

Research article

## Functional expression of NFI tumor suppressor protein: association with keratin intermediate filaments during the early development of human epidermis

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### Abstract

**Background:** NFI refers to type I neurofibromatosis syndrome, which has been linked with mutations of the large *NFI* gene. NFI tumor suppressor protein, neurofibromin, has been shown to regulate ras: the NFI protein contains a GTPase activating protein (GAP) related domain which functions as p21rasGAP. Our studies have previously demonstrated that the NFI protein forms a high affinity association with cytokeratin 14 during the formation of desmosomes and hemidesmosomes in cultured keratinocytes.

**Methods:** The expression of NFI protein was studied in developing human epidermis using western transfer analysis, indirect immunofluorescence, confocal laser scanning microscopy, immunoelectron microscopy, and *in situ* hybridization.

**Results:** The expression of NFI protein was noted to be highly elevated in the periderm at 8 weeks estimated gestational age (EGA) and in the basal cells at 8–14 weeks EGA. During this period, NFI protein was associated with cytokeratin filaments terminating to desmosomes and hemidesmosomes. NFI protein did not display colocalization with  $\alpha$ -tubulin or actin of the cytoskeleton, or with adherens junction proteins.

**Conclusions:** These results depict an early fetal period when the NFI tumor suppressor is abundantly expressed in epidermis and associated with cytokeratin filaments. This period is characterized by the initiation of differentiation of the basal cells, maturation of the basement membrane zone as well as accentuated formation of selected cellular junctions. NFI tumor suppressor may function in the regulation of epidermal histogenesis via controlling the organization of the keratin cytoskeleton during the assembly of desmosomes and hemidesmosomes.

## Background

The epidermis is derived from the embryonic ectoderm and is first detectable at 4 weeks estimated gestational age (EGA). At this time, the epidermis is composed of two distinct layers of cells. The surface layer, or the periderm, forms the outer limits of the embryo facing the amniotic fluid. Underneath the periderm lies the basal cell layer of the primitive epidermis. The maturation of the epidermis includes development of the intermediate cell layers simultaneously with the maturation of the basement membrane zone between 9 and 16 weeks EGA, shedding of the periderm layer and final maturation into stratified squamous epithelium by the beginning of the third trimester [1–3]. The development of cellular junctions takes place concomitantly with the morphological maturation [2,4–6]. These events include the formation of desmosomes, adherens junctions, tight junctions and hemidesmosomes. The formation of cellular junctions is important for the proper development of epidermis, since e.g. conditional ablation of  $\alpha$ -catenin, a cytoplasmic plaque protein of adherens junctions, in skin results in dramatic alterations in the morphogenesis and differentiation of epidermis [7]. The formation of hemidesmosomes and subsequent attachment of the keratinocytes to the underlying basement membrane is essential for the polarization of the basal cells of epidermis. The protein plaques of contact sites are connected to a specific set of cytoskeletal filaments. Specifically, the junctional proteins of desmosomes and hemidesmosomes are connected to intermediate filaments while adherens and tight junctions are linked with actin microfilaments.

NF1 refers to type 1 neurofibromatosis syndrome, which has been linked with mutations of the large *NF1* gene [8–10]. The hallmarks of NF1 include café au lait pigment spots of the epidermis, skin freckling, cutaneous neurofibromas, and Lisch nodules of the iris [9,11]. Other findings often associated with NF1 include learning disabilities, varying osseous dysplasias, optic pathway gliomas and predisposition to malignancies. Molecular cloning of the entire coding sequence of the *NF1* gene and subsequent analysis of the corresponding peptide sequence has led to recognition of NF1 protein (neurofibromin) as a regulator of ras, or p21rasGAP [8,10,12,13]. NF1 protein regulates the levels of biologically active ras-GTP. Ras activity is associated with the regulation of cell growth and differentiation, including control of cytoskeletal organization and formation of cell-cell junctions [7,14]. The NF1 protein has been referred to as a tumor suppressor as cells of malignant peripheral nerve sheath tumors of neurofibromatosis patients may display loss of heterozygosity of the *NF1* gene [15]. In addition, somatic mutations of *NF1* gene have also been found in colon adenocarcinoma, myelodysplastic syndrome, and anaplastic astrocytoma tissues of otherwise healthy persons [16,17].

Furthermore, the levels of NF1 protein and/or mRNA have been reported to be altered in certain proliferative diseases, such as transitional cell carcinoma, basal cell carcinoma, astrocytoma, pheochromocytoma, meningioma, and psoriasis [18–22].

Several studies implicate that NF1 protein has a crucial role during the development. Mice carrying a homozygous null mutation at the *NF1* locus die *in utero* apparently due to the severe malformations in heart [23]. Unlike in adult tissues, NF1 protein is ubiquitously expressed during the development. The expression level and pattern of NF1 protein have been noted to change markedly and rapidly during the development in mice and men [24,25]. These rapid changes have been considered to be related to some of the major morphological changes occurring in tissues e.g. heart [26,27].

In order to reveal the functions of NF1 protein at the molecular level, interactions of the NF1 protein with cytoskeletal and plasma membrane components have been studied. Recent keratinocyte culture studies have demonstrated that the NF1 tumor suppressor factor forms a high affinity association with cytokeratin 14 during the short period when the formation of desmosomes and hemidesmosomes takes place [28]. NF1 protein becomes rapidly associated with intermediate filament cytoskeleton (cytokeratin 14), desmoplakin, and  $\beta$ 4 integrin, when cultured human keratinocytes are induced to differentiate and form cell-cell junctions by increasing  $Ca^{2+}$  concentration of the cell culture medium. On the other hand, Li *et al.* [29] have demonstrated differential localization of the *NF1* gene product with the F-actin or microtubule cytoskeleton during the differentiation of telencephalic neurons. Furthermore, Hsueh *et al.* [30] have demonstrated that NF1 tumor suppressor can form a bipartite interaction with transmembrane proteoglycans syndecans 1–4.

The purpose of the present study was to investigate the interactions of NF1 protein with cytoskeleton during the morphogenesis of human epidermis. Developing epidermis provided a unique opportunity to visualize cytoskeletal and junctional proteins together with NF1 protein *in vivo*. The results indicated interaction of NF1 protein with cytokeratin 14 during the polarization of basal cells and the maturation of intercellular junctions.

## Methods

### Tissue samples and keratinocyte cell cultures

Skin samples from 15 normal fetuses aged 8, 10, 10, 11, 12, 12, 13, 13, 14, 14, 15, 16, 17, 18 and 21 gestational weeks were obtained from the Department of Obstetrics and Gynecology, University Hospital of Turku, Finland. A sample of neonatal skin was obtained from the Department of Pathology, University of Turku, Finland. Adult

skin samples were obtained from plastic surgical operations carried out for cosmetic reasons from healthy persons at the University Hospital of Turku, Finland. All the tissue samples were obtained with the appropriate approval of the Joint Ethical Committee of the University Hospital of Turku and the University of Turku, Finland. The tissue samples were used for the following experiments. Primary cultures of normal human keratinocytes were established from adult skin samples and cultured as described earlier by Pummi *et al.* [6].

#### Primary antibodies

A commercial polyclonal affinity-purified rabbit antibody NF1GRP(D) raised against synthetic peptide corresponding to amino acids 2798 – 2818 of the predicted *NF1* gene product was used for Western blotting, indirect immunofluorescence, immunoelectron microscopy, avidin-biotin immunolabeling, and coimmunoprecipitation (sc-67, Santa Cruz Biotechnology, Santa Cruz, CA) [8]. The dilution used for indirect immunofluorescence varied from 1:10 in neonatal and adult skin to 1:500 in fetal samples. Other antibodies specific for NF1 protein were the affinity-purified polyclonal NF1ab67 recognizing a peptide sequence encoded by exon 23a and the polyclonal antiserum NF1as159 recognizing a peptide sequence at the carboxyterminal end of NF1 protein. These antibodies have been characterized earlier by Hermonen *et al.* [20] and Koivunen *et al.* [28]. Other primary antibodies used in indirect immunofluorescence were mouse monoclonal antibodies to human E-cadherin (13-1700; Zymed Laboratories Inc., San Francisco, CA); actin (1378 996) and desmoplakin I and II (881 147) both from Boehringer Mannheim Biochemica, Mannheim, Germany;  $\alpha$ -actinin (A-5044), cytokeratin 14 (C-8791), and  $\alpha$ -tubulin (T-9026) from Sigma Chemical Company, St. Louis, MO;  $\beta$ 4 integrin (12088-019, Gibco BRL Life Technologies Inc.); and syndecan-1 (CD138, MCA681H, Serotec, Kidlington, UK).

#### Western transfer analysis

Cultured keratinocytes, adult epidermal and fetal skin samples were homogenized in RIPA buffer [1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulphate (SDS) in phosphate buffered saline (PBS)] supplemented with protease inhibitors (Complete, Mini, EDTA-free Protease Inhibitors Cocktail Tablets, Boehringer Mannheim GmbH). Protein concentrations of soluble fraction were detected with DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). 40  $\mu$ g of cultured keratinocyte and fetal preparations and 80  $\mu$ g of adult epidermal preparation were loaded on 6% SDS polyacrylamide gel. After electrophoresis, proteins were transferred to Immobilon-P filter (Millipore Corporation, Bedford, MA) and immunolabeled with NF1GRP(D) antibody at the dilution of 1:1000. Peroxidase-linked donkey-anti-rabbit (NA

934) (Amersham International plc, Little Chalfont, Buckinghamshire, England) was used as secondary antibody at the dilution of 1:10 000. Proteins were detected with ECL (Amersham Life Sciences, Little Chalfont, England) and the filter was exposed to autoradiographic film (Eastman Kodak, New York, NY).

#### Co-immunoprecipitation

Cultured keratinocytes were extracted with CB-buffer (Coimmunoprecipitation buffer: 20 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Igepal CA-630, and Complete, Mini, EDTA-free Protease Inhibitors Cocktail Tablets, Boehringer Mannheim) and the protein concentrations of soluble fraction were detected with DC Protein Assay (Bio-Rad Laboratories). 650  $\mu$ g of the cell lysate was used for the coimmunoprecipitation. The lysate was first precleared with 50  $\mu$ l of G-sepharose (Boehringer Mannheim) for 3 h and centrifuged. 10  $\mu$ l of antibody for syndecan-1 (0.1 mg/ml) was added to the supernatant and incubated for 1 h. 50  $\mu$ l of G-sepharose was added and further incubated for 12 h. The lysate was centrifuged and the pellet was washed with CB-buffer for four times. Precipitated proteins were separated from the sepharose by adding Laemmli buffer and boiling the sample for 4 min. After centrifugation, the supernatant was loaded on SDS polyacrylamide gel, electrophoresed, transferred to Immobilon-P filter, immunolabeled with NF1GRP(D), and detected with ECL as described above with Western blotting.

#### Indirect immunofluorescence and confocal laser scanning microscopy

Indirect immunofluorescence labeling of frozen fetal, neonatal, and adult skin sections and confocal laser scanning microscopy of these labelings were performed as described earlier by Pummi *et al.* [6]. Frozen sections of fetal, neonatal and adult skin were cut on silanated glass slides. Samples were fixed with 100% methyl alcohol and preincubated in PBS supplemented with 1% bovine serum albumin (BSA). The primary antibodies were diluted in 1% BSA-PBS, and incubated on the samples at 4°C for 20 h. After several washes in PBS, the slides were incubated with secondary antibodies at 20°C for 1 h. The samples were washed in PBS and mounted with Glycergel (Dako, Glostrup, Denmark). The secondary antibody used for indirect immunofluorescence was tetramethylrhodamine isothiocyanate (TRITC) conjugated swine anti-rabbit IgG (R0156, Dako A/S). In double labelings, TRITC-conjugated swine anti-rabbit IgG was mixed with fluorescein isothiocyanate (FITC)-conjugated F(ab')<sub>2</sub> fragment of goat anti-mouse immunoglobulins (F0479, Dako A/S) or Alexa Fluor™ 488 conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Alternatively, FITC-conjugated swine anti-rabbit IgG (F0205, Dako A/S) was mixed with TRITC-conjugated goat anti-mouse IgG (H+L) (115-025-

146, Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Confocal laser scanning microscopy was carried out using Leica TCS SP Spectral confocal laser scanning microscope equipped with an air-cooled Argon-Krypton ion-laser system (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) and Leica TCS NT software (Version 1.6.551 Heidelberg, Germany).

#### Immunoelectron microscopy

The immunoelectron microscopy was performed using a post-embedding method as described earlier by Pummi *et al.* [6]. In short, 1 × 1 mm pieces of fetal skin were fixed with 4% paraformaldehyde and embedded in L.R. White resin (London Resin Company Limited, Berkshire, England). NF1GRP(D) (dilution 1:100) was used as primary antibody and goat-anti-rabbit IgG coupled to 12 nm gold particles (Jackson ImmunoResearch Laboratories Inc.) was used as secondary antibody at the dilution of 1:30. The sections were examined and photographed using JEOL 1200SX electron microscope.

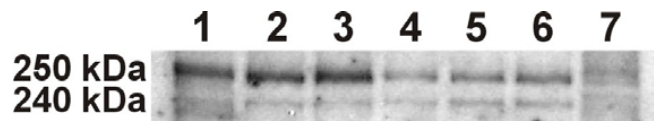
#### Avidin-Biotin immunolabeling

Formalin fixed and paraffin-embedded specimens were immunolabeled with avidin-biotin method using Histo-stain-Plus Kit (Zymed Laboratories Inc.) according to the protocol supplied with the kit by the manufacturer. NF1GRP(D) was used as the primary antibody at the dilution of 1:200. 3,3'-diaminobenzidine tetrahydrochloride (DAB-Plus Kit, Zymed Laboratories Inc.) was used to visualize the antibody localization. Slides were counterstained with Mayer's haematoxylin (Oy Reagent LTD, Kuopio, Finland).

#### In situ hybridization

*In situ* hybridization was performed as described previously in detail by Ylä-Outinen *et al.* [31]. In short, a 650 bp mouse NF1 cDNA fragment corresponding to bases 230–880 of human NF1 cDNA (acc:M89914) was used as a template for the synthesis of digoxigenin labeled sense and antisense cRNA probes. Paraffin-embedded fetal skin sections were deparaffinized, pretreated with 0.2 M HCl, washed in 2 mg/ml glycyl-PBS, and acetylated in 0.1 M triethanolamine (pH 8.0) 0.25% acetic anhydride solution. Slides were heated to 90°C for 5 min, cooled over ice, and prehybridized in solution containing 50% formamide, 0.6 M NaCl, 2 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1.0 mg/ml BSA, 0.02% PVP, 0.02% Ficoll, 10 mM DTT, 0.2 mg/ml ssDNA, and 10% dextran sulphate. Hybridization was performed at 45°C overnight in a mixture containing prehybridization solution and 300 ng of cRNA probe for each sample. All samples were prepared as duplicate and hybridized separately with sense and antisense probes.

After hybridization, the samples were washed in 4 × SSC and unbound RNA probe was removed with RNase treat-



**Figure 1**

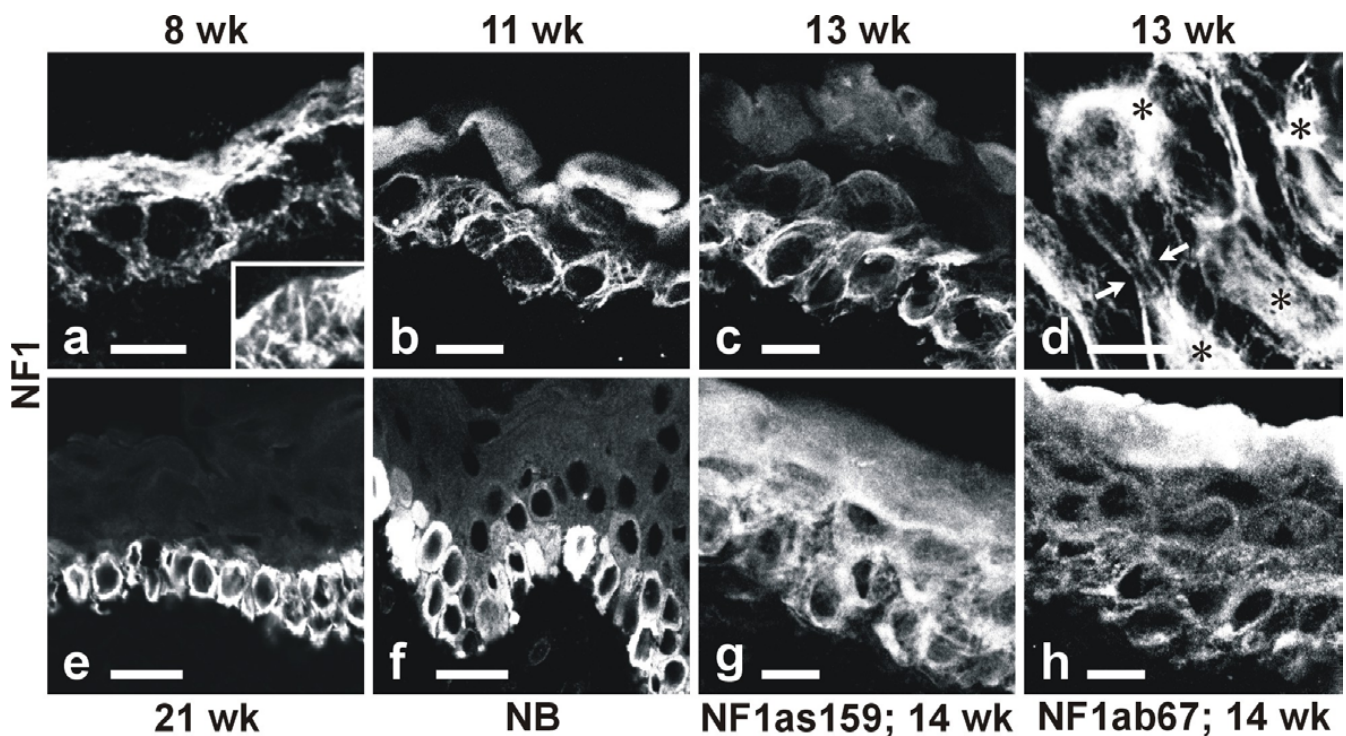
Western transfer analysis of fetal skin and adult epidermis with NF1GRP(D) antibody. Lane 1: cultured human keratinocytes (40 µg). Lanes 2–3: 12-week-old fetal skin (40 µg). Lanes 4–5: 13-week-old fetal skin (40 µg). Lane 6: 14-week-old fetal skin (40 µg). Lane 7: adult epidermis (80 µg). The analysis reveal typical 240 and 250 kDa bands representative of NF1 protein.

ment. The samples were washed to the final stringency of 0.1 × SSC, 1 mM EDTA, and 1 mM DTT at 45°C. DIG-labeled cRNA probe was detected with sheep anti-digoxigenin antibody (dilution 1:100) coupled to alkaline phosphatase (Roche Diagnostics), which was used with color forming substrate solution according to the protocol provided by manufacturer.

#### Results

The expression of NF1 protein in epidermis and in cultured keratinocytes was first assessed by western transfer analysis. The results revealed two bands of 240 kDa and 250 kDa representative of NF1 protein in all samples, although the band of 240 kDa was less prominent in cultured keratinocytes (Fig 1, lane 1) and in adult epidermis (Fig 1, lane 7) compared to the fetal skin samples (Fig 1, lanes 2–6). 240 and 250 kDa bands positive for NF1 protein in western transfer analysis have earlier been described in other tissues, also [8,27,32]. The expression level of the NF1 protein was higher in cultured keratinocytes and fetal skin samples compared to the adult epidermis.

Indirect immunofluorescence labeling and confocal laser scanning microscopy (CLSM) was effective in visualizing NF1 protein in different layers of the epidermis, and in demonstrating variable subcellular distribution patterns of the protein within individual cells. At 8 weeks EGA, epidermis consisted of two cell layers, the basal and the periderm layers. Both cell layers were intensely labeled with the C terminus-specific antibody NF1GRP(D) which revealed a fibrillar labeling pattern (Fig 2a). The periderm cells displayed a more intense immunosignal compared to the basal cells at this two-layer stage of epidermal development. A third, the intermediate, cell layer was detectable between the basal and the periderm cell layers at 11 weeks EGA (Fig 2b). All three cell layers were positive for NF1 protein. The labeling pattern remained fibrillar in the basal cells, while labeling of periderm cells had changed into a diffuse distribution within the cytoplasm. Cells of



**Figure 2**

Indirect immunofluorescence labeling and CLSM analysis of developing human epidermis for NF1 protein. (a-f) Labeling with NF1GRP(D) antibody (dilution of 1:500 in fetal samples and dilution of 1:10 in neonatal (NB) sample). (a) At 8 weeks EGA, developing epidermis consists of basal and periderm cell layers. Both cell layers are intensely labeled and reveal a fibrillar labeling pattern. *Insert*: higher magnification of cytoplasmic compartment of a periderm cell. (b) At 11 weeks EGA, an intermediate cell layer is detectable between the basal and the periderm cell layers. The basal cells show a fibrillar labeling pattern, while labeling of periderm cells is diffuse. The intermediate layer shows less intense immunoreaction for NF1 protein. (c) At 13 weeks EGA, the suprabasal epidermis consists of multiple cell layers. The basal cells show fibrillar labeling pattern for NF1 protein while the periderm cells are characterized with a diffuse and faint immunoreaction. (d) A transverse section of basal cells at 13 weeks EGA. Cells have a perinuclear web of fibrils positive for NF1 protein (\*). Some fibrils are directing towards desmosomes (arrows) (see also figure 5B, a and c). (e,f) In 21-week-old and in neonatal (NB) epidermis the basal cells show diffuse cytoplasmic labeling for NF1 protein. (g) NF1 specific antiserum NF1as159 labels all epidermal cell layers at 14 weeks EGA. The labeling is fibrillar in the basal cells. (h) Also NF1 specific antibody NF1ab67 labels all cell layers, but in a more punctuate manner. Scale bars 10  $\mu$ m in a-c, g, h; 5  $\mu$ m in d; 20  $\mu$ m in e, f. *Insert* in (a) 30  $\mu$ m.

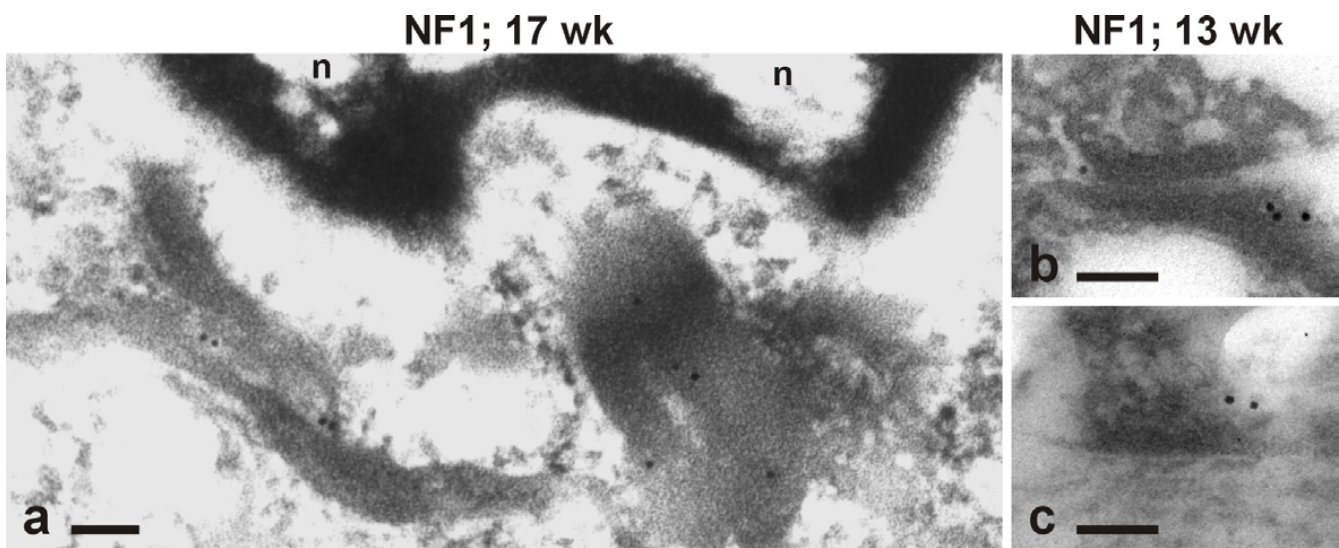
the intermediate layer showed less intense immunoreaction for NF1 protein.

At 13 weeks EGA, the basal cells retained the fibrillar labeling pattern with NF1GRP(D) antibody (Fig 2c). Analyses of the transverse sections of the basal cells were particularly informative. Specifically, the results demonstrated that NF1 antibody labeled a web of fibrils extending from a perinuclear location towards the cell periphery and desmosomes (Fig 2d; see also Fig 5B:c). At 13 weeks EGA, the periderm cells revealed a faint and a more diffuse labeling pattern for the NF1 compared to the basal cells (Fig 2c). Samples of 14 weeks EGA were also labeled with two other NF1 specific antibodies (NF1as159 and NF1ab67) (Fig 2g,2h). Both antibodies labeled all three types of epidermal cell layers. The NF1as159 antibody showed a fi-

brillar pattern in the basal cell cytoplasm (Fig 2g). NF1 isoform type II specific antibody ab67 labeled all cell layers in a more punctuate manner (Fig 2h). Between 13 and 15 weeks EGA, the expression of NF1 protein faded out in periderm cells (data not shown).

Four distinct layers of epidermis, the basal, the spinous, the granular, and the cornified cell layers were detectable at 21 weeks EGA. The periderm cells had disappeared by that time. By 21 weeks EGA and in the neonatal human epidermis, the fibrillar labeling for the NF1 protein in basal cells was no longer detectable. The labeling pattern had changed from fibrillar into more diffuse cytoplasmic staining (Fig 2e,2f). Even the highest magnifications failed to reveal the fibrillar labeling (not shown).





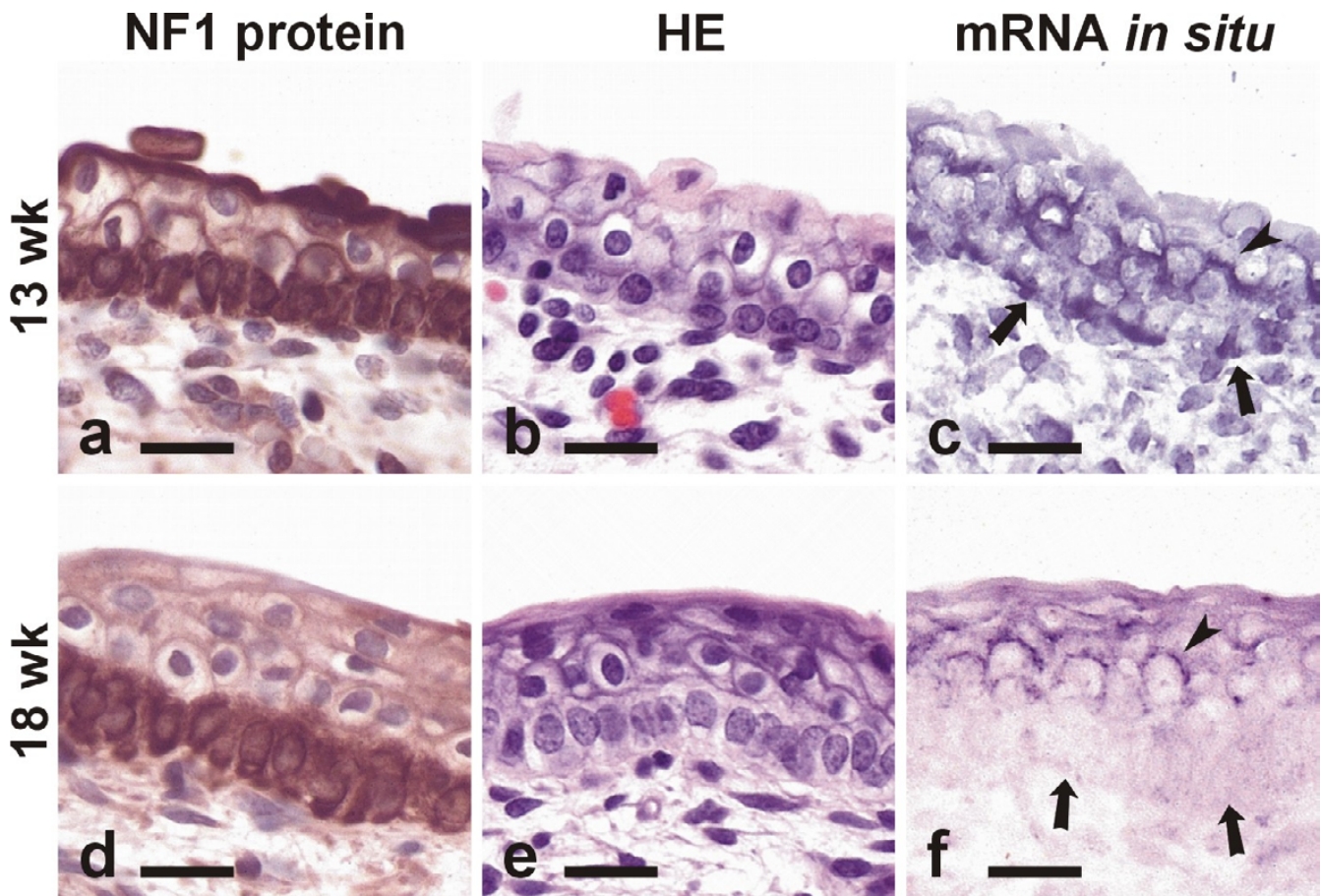
**Figure 3**  
Immunoelectron microscopy of the developing human epidermis with NF1GRP(D) antibody. Gold particles representative of NF1 protein are located on the cytokeratin filaments (a) circumscribing the nucleus (n), (b) terminating to a desmosome, and (c) to a hemidesmosome. Scale bars 100 nm.

In order to localize NF1 protein at the ultrastructural level, post-embedding immunoelectron microscopy of developing skin was performed using NF1GRP(D) antibody. The results revealed that in basal keratinocytes, the NF1 protein was located on the bundles of cytokeratin filaments, which formed a tight web around the nucleus (Fig 3a). Gold particles representative of NF1 protein were detected also on cytokeratin filaments terminating to desmosomes (Fig 3b) and hemidesmosomes (Fig 3c).

In further analyses, the expression of *NF1* gene was studied by *in situ* hybridization and avidin-biotin immunolabeling of paraffin embedded samples. Avidin-biotin immunolabeling revealed a pattern corresponding to that detected by indirect immunofluorescence. At 13 weeks EGA, the basal and the periderm cell layers were intensely labeled with the NF1GRP(D) antibody (Fig 4a). Also the intermediate cells showed positive reaction located in the cell periphery. At 18 weeks EGA, the basal cell layer revealed the most intense labeling while the other layers were less positive (Fig 4d). *In situ* hybridization revealed NF1 mRNA in developing epidermis. At 13 weeks EGA, NF1 mRNA was noted in the basal and intermediate cell layers (Fig 4c). Subcellularly, NF1 mRNA was located at the basal aspect of basal cells and at the cell periphery of intermediate cells. At 18 weeks EGA, NF1 mRNA was detectable near the cell membranes in the suprabasal cell layers, while the basal cells expressed NF1 mRNA at a very low level (Fig 4f).

To investigate the fibrillar labeling pattern of NF1 protein in early developing epidermis in more detail, double immunolabelings with NF1 protein and cytoskeletal marker proteins were carried out. At 13 weeks EGA, cytokeratin 14 antibody labeled cytoskeletal intermediate filaments of the basal cells (Fig 5A:a,d). Some labeling was also noted in the periderm. Immunosignals for the NF1 protein and cytokeratin 14 showed marked colocalization on many locations in basal cells (Fig 5A:a,d). Actin antibody labeled the peripheral cytoplasm of all epidermal and periderm cells (Fig 5A:b,e). As the NF1 protein positive fibrils were located closer to the nuclei and actin labeling was detected more peripherally, these two components showed separate labeling (Fig 5A:b,e).  $\alpha$ -tubulin antibody labeled also all epidermal cell layers, but did not colocalize with NF1 protein (Fig 5A:c,f).

In order to evaluate the spatial relationship of NF1 positive cytokeratin fibrils with desmosomes and hemidesmosomes, double immunolabelings of NF1 protein with desmosomal and hemidesmosomal marker proteins were carried out. At 13 weeks EGA, desmoplakin, a desmosomal cytoplasmic plaque protein, was present in a typical punctuate pattern at the cell-cell junction sites in all cell layers (Fig 5B:a). However, the intercellular junction sites of basal cells showed weaker labeling for desmoplakin. Higher magnification revealed that NF1 positive cytokeratin fibrils terminated to dots of desmoplakin (Fig 5B:c). Antibody for  $\beta$ 4 integrin, a transmembrane component of hemidesmosomes, labeled the basal aspects of the basal cells as distinct dots (Fig 5B:b,d). Higher magnification re-



**Figure 4**

Avidin-biotin immunolabeling of NF1 protein and *in situ* hybridization of NF1 mRNA in developing human epidermis. (a) At 13 weeks EGA, the basal and the periderm cell layers are intensely labeled with the NF1GRP(D) antibody. Also intermediate cells show positive reaction near cell membranes. (b) Hematoxylin-eosin staining of developing epidermis at 13 weeks EGA. (c) NF1 mRNA is noted in the basal and intermediate cell layers at 13 weeks EGA. Subcellularly, NF1 mRNA is located at the cell periphery of intermediate cells (arrowhead). Note the positive NF1 mRNA signal at the basal aspect of basal cells at this stage of development. Arrows point to the basement membrane zone. (d) At 18 weeks EGA, the basal cells display the most intense immunoreaction with NF1GRP(D) antibody. (e) Hematoxylin-eosin staining of 18-week-old epidermis. (f) At 18 weeks EGA, NF1 mRNA is detectable only in the suprabasal cell layer by *in situ* hybridization. The positive NF1 mRNA signal is located near cell membranes (arrowhead). Arrows point to the basement membrane zone. Scale bars 20  $\mu$ m.

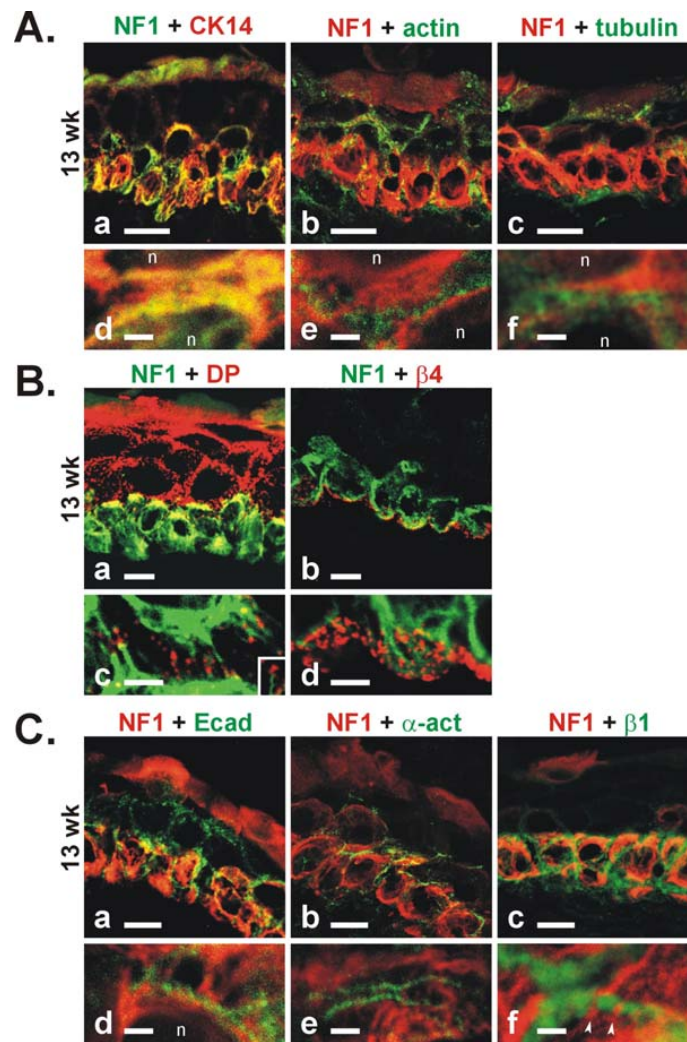
vealed that NF1 positive cyokeratin fibrils were directed towards the  $\beta$ 4 integrin dots (Fig 5B:d). By 16 weeks EGA, labeling of  $\beta$ 4 integrin was intensified and changed into a more linear labeling pattern (not shown).

The localization of NF1 protein and selected adherens junction proteins were investigated using double immunolabelings in developing epidermis at 13 weeks EGA. E-cadherin antibody revealed the cell-cell junction sites of the basal and intermediate cells (Fig 5C:a), but high magnification showed that the immunosignals for E-cadherin and NF1 protein were not colocalized (Fig 5C:d). Also,  $\alpha$ -actinin and NF1 protein were mutually exclusive (Fig 5C:b,e).  $\beta$ 1 integrin labeled the cell membranes of the ba-

sal cells, including the basal aspect of basal cells (Fig 5C:c). Higher magnification showed that NF1 protein positive fibrils were terminating between the  $\beta$ 1 integrin labeling at cell-cell junction sites (Fig 5C:f). Thus, immunosignals for NF1 protein and adherens junction components were separate.

Recent data by Hsueh *et al.* [30] demonstrated interaction of NF1 protein and syndecans 1–4. To evaluate whether these proteins were in close contact in keratinocytes, double immunolabeling was performed. In developing epidermis at 14 weeks EGA, syndecan-1 was localized to the plasma membrane of keratinocytes in all epidermal layers (Fig 6a). Potential colocalization or close spatial relation-

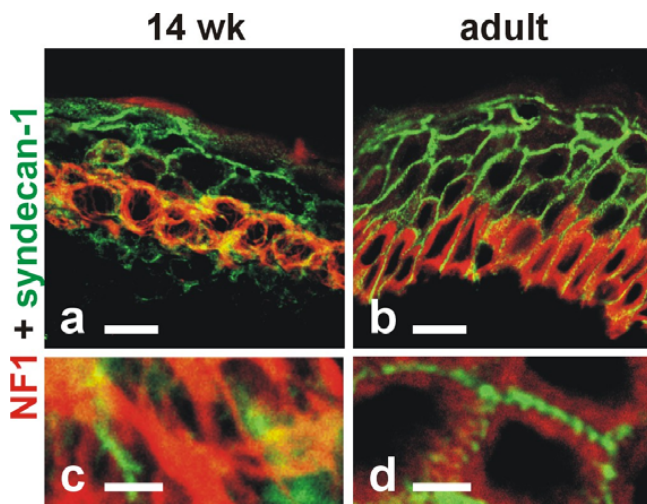




**Figure 5**

**A.** Double immunolabeling and CLSM analysis of NF1 protein with NF1GRP(D) antibody and cytoskeletal marker proteins at 13 weeks EGA in developing human epidermis. (a) NF1 protein (green) and cytokeratin 14 (red) show colocalization (yellow) on many locations of the basal cells. (d) High magnification reveals a marked degree of colocalization of NF1 protein and cytokeratin 14 labeling patterns. (b) NF1 protein (red) and actin (green) antibodies show separate labeling patterns. (e) Higher magnification reveals that the NF1 positive fibrils (red) are located closer to the nuclei (n), while actin (green) is located more peripherally. (c) NF1 protein (red) and  $\alpha$ -tubulin (green) antibodies show separate labeling. (f) High magnification of labeling for  $\alpha$ -tubulin and NF1 protein. Scale bars 10  $\mu$ m in a-c, 1  $\mu$ m in d-f. **B.** Double immunolabeling and CLSM analysis for NF1 protein with NF1GRP(D) antibody and desmoplakin or  $\beta 4$  integrin in developing human epidermis at 13 weeks EGA. (a) Desmoplakin (red) is present in all epidermal cell layers, although the basal cells show weaker labeling. Instead, NF1-specific antibody labels the basal and the periderm cells. (c) Higher magnification reveals that cytokeratin fibrils associated with NF1 protein (green) terminate to spots of desmoplakin (red) indicating the location of desmosomes. Insert in (c) show the location of a desmoplakin dot (red) at the end of a NF1 positive fibril (green). (b)  $\beta 4$  integrin (red) label the basal aspect of the basal cells, while NF1 protein (green) label the basal cell cytoplasm. (d) Higher magnification reveals that  $\beta 4$  integrin dots (red) are located at the end of NF1 positive fibrils (green). Scale bars 10  $\mu$ m in a, b; 2  $\mu$ m in c, d. **C.** Double immunolabeling and CLSM analysis of NF1 protein with adherens junction proteins and  $\alpha$ -actinin in developing human epidermis at 13 weeks EGA. (a) E-cadherin antibody (green) labels the cell-cell junctions of the basal and intermediate cells. NF1GRP(D) antibody (red) show basal and periderm cell cytoplasmic labeling. (d) Higher magnification reveals that immunosignals for E-cadherin (green) and NF1 protein (red) are not colocalized. (b and e) Also  $\alpha$ -actinin (green) and NF1 (red) are seen mutually exclusive. (c)  $\beta 1$  integrin (green) labels the cell membranes of the basal cells, while NF1 protein (red) labeling is cytoplasmic. (f) In higher magnification, NF1 positive fibrils (red; arrowheads) are terminating between the  $\beta 1$  integrin labeling (green) at cell-cell junction sites. n, nucleus. Scale bars 10  $\mu$ m a-c; 1  $\mu$ m in d-f.



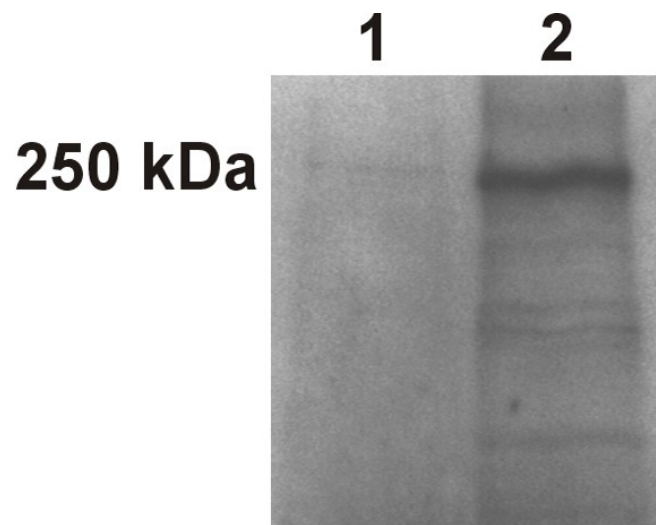


**Figure 6**  
Double immunolabeling and CLSM analysis of developing and adult epidermis for NF1 protein and syndecan-1. (a) At 14 weeks EGA, the NF1 protein (red) shows fibrillar pattern in basal cells while syndecan-1 (green) is localized to the plasma membrane of keratinocytes in all epidermal layers. Close spatial distribution or colocalization (yellow) is detected on some basal cells. (b) In adult epidermis the immunosignals for the NF1 protein (red) and syndecan-1 (green) are completely separate. Scale bars 10  $\mu$ m in a,b, 2  $\mu$ m in c,d.

ship of syndecan-1 with NF1 protein was noted on some basal cells (Fig 6a,6c). However, in adult epidermis, immunosignals for the NF1 protein and syndecan-1 were entirely separate (Fig 6b,6d). Immunoprecipitation of keratinocyte culture lysates with syndecan-1 specific antibody and subsequent western analysis with NF1GRP(D) antibody demonstrated that syndecan-1 was connected to a small fraction of NF1 protein (Fig 7).

### Discussion

The results of the present study demonstrate an early fetal period when the NF1 tumor suppressor is abundantly expressed and associated with bundles of intermediate filaments of the basal keratinocytes. This emphasizes that the *NF1* gene is developmentally regulated also in human skin. These findings also strengthen the view that the large NF1 protein exerts its functions through multiple molecular interactions. Specifically, NF1 tumor suppressor factor associates and functions with proteins located with the plasma membrane (p21ras, syndecans), or with cytoskeletal components associated with selected cellular junctions (desmosomes, hemidesmosomes) [12,28,30]. The action of NF1 protein may be analogous to e.g. fatty acid synthase, which conducts its tasks using different sites of the same molecule. It should also be noted that the activity of ras is associated with the regulation of cell growth



**Figure 7**  
Coimmunoprecipitation of syndecan-1 and NF1 protein. Lane 1: Keratinocyte cell lysate was immunoprecipitated with syndecan-1 antibody. Western transfer analysis was carried out using NF1GRP(D) antibody. Lane 2: Parallel sample as in lane 1, but without immunoprecipitation. Note typical ~250 kDa band representative of NF1 protein in both samples.

and differentiation, including control of cytoskeletal organization and formation of cell-cell junctions [7,14].

Western transfer analysis, immunolocalization and *in situ* hybridization showed that the *NF1* gene was abundantly expressed in developing human epidermis. In contrast to adult epidermis, fetal basal cells displayed a fibrillar labeling pattern for NF1 protein between 8 and 14 weeks EGA. Later during the development, the fibrillar labeling of basal cells changed into a more diffuse cytoplasmic pattern. The fibrillar labeling pattern suggested that the NF1 protein was associated with cytoskeleton. Double immunolabelings demonstrated a relatively high degree of colocalization of NF1 protein and cytokeratin 14 at light microscopic level, and immunoelectron microscopy located NF1 protein on perinuclear keratin bundles. In developing human epidermis, NF1 protein did not show detectable association with actin microfilaments or tubulin, although in other studies and conditions, NF1 protein has been shown to interact with these cytoskeletal components [29].

Concomitantly with the association of NF1 protein to the cytokeratin, the epidermis undergoes profound changes: the stratification of epidermis, maturation of basement membrane zone, and the formation of desmosomes and hemidesmosomes is activated. At 9–16 weeks EGA, keratin filaments become more prominent, and the number of desmosomes increase [4]. First hemidesmosomes have

been noted at ~9 weeks EGA. Between 9 and 15 weeks EGA the number of hemidesmosomes increases by about fourfold, and the morphology of hemidesmosomes matures simultaneously with increased contact with intermediate keratin filaments [2]. NF1 protein was detected also near the desmosomes and hemidesmosomes as indicated by both immunoelectron microscopy and double immunolabeling of NF1 protein with desmoplakin and  $\beta$ 4 integrin. These *in vivo* observations are in good agreement with the earlier *in vitro* study by Koivunen *et al.* [28]. They showed the association of NF1 protein with CK14-containing keratin intermediate filaments during the formation of desmosomes in cultured human keratinocytes. The present study demonstrates a similar association of NF1 protein and intermediate filaments during the formation of intercellular junctions *in vivo*. Developing epidermis thus provides a good model to study differentiation of keratinocytes *in vivo*, since the interactions of various cytoskeletal and junction proteins can be readily visualized.

The finding that NF1 specific antibodies raised against different peptide segments of the protein results in different labeling is in agreement with earlier studies [20,21,28,33]. Specifically, NF1GRP(D) antibody labeled most intensely the basal cells, while other antibodies used, NF1ab67 and NF1as159, labeled all the developing cell layers indicating that NF1 protein is present in the intermediate cell layers, too. One plausible explanation is that NF1GRP(D) antibody may recognize only a specific type of NF1 protein conformation.

Expression of NF1 mRNA was studied at 13 and 18 weeks EGA using *in situ* hybridization. At 13 weeks, the localization of NF1 mRNA corresponded with the immunosignal for the NF1 protein. However, at 18 weeks, NF1 mRNA and protein displayed differential spatial distribution within the epidermis. Specifically, NF1 mRNA was detected in the suprabasal cells but not in the basal layer. Although NF1 protein remained detectable in the basal cells at 18 weeks EGA, the labeling pattern for NF1 protein in the basal cells had changed to a more diffuse cytoplasmic staining. It is thus possible that the more diffuse localization of NF1 protein in the basal cells is associated with downregulation of the NF1 mRNA level. These results are in concordance with previous studies detecting NF1 mRNA in suprabasal cell layers in late developing rat epidermis while basal cells contained no detectable NF1 mRNA at this stage of rat epidermal development [33].

NF1 mRNA was intracellularly distributed to the basal aspects of the basal cells and to the cell peripheries of intermediate cells at 13 weeks EGA. These are the sites where desmosome and hemidesmosome formation takes place during this stage of development. Previously, Ylä-Outinen *et al.* [31] have shown that NF1 mRNA is targeted to cell-

cell contact zone in cultured human keratinocytes when they are induced to differentiate. This might be true also *in vivo*, since NF1 mRNA was accounted at cell-cell and cell-basement membrane contact zones.

Periderm cells showed profound changes in the expression pattern of NF1 protein. Our study revealed that, at 8 weeks EGA, when the desmosome formation is active in the periderm, the periderm cells showed fibrillar labeling for NF1 protein. By the 11th week of EGA, labeling for NF1 protein revealed a more diffuse pattern in the periderm cells, diminished thereafter, and disappeared by the 16th week EGA. This correlates with the cessation of periderm cell proliferation at about 12 weeks EGA [3] and disruption of intermediate keratin filaments at 15–17 weeks EGA [4]. After the periderm cells cease dividing, they synthesize little if any protein and are gradually shed into the amniotic fluid by the end of the third trimester [3]. The expression of NF1 protein in the periderm thus correlates well with the periderm cell proliferation and cell junction forming activity. This is also in concordance with previous study on developing rat skin demonstrating NF1 protein first in the basal and periderm cells, but later mostly in the spinous cell layer [33].

Even though a bipartite interaction between transmembrane syndecans and NF1 protein has been characterized in neurons by two hybrid method [30], the results of the present study demonstrated only little of potential colocalization of NF1 protein and syndecan-1 in epidermal keratinocytes during the development and in adult. Thus, the conditions when NF1 protein and syndecans may interact, and the cell biological significance of this interaction remain to be elucidated.

#### List of abbreviations

BSA, Bovine serum albumin; CLSM, confocal laser scanning microscopy; EGA, estimated gestational age; GAP, G-protein activating protein; NF1, type 1 neurofibromatosis; PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate.

#### Competing interests

None declared.

#### Authors' Contributions

MM carried out the Western transfer analysis, indirect immunofluorescence labelings, confocal laser scanning microscopy, immunoelectron microscopy, and avidin-biotin immunolabelings. She also participated in drafting the manuscript. JK carried out keratinocyte cell cultures and coimmunoprecipitation, and participated in Western transfer analysis. SP and JP conceived of the study, drafted the manuscript, and participated in the design of the study. All authors read and approved the final manuscript.

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