytokeratin filaments after calcium-induced keratinocyte differentiation. Consequently, one can speculate on the role of the NF1 protein in the re-organization of cytokeratin filaments or in the formation of desmosomes by affecting the transport of desmoplakin to the cell surface. Another

possibility is that this phenomenon could be part of the regulation of Ras signaling by withdrawing the NF1 protein from the cell membrane. Interestingly, previous studies have shown that the p53 tumor suppressor associates with the actin cytoskeleton in response to increased calcium concentrations (Metcalfe *et al.* 1999).

The NF1 protein was also shown to be associated with cytokeratin filaments in skin *in vivo*. Immunoelectron microscopy showed NF1 protein to be in close association with cytokeratin filaments in basal keratinocytes, but not in the more superficial layers of the epidermis. Furthermore, immunogold particles representing the NF1 protein were commonly found to locate a specific distance apart. In keratinocytes, the average minimum distance between any two immunogold particles was shown to be ~48nm, which is the length of an intermediate filament monomer (Alberts 2002). Interestingly, in fibroblasts, the minimum distance between any two immunogold particles was also ~48nm, suggesting that the NF1 protein could associate with intermediate filaments even in these cells. The location of immunogold particles in the nucleus or close to the cell membrane was also evaluated due to the previous suggestions that the NF1 protein could be located in the nucleus and near the plasma membrane (Malhotra & Ratner 1994, Hsueh *et al.* 2001, Li *et al.* 2001). Only a fraction of the immunogold particles were detected in close proximity to the plasma membrane, and no immunogold was found in the nucleus. The findings suggest that the NF1 protein mainly does not locate inside the plasma membrane or in the nucleus in keratinocytes or fibroblasts. Another possibility is that the processing of the samples through fixation could destroy the antigen epitopes, resulting in poor labeling.

Keratinocytes cultured from NF1 patients were used as a model for NF1 gene deficiency and analyzed for possible alterations in cell differentiation caused by this deficiency. NF1 keratinocytes displayed altered morphology and differentiation responses compared to control keratinocytes. Specifically, NF1 keratinocytes were larger in size and displayed greater variation in cell size. The alterations were more pronounced in differentiating keratinocytes, which showed the co-localization of NF1 protein and cytokeratin-positive filaments. This emphasizes that the NF1 gene has a role in cytoskeletal re-organization and cell contact formation. The cytoskeleton and cell adhesions have a central role in regulating cell size and shape (Braga 2000, Pedersen *et al.* 2001, Runswick *et al.* 2001, Goldmann 2002). Furthermore, keratinocytes cultured from NF1 patients were immunolabeled for NF1 protein. These cells displayed a lower number of NF1 protein-positive filaments representing the association between NF1 protein and cytokeratin filaments.

Additional studies are needed to shed further light on the association between the NF1 protein and cytokeratin filaments and its link with the function of the NF1 gene and the altered morphology of keratinocytes cultured from NF1 patients. Keratinocytes homozygous (-/-) for NF1 would be a novel approach to studying these phenomena. Keratinocytes cultured from NF1 patients have a heterozygous (+/-) genotype and are less informative for the characterization of the function of the NF1 gene. Homozygous keratinocytes for NF1 can be generated by a conditional knockout mouse, in which the two copies of the NF1 gene can be removed in cultured cells or in specific tissues. Another possibility to generate NF1 gene-deficient cells would be the RNA interference (RNAi) technology, which has recently been successfully used in gene expression silencing in cell cultures. RNAi uses double-stranded RNAs 21-25 nucleic acids long,

which silence a specific gene expression with an identical nucleic acid sequence. Double-stranded RNAs can be generated by chemical synthesis or by DNA vector-based strategy (Hannon 2002). The DNA vector-based strategy allows gene silencing over extended periods of time and would be more suitable for the NF1 gene due to the long half-life of the NF1 protein.

6.2.2 NF1 tumor suppressor in psoriasis in vivo and in vitro

Psoriatic epidermis is marked by hyperproliferation of keratinocytes and their abnormal maturation. Inflammation and inflammatory cytokines are thought to have an important role in the development of the psoriatic phenotype. However, it is still under debate whether psoriasis is a primary disorder of keratinocytes or whether the changes in keratinocytes are caused by the activation of the immune system. NF1 protein has previously been shown to be downregulated in psoriatic lesions (Peltonen *et al.* 1995). Furthermore, elevated levels of active Ras have been demonstrated in psoriatic lesions (Lin *et al.* 1999), and animal models for psoriasis display increased MAPK activity (Haase *et al.* 2001).

The results of the present study displayed downregulation of both the NF1 mRNA and protein in psoriatic lesions compared to apparently healthy psoriatic epidermis. The results showed that in the healthy psoriatic epidermis, NF1 protein was expressed in the whole epidermis and the most intense signal was seen basally. This finding differed slightly from previous studies made with frozen sections (Hermonen *et al.* 1995, Peltonen *et al.* 1995, Koivunen *et al.* 2002), which have displayed NF1 protein expression to be more restricted to the basal layer of the epidermis. This might be due to the differences in the epitope availability caused by different fixation protocol or by the differences in fluorescence or color reactions based detection systems. In the psoriatic lesion, the NF1 protein expression decreased compared to the healthy looking epidermis. Interestingly, NF1 mRNA expression in healthy psoriatic epidermis displayed different localization compared to the NF1 protein expression. The most intense signal for the NF1 mRNA was seen suprabasally compared to the NF1 protein expression which was more restricted to the basal layer. This could be caused by post-translational regulation of the NF1 protein half-life which is known to occur (Kaufmann *et al.* 1999a, Cichowski *et al.* 2003). Furthermore, NF1 mRNA and protein expressions are known not to be upregulated together (Kaufmann *et al.* 1999a). In the psoriatic lesions, NF1 mRNA expression decreased and no clear difference in the expression was evident between the different layers of the epidermis.

In vitro, keratinocytes cultured from psoriatic patients revealed decreased levels of NF1 mRNA and protein in both lesional and non-lesional cells. It was also noted that cultured psoriatic keratinocytes had more pronounced downregulation of NF1 mRNA compared to the NF1 protein. The downregulation of NF1 gene expression suggests that cultured psoriatic keratinocytes are able to retain their altered NF1 expression after removal of the cells from the presence of lymphocytes or fibroblasts. This favors the assumption of the primary role of keratinocytes in the pathogenesis of psoriasis. It should

be emphasized that decreased NF1 gene expression could be a subclinical condition that occurs prior to the clinical manifestations. Previous studies have suggested that cultured psoriatic keratinocytes can maintain their altered phenotype and function (Baden *et al.* 1981, Baker *et al.* 1988, Szabo *et al.* 2002). In addition to the quantitative studies on NF1 gene expression in cultured psoriatic keratinocytes, subcellular localization analysis was done by using immunostaining and *in situ* hybridization. Immunostaining for NF1 protein in differentiating keratinocytes displayed less intense labeling on the intermediate filament network. Non-radioactive *in situ* hybridization methods with digoxigenin-labeled probes have made it possible to identify subcellular distribution of certain mRNAs (Yla-Outinen *et al.* 2002), and this method was used in the current study to identify the localization of NF1 mRNA. Localization of NF1 mRNA in differentiating cells displayed less marked re-distribution of NF1 mRNA in psoriatic cells compared to controls.

One can speculate about the role of the NF1 gene and the mechanism and effect of its downregulation in psoriasis. Increased amounts of active Ras could lead to increased consumption of NF1 mRNA/protein. Furthermore, it has been shown that NF1 mRNA is redistributed towards the cell-cell adhesion area via actin filaments (Yla-Outinen *et al.* 2002). Previous studies have demonstrated alterations in the cytoskeleton and cell adhesions in psoriasis (Jahn *et al.* 1988, Pellegrini *et al.* 1992, De Luca *et al.* 1994, Cavicchini *et al.* 1996, Magaouda *et al.* 1997). Alterations in cytoskeleton and cell adhesions could lead to improper transport and faster breakdown of NF1 mRNA, resulting in decreased levels of NF1 protein. NF1 deficiency was shown in the first part of the current study to result in an altered phenotype of keratinocytes. Whether NF1 gene deficiency has a role in the formation of psoriatic phenotype or whether it is just a consequence of the alterations in psoriatic keratinocytes needs to be further studied.

6.2.3 NF1 and epidermal wound healing

It has been suggested that trauma could play a part in the formation of the skin manifestations associated with neurofibromatosis, such as neurofibromas and CALMs (Friedman & Riccardi 1999, Karvonen *et al.* 2000). Furthermore, the NF1 gene is expressed in keratinocytes and fibroblasts, which are the most important elements in the process of wound healing (Malhotra & Ratner 1994, Hermonen *et al.* 1995, Yla-Outinen *et al.* 1998). One can hypothesize that the NF1 gene may have a role in wound healing, and that abnormal wound healing could result in skin manifestations associated with the disease. Previous studies have addressed the connection between wound healing and the NF1 gene. Specifically, NF1 gene expression is increased in fibroblasts of scars and cultured fibroblasts exposed to various growth factors operative during wound healing (Yla-Outinen *et al.* 1998). Mice heterozygous for the NF1 gene have shown defects in wound healing after a deep dermal wound (Atit *et al.* 1999).

The current study investigated, for the first time, experimental wound healing and NF1. Suction blisters were used to generate epidermal injuries, and their healing was followed. In normal epidermal wound healing, NF1 gene expression increased both in

epidermal keratinocytes and in dermal fibroblasts in response to wounding, suggesting that the gene may have a role in the process. Consequently, wound healing efficiency was studied in NF1 patients. All the patients were first evaluated retrospectively for any abnormalities in wound healing. Clinical evaluation of all the scars on NF1 patients showed that two patients had neurofibromas rising directly from scars. This suggests that neurofibromas or CALMs mainly do not co-localize with scars. The suction blister method was used to study the efficiency of epidermal wound healing. In the early stages of wound healing, no significant differences between NF1 patients and healthy controls were found as determined by clinical evaluation, TEWL measurements or histological analysis. Potential long-term effects of wounding were studied clinically by re-evaluating the blister and biopsy areas three years after wounding. There were no long-term effects of the wounding.

In conclusion, epidermal wound healing and the healing of biopsy scars seem to be equally effective in NF1 patients as in controls. Furthermore, trauma caused by epidermal or deep skin injury does not appear to play any major role in the formation of skin manifestations associated with NF1.

6.2.4 NF1 gene and the Ras-MAPK pathway

The best characterized function of the NF1 gene is its function to act as a Ras-GAP and to catalyze the hydrolysis of active Ras-GTP into inactive Ras-GDP (Ballester *et al.* 1990, Martin *et al.* 1990, Xu *et al.* 1990a, Bollag & McCormick 1991, Basu *et al.* 1992, Bollag *et al.* 1996). The most important downstream target of Ras-GTP signaling is the Raf-MEK-ERK cascade, which leads to increased cell growth and proliferation in most tissues (Kolch 2000). It is still under debate whether the most important function of NF1 gene is to regulate Ras signaling, or whether it has some other functions. Previous studies have shown that NF1 deficiency/functional insufficiency may lead to increased Ras-MAPK activities in various tissues (Bollag *et al.* 1996, Sherman *et al.* 2000, Gitler *et al.* 2003). However, NF1 deficiency and Ras-GTP levels do not go together in all tissues (Johnson *et al.* 1994, Boddlich *et al.* 1995, Griesser *et al.* 1995, Sherman *et al.* 2000). Furthermore, NF1 gene impairment may lead to alterations in cells without changes in Ras-GTP levels (Jimbow *et al.* 1973, Ishida & Jimbow 1987, Griesser *et al.* 1995, Atit *et al.* 1999, Sherman *et al.* 2000), and the NF1 gene may carry out its function, such as the regulation of cell growth, independently of Ras signaling (Johnson *et al.* 1994, Li & White 1996, Guo *et al.* 1997, The *et al.* 1997).

The present study investigated the behavior and function of the NF1 gene in epidermal keratinocytes. The NF1 protein is known to function in epidermal keratinocytes because mice heterozygous for the NF1 gene display increased susceptibility to chemical carcinogens. However, it is not clear whether this susceptibility is caused by impaired GAP activity, although Ras signaling and NF1 gene impairment seemed to have a synergistic effect on it (Atit *et al.* 2000). The results of the current study favor the theory that the NF1 gene has functions other than acting as a GAP protein in keratinocytes. The NF1 protein specifically adhered to the intermediate filament network in differentiating

cells rather than localized close to the plasma membrane both *in vitro* and *in vivo*. Active Ras is attached to the plasma membrane through a farnesyl residue, and the prerequisite for the GAP function of the NF1 protein is a close spatial relationship with the plasma membrane. Furthermore, NF1 patients did not display an increased number of cells with active MAPK in normal, hyperproliferative or migrative epidermis. In psoriasis, increased Ras activity of keratinocytes has previously been reported (Lin *et al.* 1999). Further studies are needed to investigate the link between decreased expression of the NF1 gene in psoriatic keratinocytes and elevated Ras-GTP levels.

Dermal tissues were also studied for the activity of the Ras-MAPK pathway. In fibroblasts, there was no difference in the number of cells active for MAPK between NF1 patients and healthy controls. This is in agreement with the previous results showing that fibroblasts cultured from NF1 patients have normal Ras-GTP levels (Sherman *et al.* 2000). Unexpectedly, an increased number of MAPK-active cells were seen in the periarterial area of NF1 patients. This correlated with the increased cell proliferation detected by KI-67 immunostaining. The periarteriolar area is located just underneath the endothelium and is mainly composed of smooth muscle cells or pericytes. It is likely that the active cells were smooth muscle cells because smooth muscle cell actin positive cells were located roughly on the same area as the positive cells, and NF1 patients displayed increased amount of smooth muscle cells around the arterioles. Previous studies have suggested that vasculopathy related to neurofibromatosis is caused by proliferative smooth muscle cells in the vessel media. Increased smooth muscle cell proliferation may result from failure of the endothelium to regulate smooth muscle cells or abnormal smooth muscle cell responses to endothelial signals (Greene *et al.* 1974, Lehrnbecher *et al.* 1994, Hamilton & Friedman 2000, Riccardi 2000). The NF1 gene has previously been identified in the endothelium and the smooth muscle layer of blood vessels (Norton *et al.* 1995). Furthermore, a recent study with conditional NF1 knockout mice has shown the importance of the NF1 gene as a downregulator of Ras-MAPK signaling in the endothelium (Gitler *et al.* 2003). It is interesting to note that HMG-CoA inhibitors (statins), which are used widely to lower high cholesterol and to treat atherosclerosis, do have other functions apart from inhibiting cholesterol synthesis. Furthermore, not all of the beneficial effects of statins can be explained by the cholesterol-lowering action. Statins also inhibit the synthesis of the farnesyl moiety, which is essential for the Ras protein function. It has been suggested that some of the beneficial effects of statins could be caused by Ras inhibition (Libby & Aikawa 2002). One can speculate about the role of the Ras-MAPK pathway in atherosclerosis and neurofibromatosis associated vasculopathy, which both share similar features, such as distribution of the lesions and increased smooth muscle cell proliferation of the vessel wall.

In conclusion, the present study supported the theory that the NF1 gene functions as Ras-GAP, but it also has other functions. The results suggest that NF1 deficiency results in increased Ras-MAPK signaling only in some tissues, such as smooth muscle cells, but not in keratinocytes or fibroblasts.

7 Conclusions

The present study investigated the behavior and role of the NF1 gene during keratinocyte differentiation and wound healing. The following conclusions were made on the basis of the current study:

- The NF1 gene is likely to have a function in the re-organization of the cytoskeleton and/or cell adhesion formation because NF1 protein was shown to be associated with the intermediate filament network shortly and transiently during keratinocyte differentiation *in vitro*, and NF1 gene-deficient keratinocytes showed abnormalities in these processes.
- The NF1 protein associates with intermediate filaments not only *in vitro* but also *in vivo* in human skin.
- The NF1 gene may have a role in the formation of the psoriatic phenotype, since NF1 gene expression is downregulated in psoriatic epidermis and in cultured psoriatic keratinocytes.
- Upregulation of the NF1 gene by the resident cells is part of normal tissue repair. However, the processes of wound epithelialization and healing show no apparent alterations between NF1 patients and controls.
- The NF1 gene seems to act as a Ras-GAP in only some tissues, such as smooth muscle cells.

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