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## Neuropilin 1 expression correlates with differentiation status of epidermal cells and cutaneous squamous cell carcinomas

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### Abstract

Neuropilins (NRP) are cell surface receptors for VEGF and SEMA3 family members. The role of NRP in neurons and endothelial cells has been investigated, but the expression and role of NRP in epithelial cells is much less clear. Herein, the expression and localization of neuropilin 1 (NRP1) was investigated in human and mouse skin and squamous cell carcinomas (SCC). Results indicated that NRP1 mRNA and protein was expressed in the suprabasal epithelial layers of skin sections. NRP1 staining did not overlap with that of keratin 14 (K14) or proliferating cell nuclear antigen, but did colocalize with staining for keratin 1, indicating that differentiated keratinocytes express NRP1. Similar to the expression of NRP1, VEGF-A was expressed in suprabasal epithelial cells, whereas Nrp2 and VEGFR2 were not detectable in the epidermis. The expression of NRP1 correlated with a high degree of differentiation in human SCC specimens, human SCC xenografts, and mouse K14-HPV16 transgenic SCC. UVB irradiation of mouse skin induced *Nrp1* upregulation. *In vitro*, Nrp1 was upregulated in primary keratinocytes in response to differentiating media or EGF-family growth factors. In conclusion, the expression of NRP1 is regulated in the skin and is selectively produced in differentiated epithelial cells. NRP1 may function as a reservoir to sequester VEGF ligand within the epithelial compartment, thereby modulating its bioactivity.

### Keywords

squamous cell carcinoma; skin cancer; angiogenesis; differentiation; epithelium; neuropilin; semaphorin

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Neuropilins (NRP, human; Nrp, mouse) are type I transmembrane receptors for two distinct ligand families—the vascular endothelial growth factor (VEGF) family of angiogenic molecules and the class 3 semaphorin (SEMA3) family of guidance proteins (1, 2). The two neuropilin genes, *NRP1* and *NRP2*, are located on different chromosomes (3). Both NRP

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proteins are similarly sized and have analogous domain structures (4, 5). The two NRP receptors share some ligands including VEGF-A (hereafter called VEGF), but differ in their specificity for other VEGF family members (6-8); for example, NRP1 binds placenta growth factor 2 (PlGF2) (9), whereas NRP2 binds VEGF-C and VEGF-D (10).

NRPs were first characterized as neuronal guidance receptors (11, 12), and their role in the vasculature has also been widely studied. Endothelial cells express NRP with some degree of specificity during development such that arteries express Nrp1 and veins and lymphatics express Nrp2 (13-15). In contrast, the expression, regulation and function of NRPs in epithelial cells are unclear (16). Previously, we reported that human skin expresses full-length NRP1 but not soluble NRP1 (sNRP1) (17). Additionally, we have demonstrated the upregulation of NRP1 in keratinocytes by growth factors such as epidermal growth factor (EGF), and down regulation by transcription factors such as the neuron restrictive silencer factor (NRSF) (18).

Genetically-engineered mice lacking Nrp1 (knockout) are embryonic lethal at E12.5-13.5 (19). Tissue-specific deletion of Nrp1 in epithelial cells, achieved by crossing K14-cre mice and Nrp1-floxed mice, yield mice that are viable and show no obvious phenotype (20). Yet, mice lacking Nrp1 in the epidermis are more sensitive to UVB irradiation and displayed increased apoptosis following irradiation (20). Using mice overexpressing sNRP1 in the epidermis (K14-sNRP1), we demonstrated that sNRP1 could diffuse through the basement membrane of the epidermal/dermal junction and inhibit dermal blood vessels and vascular permeability (21).

Within the skin epithelium, the VEGF protein is localized in suprabasal layers— farthest from dermal vessels (22). The VEGF ligand has been shown to be upregulated in the epidermis in association with ischemia, wound healing or hyperplasia (23, 24). Moreover, VEGF is highly expressed by invasive and metastatic human squamous cell carcinomas (SCC) (25), yet its expression also correlates with the degree of differentiation in cutaneous human SCC (26). In fact, overexpression of VEGF by lowgrade SCC cells (SCC13) increased their growth rate and invasiveness (27). In order to clarify some of these findings and further our understanding of the potential function of VEGF in the epithelium, we interrogated the expression profile of neuropilin 1 in the skin and investigated its regulation both *in vitro* and *in vivo* under normal physiological conditions as well as in SCC models.

## Materials and Methods

### Cell culture

A431 human epidermoid SCC cells, originally isolated from an 85-year old woman (28), were purchased from American Type Culture Collection (ATCC). DJM1 cells, originally isolated from a 54-year-old woman with metastatic SCC (29), were obtained from Dr. Misuzu Seo (Kyoto Sangyo University, Japan). SCC13 cells, originally isolated from a 56-year-old female with facial SCC that was previously treated with radiation (30), were obtained from Dr. James Rheinwald (Harvard Medical School). HaCat, spontaneously immortalized human keratinocytes (31), and all human SCC lines were cultured in minimal essential media (MEM, Life Technologies) supplemented with 10% fetal bovine serum

(FBS) and 1% glutamine-penstrep (GPS, Life Technologies). HaCat were incubated with EGF (10 ng/ml) for indicated times. Primary normal human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in EGM2 (Lonza). Porcine aortic endothelial (PAE) cells overexpressing human NRP1 or NRP2 were obtained from Dr. Michael Klagsbrun (Harvard Medical School) and cultured in Ham's F12 media (Life Technologies) supplemented with 10% FBS and 1% GPS. Mouse hemangioendothelioma EOMA cells (32) were purchased from ATCC and maintained in high glucose DMEM (Life Technologies) with 10% FBS and 1% GPS.

### Animal studies

**Tumor Inoculation**—Adult (8-wk) female Balb/c Nude (nu/nu) mice were purchased from Massachusetts General Hospital. Human SCC cells ( $1 \times 10^6$ ) were injected subdermally (33) on the right dorsal flank. Thirty days later, mice were euthanized; tumors were removed, fixed in formalin, and embedded in paraffin.

**Ultraviolet B (UVB) Irradiation**—Adult (8-wk) female C57Bl/6 mice were purchased from Charles River Laboratories and exposed to a single  $15\text{kJ/m}^2$  dose of UVB irradiation as previously described (34). Mice were euthanized at various time points; and their exposed ears were resected, fixed in formalin, and embedded in paraffin. Age-matched, unirradiated mice served as normal controls.

**Transgenic/knockin mice**—Ears from adult (3-month) heterozygous  $\text{VEGFR2}^{+/LacZ}$  (also called  $\text{Flk1}^{+/LacZ}$ ) mice (35) were resected under anesthesia, frozen in OCT compound, and stained with X-gal reagent to detect  $\beta$ -galactosidase activity. Heterozygous  $\text{Nrp2}^{+/LacZ}$  mice (36, 37) were euthanized at P1 or P42 (6 wk). Dorsal skin was shaved, removed, frozen in OCT compound, and stained with X-gal reagent. Transgenic mice expressing the human papillomavirus type 16 early region genes under the control of the keratin 14 promoter (K14-HPV16 mice) (38, 39) were euthanized at various time points during their disease progression from hyperplastic to dysplastic to squamous cell carcinoma. Ears were resected, fixed in formalin, and embedded in paraffin.

### Keratinocyte isolation

Adult lactating female Balb/c mice with new litters were purchased from Charles River Laboratories. P3 pups were euthanized, and skin tissue was used for primary epidermal cell isolation as described (38). Cells ( $1 \times 10^6$ ) were maintained in MEM with 8% calcium-chelated FBS and cultured in low calcium (0.05 mM  $\text{CaCl}_2$ ) media or differentiated in high calcium (0.12 mM  $\text{CaCl}_2$ ) media or retinoic acid (3 mM) media for various time points (40). Alternately, cells were incubated for various times in media containing HB-EGF (10 ng/ml) or SEMA3A (640 ng/ml).

### Northern blot analysis

Cells were incubated with growth factors or differentiating agents and cellular mRNA purified using the FastTrack mRNA isolation kit (Life Technologies). mRNA was separated on formaldehyde/agarose gels, transferred to nylon membranes and hybridized with  $^{32}\text{P}$ -labeled cDNA-probes corresponding to 838-bp mouse NRP1 b domain generated with

primers 5'CCTGAACTACCCTGAAAATGG and 3'GATGACCCGACACTTCACCTT (21) or 950-bp human NRP1 b domain generated with primers 5'GAAGATTTCAAATGTATGGAAG and 3'GGCTTCCACTTCACAGCCCAG (17). Probes were labeled with Rediprime II, random primed synthesis kit (GE Healthcare). Blots were washed and exposed to Kodak film. Blots were stripped and hybridized with a  $\beta$ -actin probe to normalize RNA loading.

### Western blot analysis

Cells were lysed in RIPA buffer (Boston Bioproducts) and complete protease inhibitor cocktail (Roche). Proteins in either reducing or non-reducing sample buffer were run on 7.5% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with nonfat milk and incubated with rabbit polyclonal anti-human NRP1 (44-2) (recognizing amino acids DDSKRKAKSFEGNNYD in the b2 domain; not commercial) (18) or goat anti-rat/mouse Nrp1 (R&D Systems). Membranes were incubated with HRP-linked secondary antibodies, donkey anti-rabbit (GE Healthcare) or donkey anti-goat (R&D Systems), and detected with Western Lightning Plus ECL (PerkinElmer). Blots were stripped with Reblot Plus (Millipore) and re-probed with rabbit anti-GAPDH (Abcam) to normalize for protein loading.

### VEGF cross-linking

HaCat or HUVEC were pretreated with HB-EGF (10 ng/ml) for 24 hrs to upregulate NRP1. VEGF<sub>165</sub> (carrier-free, R&D Systems) was radioiodinated using IODO-BEADS as previously described (1). Cells were incubated on ice for 2 hr in the presence of I<sup>125</sup>-VEGF<sub>165</sub> and heparin (1 mg/ml) or SEMA3A and cross-linked with disuccinimidyl suberate (DSS, Pierce). Cross-linked complexes were resolved by 6% SDS-PAGE, and gels were exposed to X-ray films.

### Immunocytochemistry (ICC)

PAE cells or primary mouse keratinocytes were grown on glass slides and fixed with 4% paraformaldehyde in PBS. Endogenous peroxidase was quenched with 3% H<sub>2</sub>O<sub>2</sub> in methanol, proteins blocked with TNB (PerkinElmer), and incubated with either rabbit anti-human NRP1 (44-2; not commercial) or rabbit anti-mouse keratin 1 (AF109; Covance). Sections were incubated with biotinylated goat anti-rabbit (Vector), Vectastain Elite HRP-linked avidin (Vector), and diaminobenzidine (DAB, Vector). Cells were counterstained in hematoxylin (H) (Sigma-Aldrich) and mounted with Permount (Fisher Scientific).

### Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and rehydrated through a graded series of alcohols to water. Antigen retrieval included proteinase K (20  $\mu$ g/ml) for CD31 and mouse Nrp1 and heat-induced epitope retrieval for PCNA. Staining with the other antibodies did not require antigen retrieval.

Endogenous peroxidase was inhibited with 3% H<sub>2</sub>O<sub>2</sub> in methanol, and proteins were blocked in TNB. Sections were incubated in primary antibodies overnight at 4°C including rabbit anti-human NRP1 44-2 (not commercial), rabbit monoclonal anti-mouse Nrp1 (clone

EPR3113; detects C terminus and not sNrp1; Epitomics), monoclonal anti-PCNA (clone PC10; Dako), rabbit anti-human keratin 1 (AF87; Covance), rabbit anti-human keratin 14 (AF64; Covance), and monoclonal rat anti-mouse CD31 (clone MEC13.3; BD Pharmingen). Sections were incubated in secondary antibodies including HRP-conjugated rat anti-mouse IgG2a (Serotec), biotinylated goat anti-rabbit (Vector), biotinylated goat anti-rat (mouse absorbed; Vector), Vectastain Elite HRP-linked avidin, and DAB. Cells were counterstained with hematoxylin and mounted with Permount.

### Human SCC array and scoring

Human skin cancer tissue microarray slides (A216) containing 50 cases/2 spots each were purchased from AccuMax Array. Sections were stained for human NRP1 as described above and then scored by a pathologist. Staining was ranked according to area of staining (1-3:1 = 1-10%, 2 = 11 -50%, 3 = >50%) and intensity of staining (1-3, with 3 for greatest intensity of staining). Staining Index was calculated as area  $\times$  intensity (9 = highest, 0 = lowest).

### X-gal staining

For detection of  $\beta$ -galactosidase activity, cryosections from VEGFLacZ, VEGFR2<sup>+/LacZ</sup> or Nrp2<sup>+/LacZ</sup> mice were fixed in methanol and incubated (37°C) in X-gal [1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) in DMSO; 5 mM K<sub>3</sub>Fe(CN)<sub>6</sub>; 5Mm K<sub>4</sub>Fe(CN)<sub>6</sub>; 2 mM MgCl<sub>2</sub> in PBS; pH 6.5]. Sections were counterstained with eosin (E) (Sigma) and mounted with Permount.

### In situ hybridization

Sections were de-waxed, rehydrated, and digested with proteinase k; then, post-fixed, dehydrated, and air-dried. Anti-sense human NRP1-specific riboprobe and sense probe were synthesized from 750-bp 3'UTR-derived cDNA using a digoxigenin RNA-labeling kit (Roche) as previously described (17, 41). Anti-sense mouse Nrp1-specific riboprobe and sense probe were created similarly from a 500-bp cDNA (41). Probes were hybridized and washed in SSC. Alkaline phosphatase-labeled anti-digoxigenin and BM Purple (Roche) were used to visualize the reaction.

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total mRNA was isolated from primary mouse keratinocytes (1'MK) or EOMA cells using RNeasy mini kit (Qiagen) with RT reaction and PCR performed as previously described (42, 43). The following primer pairs were used: mouse NRP1 a domain (404bp) F5'-GGC TGC CGT TGC TGT GCG-3' and R5'-ATA GCG GAT GGA AAA CCC-3'; VEGF (amplifying all known isoforms of mouse VEGF-A) F5'-GGG TGC ACT GGA CCC TGG CTT TAC-3' and R5'-CCT GGC TCA CCG CCT TGG CTT GTC-3'; VEGFR-1 (625 bp) F5'-GGC TCA GGG TCG AAG TTA AAA GTG CCT-3' and R5'-TAG GAT TGT ATT GGT CTG CCG ATG GGT-3'; VEGFR-2 (408 bp) F5'-CTC TGT GGG TTT GCC TGG CGA TTT TCT-3' and R5'-GCG GAT CAC CAC AGT TTT GTT CTT GTT-3'; GAPDH F5'-ACC CCT TCA TTG ACC TCA ACT-3' and R5'-CCACCA CCCTGTTGCTGTAG-3'.

## Results

### NRP1 expression in normal skin

We previously described high NRP1 expression in human skin (17, 18). Herein, we examined NRP1 protein localization in human paraffin skin sections at a higher resolution using IHC. NRP1 protein was highly expressed in all suprabasal epithelial cells as well as in dermal endothelial cells (Figure 1A, brown color). The negative control, secondary antibody alone, showed no specific staining in the skin (Figure 1B). The NRP1 antibody was specific to NRP1 and did not react with human NRP2 as shown by western blot or ICC analyses (Figure S1A-B). *NRP1* mRNA was also found exclusively in suprabasal epithelial cells and dermal blood vessels as demonstrated by *in situ* hybridization (Figure 1C-D, purple color). Double IHC with antibodies to human NRP1 and proliferating cell nuclear antigen (PCNA) (Figure 1E) or NRP1 and K14 (Figure 1F) demonstrated that basal cells in the epidermis did not express NRP1.

### NRP1 in human SCC specimens

We next examined the expression and localization of NRP1 protein in human cutaneous SCC specimens. Human SCC tissue microarrays (#A216) were stained by IHC for NRP1 (Figure 2A) and results were recorded by a staining index as described in the methods. Table 1 summarizes the differentiation status of the SCC specimens, NRP1 staining index, sex, age, tumor stage, and tumor size. All cases were positive for NRP1, and representative stainings of normal skin (NR), highly differentiated (HD), moderately differentiated (MD), and poorly differentiated (PD) SCC are shown in Figure 2C. NRP1 staining index scores ranged from 6-9 for HD samples, from 3-6 for MD samples, and from 2-3 for PD samples (Table 1). Highly differentiated human SCC samples with surrounding normal cutaneous tissues were also stained for NRP1 using IHC. NRP1 protein was upregulated in SCC tumor tissue and also found in adjacent hyperplastic suprabasal epithelium (Figure 2B). Strong NRP1 protein expression in SCC correlated with high differentiation status but did not correlate with age, sex, size or tumor stage (Table 1, Figure 2 A-C).

### NRP1 in human SCC xenografts

Three human SCC cell lines with varying differentiation status were injected subdermally in nude mice, allowed to grow for one month, and resected. Paraffin sections of tumors were stained by hematoxylin and eosin (H&E), and entire cross-sections were scanned and shown in Figure S2A-C. SCC13 tumors are highly differentiated (Figure S2A), DJM1 tumors are moderately differentiated (Figure S2B), and A431 tumors are poorly differentiated (Figure S2C). Tumor sections were also stained by IHC to detect human NRP1 (Figure 3A-C), to detect human K1 in differentiated SCC cells (Figure 3D-F), and to detect (human) K14 in all human epithelial cells (Figure 3G-I). All tumors expressed K14. As expected, the majority of SCC13 strongly expressed K1, confirming their high differentiation status (Figure 3D). DJM1 expressed K1 in a heterogeneous pattern throughout the tumor (Figure 3E), whereas A431 lacked K1 staining (Figure 3F). NRP1 staining paralleled that of K1 in all tumors; NRP1 was high in SCC13, moderate in DJM1 and absent in A431 (Figure 3A-C). In the SCC13 tumors, basal/proliferating cells (arrow) lacked NRP1 and K1 (Figures 3A and 3D, respectively).



## NRP1 in transgenic SCC

Ears from adult K14-HPV16 mice were evaluated histologically at various stages including hyperplastic (baseline) (Figure 4A, D, G), papillomatous and/or dysplastic (Figure 4B, E, H), and at time points when SCC was observed (Figure 4C, F, I). Ear thickness dramatically increased during tumor progression (compare width from central cartilage layer to outer layer of epidermis, Figure 4 A vs. B). Thickness of the ear in tumor areas exceeded the field of view (compare Figure 4 A vs. C). Nrp1 protein expression increased in dysplastic and SCC samples as compared to baseline/control ears with Nrp1 localization remaining in differentiated cells at all stages (Figure 4D-F). Microvessel density, as identified by CD31 IHC, also increased with tumor progression (Figure 4G-I), yet tumor vessels did not express Nrp1.

## Localization of VEGF and VEGF receptors using transgenic mice

Cryosections from VEGFR2<sup>+/LacZ</sup> mouse ears or Nrp2<sup>+/LacZ</sup> mouse skin were stained using X-gal reagent to detect  $\beta$ -galactosidase activity. VEGFR2 expression (as detected by LacZ) was detected only in dermal blood vessels and not in epithelial cells (Figure 5A-B). Nrp2 expression (also detected by LacZ) was observed in dermal vessels and in melanocytes of hair follicles in P1 skin and in melanocytes and arrector pili muscle (not shown) in adult skin (Figure 5C-D); epidermal keratinocytes (arrowheads) lacked VEGFR2 and Nrp2.

Primary mouse keratinocytes (1'MK) were isolated from P3 mice and cultured *in vitro*. Messenger RNA was isolated from the keratinocytes and mouse endothelioma cells (EOMA) as a control, and RT-PCR was used to examine the levels of VEGF receptor message (Figure S2D). 1'MK cells expressed only Nrp1 whereas EOMA expressed VEGFR1, VEGFR2, and Nrp1. 1'MK also expressed VEGF mRNA, but to a lesser extent than EOMA (Figure S2D).

Suprabasal epithelial cells have been shown by IHC and ISH to express VEGF-A (22, 23). Since VEGF is a secreted protein that can bind heparan sulfate proteoglycans and may be sequestered on the cell surface or in the extracellular matrix, IHC will not provide reliable information about its source. Thus, we examined VEGF production in the skin using transgenic mice in which the VEGF gene was tagged with LacZ with a nuclear translocation signal (44). Blue nuclei (VEGF-producing cell) were detected primarily in suprabasal keratinocytes and in hair follicles of mouse skin (Figure 5E).

## Regulation of Nrp1 in vivo

UVB was previously shown to induce epidermal hyperplasia and dermal angiogenesis (23, 34). Mice lacking Nrp1 in the epithelium are more sensitive to the apoptotic effects of UVB (20), therefore Nrp1 regulation was evaluated following UVB *in vivo*. Ear thickness increased following UVB exposure (primarily on the outer ear surface) and the epidermis increased from 1-2 cells to 6-8 cells thick (compare Figure 6 A vs. C). Levels of *Nrp1* mRNA were increased in the suprabasal epithelial cells after just one day (not shown) but were markedly increased by five to seven days post-UVB (Figure 6 E-F). There was little Nrp1 staining in control ear sections at average chromogen incubation times (8 hrs) (Figure 6D), but Nrp1 expression was increased in outermost layers at longer incubation times (24



hrs) (not shown). Due to the intense increase in Nrp1 expression following UVB, only short exposure of ISH to chromogen was necessary.

### Regulation of Nrp1 in vitro

To further investigate the regulation of Nrp1 expression in normal skin and epidermal cells, we turned to an *in vitro* system. I'MK were cultured under low calcium (non-differentiating) or high calcium (differentiating) conditions with longer exposure of the cells to high calcium media leading to a more dramatic change in cell shape (Figure 7A-C) and a higher level of differentiation as evidence by expression of keratin 1 (K1) (Figure S3 A vs. E).

Differentiated cells expressed increased levels of Nrp1 protein (Figure 7D) as detected by western blot following ConA-sepharose pull-down and mRNA (Figure 7E) as detected by northern blot. Similarly, I'MK cells, differentiated in media containing retinoic acid, revealed increased levels of *Nrp1* mRNA as the cells differentiated (Figure 7F). Treatment of the cells with SEMA3A alone (data not shown) or in combination with high calcium media did not affect the differentiation of I'MK cells (Figure S3A-D).

To investigate the regulation of Nrp1 under proliferative conditions such as those associated with UVB irradiation or with wound healing, I'MK cells or human HaCat cells were treated with HB-EGF or EGF. Additions of either growth factor led to the upregulation of NRP1 in both mouse and human epithelial cells following treatment (Figure 7 G-H). The functionality/binding ability of the NRP1 receptor protein was demonstrated by crosslinking radioactive VEGF ligand. Specifically, the treatment of HaCat cells with HB-EGF led to the upregulation of NRP1 which then bound more I<sup>125</sup>-VEGF<sub>165</sub> as compared to untreated control cells (Figure 7I, left panel). Unlabeled SEMA3A competed the binding of I<sup>125</sup>-VEGF<sub>165</sub> to both HaCat (Figure 7I, right panel) and to HUVEC (Figure S2E).

### Discussion

Our previous studies indicated that NRP1 was expressed on epithelial cells at levels even higher than on endothelial cells (17, 18). Yet, its expression pattern and regulation within the epithelium was unknown. Herein, we demonstrate that NRP1 is found exclusively in suprabasal (differentiated) epidermal cells in both mouse and human normal skin sections. The NRP1 ligand, VEGF, was also expressed by differentiated cells (22, 23). Primary keratinocytes cultured in normal growth media (low calcium), which does not support cell differentiation, expressed barely detectable levels of Nrp1. Yet, switching the media to high calcium concentrations or the addition of retinoic acid, which induced cell differentiation (detected by the expression of keratin 1) was associated with the upregulation of Nrp1. The expression of Nrp1 was not necessary for epidermal cell differentiation *in vivo*, since epidermal-specific deletion of Nrp1 still express the differentiation markers keratin 10 and loricrin (20).

Exposure of mouse skin to an acute dose of UVB irradiation (a typical physiological insult) led to epithelial thickening (hyperplasia/proliferation) and increased expression of Nrp1. Nrp1 localization was not altered in hyperplastic tissues where its expression remained restricted to the suprabasal layers. UVB irradiation also increases VEGF expression in the

epidermis (23). Mice with epidermal-specific deletion of *Nrp1* showed increased apoptosis in response to UVB irradiation (20), suggesting that increased *Nrp1* in the epidermis is necessary for their survival. Exposure of cultured keratinocytes to EGF or HB-EGF, which act as mitogens, also led to an upregulation of NRP1 in a time- and dose-dependent fashion. This data is consistent with our previous reports (18, 45) and with those of others (46, 47). Taken together, we conclude that NRP1 is generally expressed by differentiated, quiescent epithelial cells; but that certain conditions (injury or growth) lead to the increased expression of NRP1.

Since NRP1 is expressed by most epithelial cells (16), it was not surprising to find NRP1 in the majority of carcinoma cells examined (7, 41). In the United States, skin carcinoma is the most common form of cancer diagnosed (48). The yearly incidence of skin cancer is higher than that of breast, prostate, lung and colon cancer combined (49). SCC a common form of skin cancer, second only to basal cell carcinoma (BCC). Whereas BCC is rarely aggressive, SCC can be invasive. In this study, we examined the expression of NRP1 in samples of human SCC from tumors of various stages and sizes all on a single tissue microarray so as to fairly compare NRP1 protein expression between samples. We found that levels of NRP1 were positively correlated with differentiation. In addition, using orthotopic xenograft models of human SCC injected into mice, we discovered differing patterns of NRP1 protein expression depending upon the tumor cell line used with NRP1 levels the strongest (or in the greatest percentage of tumor cells) in SCC13. SCC13 are slow growing tumors with most of the cells highly differentiated. In contrast, A431 SCC tumors are rapidly growing, contain poorly differentiated tumor cells, and do not express NRP1; whereas, DJM1 tumors were moderate in differentiation status and NRP1 levels.

Human and mouse skin differ in many characteristics including overall thickness, compliance/ stiffness, and adhesion to underlying tissues (50). Epithelial carcinogenesis also varies between human and mouse, but is similar in the step-wise transition from normal to dysplasia to neoplasia. K14-HPV16 mice undergo transformation and tumor progression in phases similar to human SCC disease progression with stages of hyperplasia and dysplasia prior to squamous cell carcinoma (39). Using this model, we found that *Nrp1* was expressed in differentiated cells during all stages of progression and was upregulated during hyperplastic and dysplastic phases. In human SCC, VEGF expression also correlated with the degree of differentiation in cutaneous SCC (26). Taken together, these data, in both a mouse model and human cells, indicate that differentiated, but not undifferentiated, tumor cells express NRP1.

The tyrosine kinase VEGF receptors, VEGFR1, R2, and R3, are expressed by endothelial cells of blood and lymphatic vessels and bind members of the VEGF ligand family. Most VEGF functions are thought to result from VEGFR2 signaling (51). A few studies have reported expression of VEGFR2 on cutaneous epithelial cells (52, 53), while others do not find VEGFR2 expression in the epidermis (54, 55). We therefore chose to use knockin VEGFR2<sup>+LacZ</sup> mice to track the expression of VEGFR2/flk1 in the skin and avoid antibody staining. Our results clearly demonstrate that epidermal cells do not express VEGFR2. In addition, we did not see expression of VEGFR1 or VEGFR2 in cultured primary mouse keratinocytes by RT-PCR, in agreement with our previous studies using HaCat keratinocytes

(18); others have found VEGFR1 on epidermal cells (55). NRP1, on the other hand, is expressed in many epithelial cell types including the skin epidermis (16-18, 20, 21). Nrp2 is not expressed by keratinocytes (Figure 5), but is upregulated in some carcinomas (7, 16, 41). We previously published that HaCat keratinocytes do not express VEGFR1, R2, or R3 (18), yet herein we demonstrate that they bind VEGF and express NRP1 (Figure 7).

In light of the fact that the NRP1 receptor does not have any intrinsic kinase activity, the function of NRP1 in epithelial cells requires a more detailed analysis of keratinocytes and their signal transduction pathways. That said, there are several potential roles for NRP1 in the epidermis. First, NRP1 may directly signal via its C-terminal 3 amino acid (SEA) domain that has been shown to bind to GIPC (56-58). Recent data suggests that PlGF signaling in medulloblastoma requires this NRP1 C-terminal domain (59). Secondly, NRP1 may bind and sequester VEGF protein for later use, for instance in wound healing. It is possible that tumors with high NRP1 will bind more VEGF on their surface (as we show in Figure 7) and, therefore, create a chemogradient of VEGF protein within the tumor to attract new blood vessels into the tumor microenvironment. We have not shown this directly for SCC, but this “reservoir effect” of Nrp1 for VEGF was demonstrated in prostate cancer cells transfected with Nrp1 (60). Thirdly, there is also the possibility that NRP1 in the epithelium might function as a receptor for other ligands such as SEMA3A, PlGF or HGF (9, 61, 62). Consistent with this possibility, we previously showed that SEMA3A could inhibit keratinocyte migration *in vitro* (18).

In conclusion, our data indicate that NRP1 is expressed in the normal epidermis and as such is the sole VEGF receptor in these cells. NRP1 expression correlates with the level of keratinocyte differentiation both *in vitro* and *in vivo*. NRP1 is regulated by physiologic stimuli that cause hyperplasia such as UVB irradiation as well as pathologic stimuli that cause dysplasia/neoplasia such as HPV. SCC (both human and mouse) express NRP1 in a differentiation-dependent manner such that highly differentiated tumor cells express more NRP1.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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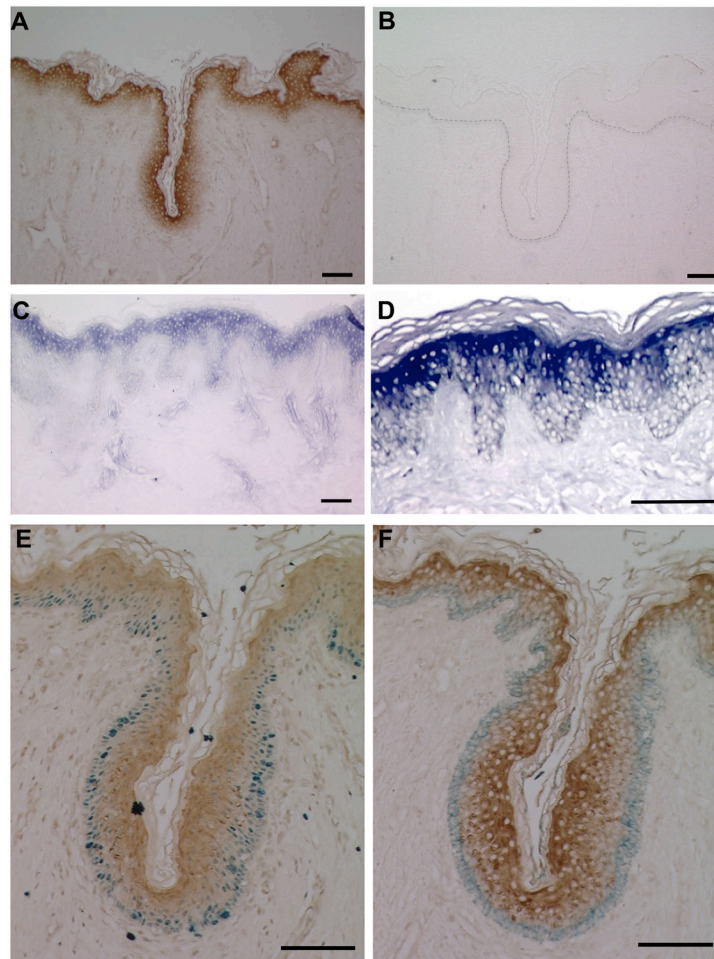
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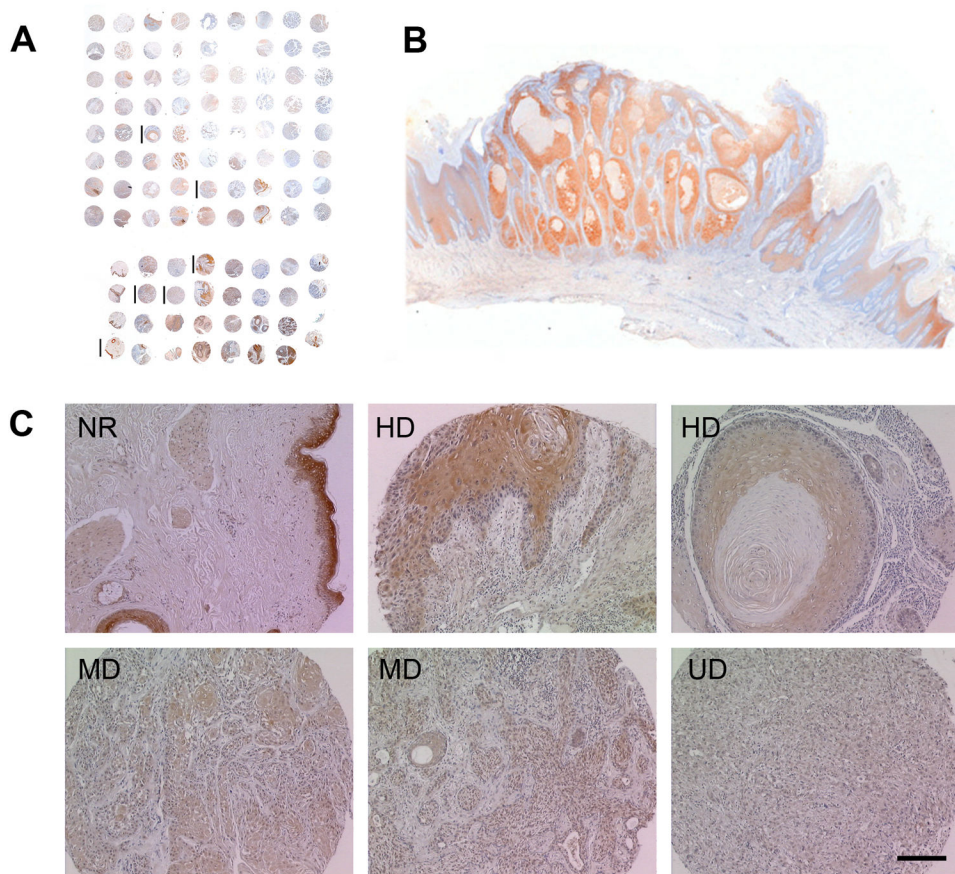
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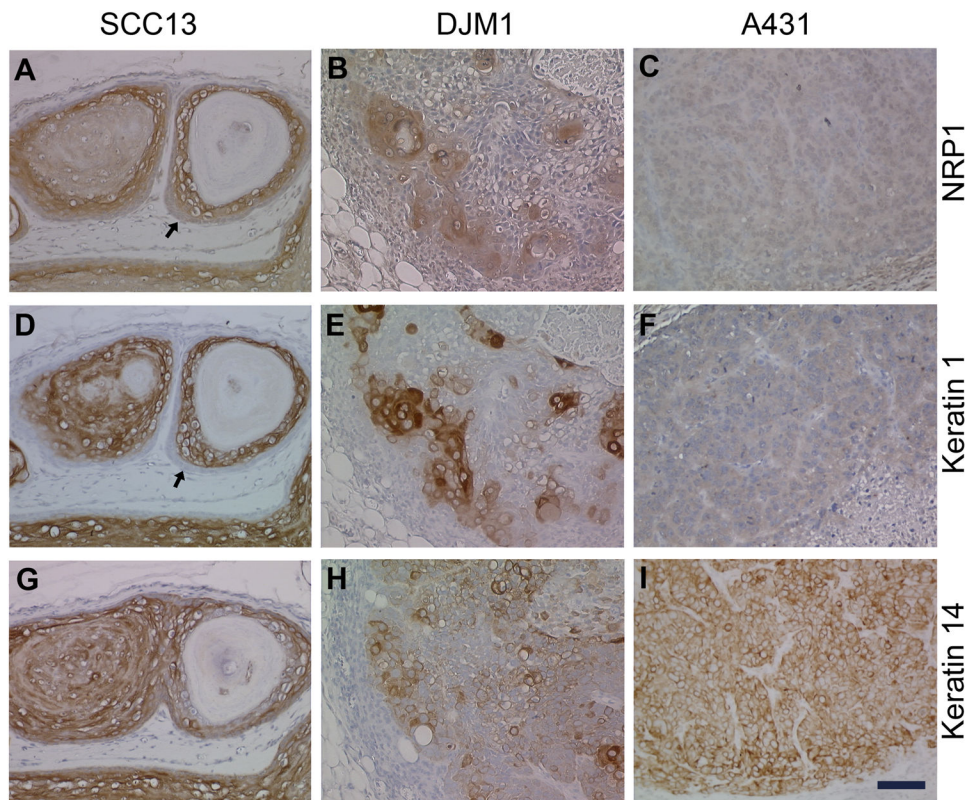
**Figure 1. NRP1 mRNA and protein is expressed in suprabasal layers of the epidermis**  
 (A) Paraffin sections of human skin were stained by IHC using anti-human NRP1 antibody. Staining (brown color) was observed in suprabasal epidermis and dermal endothelium. (B) Control sections, receiving all treatments except primary antibody, were negative for staining. The epidermal-dermal junction is outlined with a dotted line. (C-D) Paraffin sections of human skin were stained by ISH with probes to human NRP1 mRNA. Staining (purple color) was observed in suprabasal epidermis and dermal endothelium. Basal epidermis lacked staining. (E-F) Double IHC staining for human NRP1 (brown color) and PCNA (nuclear blue color) (E) or K14 (blue color) (F). NRP1 expression was not detected in dividing cells or basal cells. All scale bars = 100  $\mu$ m.



**Figure 2. NRP1 expression in human SCC patient samples correlates with degree of differentiation**

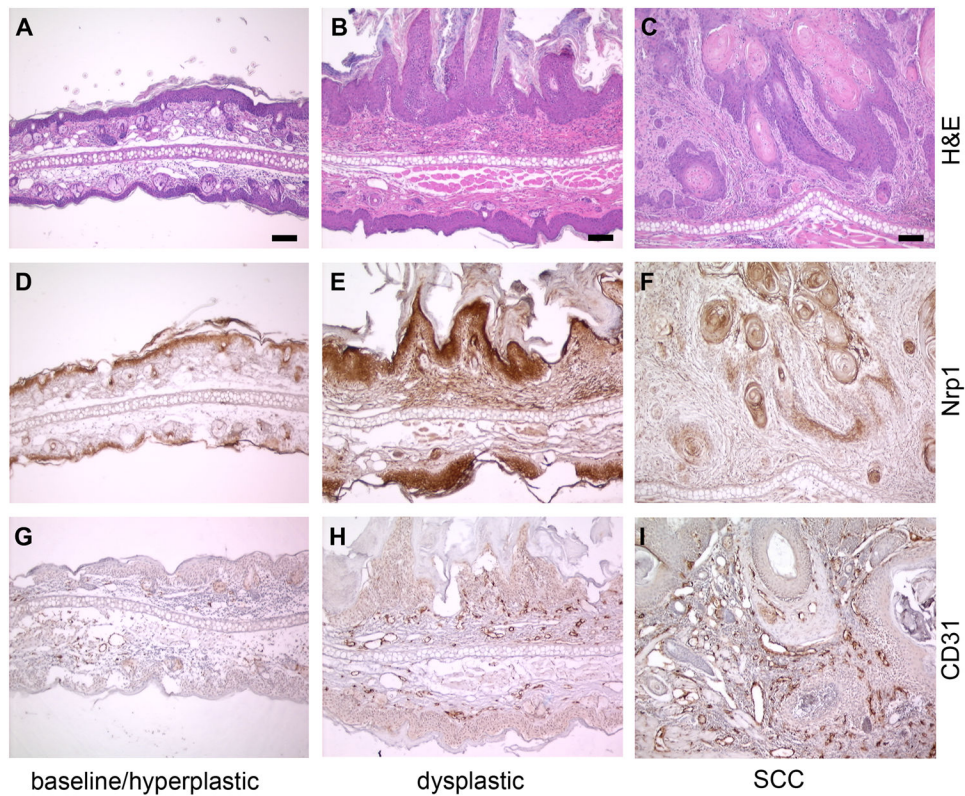
(A) Human skin cancer tissue microarray A216 was stained by IHC for NRP1 protein expression. Illustrated is the scan of entire slide showing 102 sections/biopsies. Six samples are marked with a line on the left and are shown at higher magnification below. (B) Scan of entire section of highly differentiated human SCC sample stained by IHC for NRP1. Tumor shows high expression of NRP1 (brown color). (C) Selected samples from microarray in A demonstrate high NRP1 expression in normal (NR) epidermis and highly differentiated (HD) SCC samples, medium NRP1 expression in moderately differentiated (MD) samples, and the lack of NRP1 expression in poorly differentiated (PD) samples. Scale bar = 200  $\mu\text{m}$ .





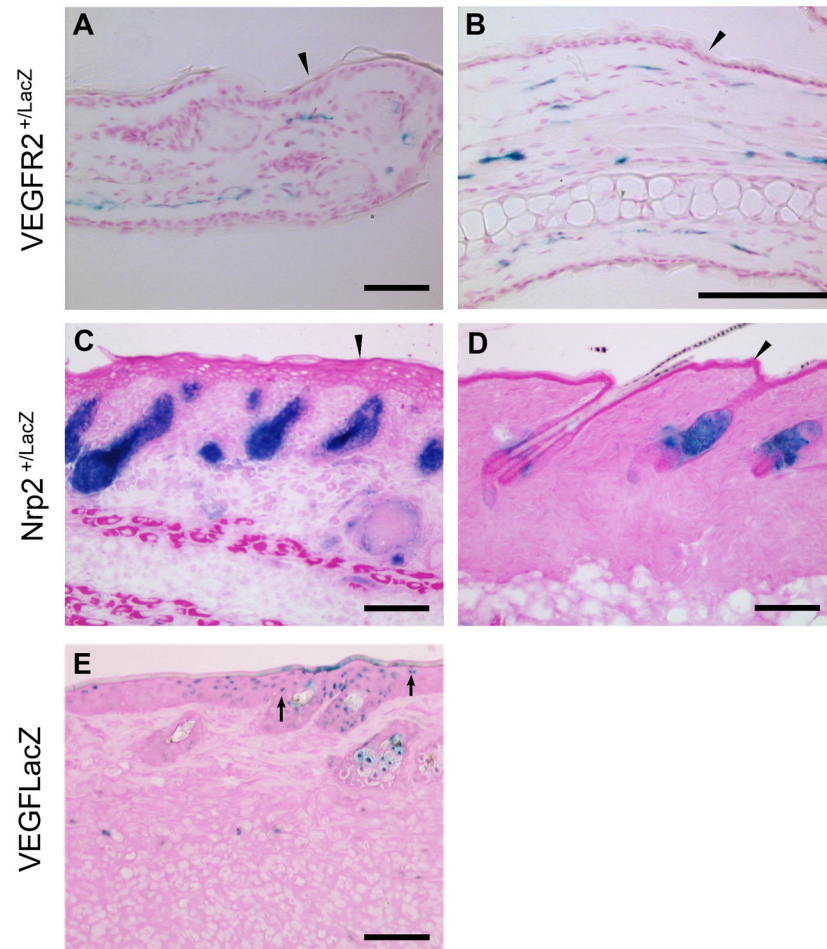
**Figure 3. Differentiated cells of human SCC xenografts in mice express the highest levels of NRP1**

Three human SCC cell lines were grown in nude mice: well-differentiated SCC13 (A, D, G), moderately differentiated DJM1 (B, E, H), and poorly differentiated A431 (C, F, I). Paraffin sections of tumors were stained by IHC for human NRP1 (A-C), human K1 (D-F), and human K14 (G-I). Note that human NRP1 antibodies do not stain mouse blood vessels. NRP1 is absent from poorly differentiated human SCC cells (arrows in A, D). Scale bar = 100  $\mu$ m.



**Figure 4. Nrp1 is expressed in normal mouse epithelium and murine SCC**

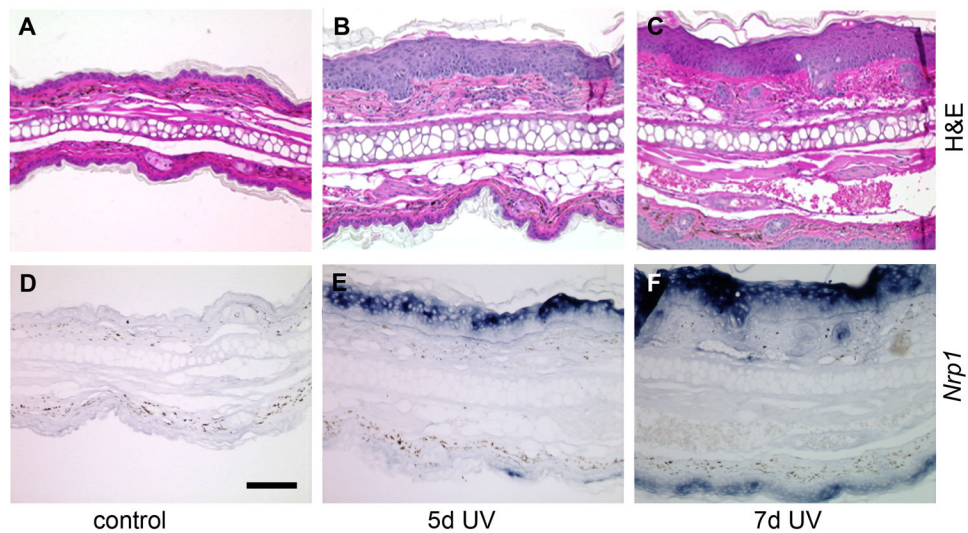
Transgenic K14-HPV16 mice develop spontaneous and progressive dysplasia and SCC. Paraffin sections of ears from K14-HPV16 mice were obtained before overt disease (normal) (A, D, G), from dysplastic ears (B, E, H), or from SCC tumors (C, F, I). Sections were stained with H&E (A-C) or immunostained for mouse Nrp1 (brown color) (D-F) or mouse CD31 (brown color) (G-I). Nrp1 expression increased in dysplastic lesions (E) and was expressed in differentiated areas of SCC (F). Microvessel density progressively increased from normal/hyperplastic epidermis (G) to dysplasia (H) to SCC (I). Some sections (D-I) were counterstained with hematoxylin (blue color). All images were taken at 200 $\times$  magnification; scale bars = 100  $\mu$ m. Notice the ears increase dramatically in thickness.



**Figure 5. Murine keratinocytes express VEGF but not VEGFR2 or Nrp2**

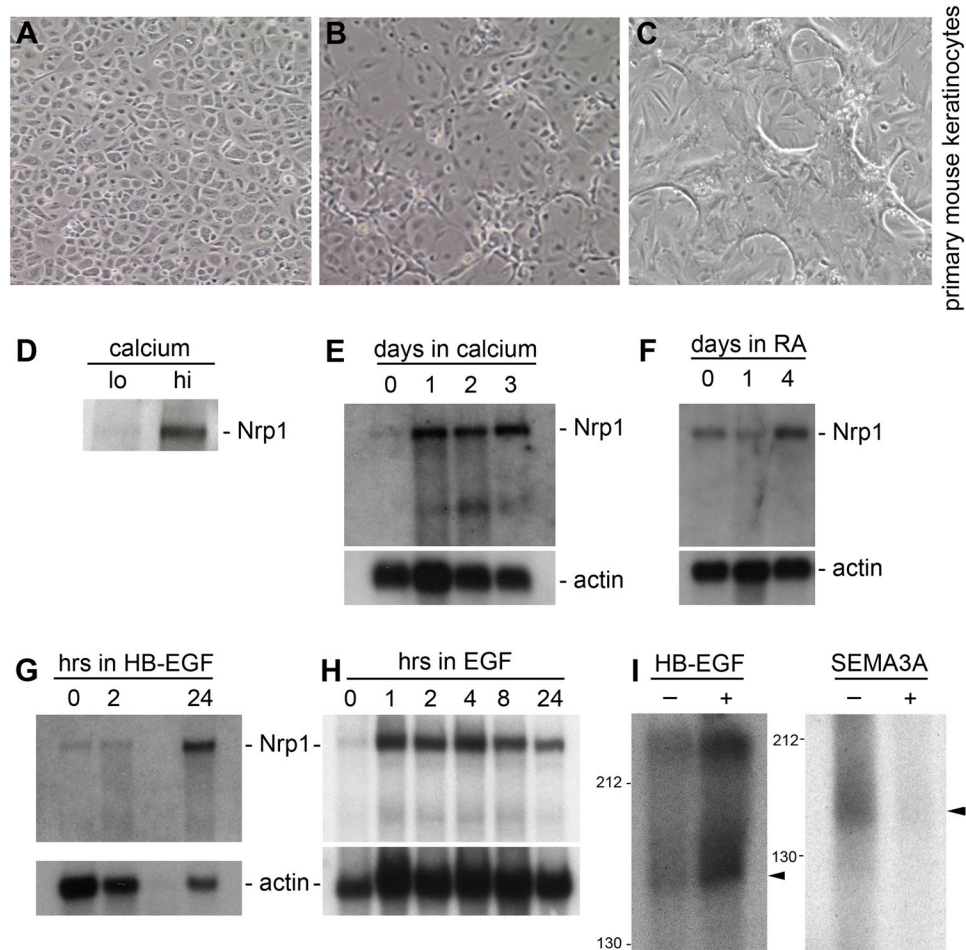
Cryosections of VEGFR2<sup>+/LacZ</sup> mouse ears (A, B), Nrp2<sup>+/LacZ</sup> mouse skin (C, D) and VEGFLacZ mouse skin (E) were stained with X-gal reagent to detect β-galactosidase. (A-B) Blue staining shows VEGFR2 expression in dermal blood vessels but not in epithelial cells (arrow heads). (C-D) Blue staining demonstrates Nrp2 expression in melanocytes of hair follicles and some dermal blood vessels, but not in the epidermis (arrow heads) of P1 mouse skin (C) or P42 mouse skin (D). Scale bars in A-D = 100 μm. (E) Blue staining nuclei denote cells producing VEGF (arrows). Keratinocytes in suprabasal layers and hair follicles make VEGF. Sections were counterstained with Eosin (pink color). Scale bar in E = 50 μm.





**Figure 6. Nrp1 mRNA expression increased following UVB irradiation**

Paraffin sections of mouse ears from normal, non-irradiated (control) (A, D) or UVB irradiated mice (B, C, E, F). Sections were stained with H&E (A-C) or by ISH with probes to mouse Nrp1 mRNA (purple color). Nrp1 expression was increased in epidermal cells and dermal vessels after UVB irradiation (E, F). Nrp1 mRNA expression was seen only in suprabasal cells of the epidermis. There was low Nrp1 expression in normal ears at short chromogen incubations (D) but expression was detectable when incubation time was increased (not shown). All images were taken at 200 $\times$  magnification; scale bars = 100  $\mu$ m. Notice the ears increase dramatically in thickness.



**Figure 7. NRP1 expression is regulated by differentiation and growth factors in vitro**  
 (A-C) Primary mouse keratinocytes were isolated and cultured from P3 Balb/c mice. Cells were grown in (A) low calcium growth media or differentiating high calcium media for (B) one to (C) three days. Phase contrast microscopy demonstrated changes in cell shape and morphology. (D) Western blot of protein lysates from primary mouse keratinocytes cultured in low (lo = 0.05mM) or high (hi = 0.12mM) calcium media. Nrp1 protein is upregulated in cells grown in the differentiating media. (E-F) Northern blot of mRNA from primary mouse keratinocytes cultured in (E) high calcium media or (F) retinoic acid (RA) for various time points. *Nrp1* was induced in cells grown for one day in high calcium media or four days in RA. (G-H) Northern blot of mRNA from primary mouse keratinocytes (G) or human Hacat cells (H) incubated for various times in growth factor. Addition of HB-EGF or EGF upregulated NRP1 expression in keratinocytes. (I)  $I^{125}$ -VEGF<sub>165</sub> crosslinking to NRP1 on Hacat cells increased after pretreatment in HBEGF (left panel).  $I^{125}$ -VEGF<sub>165</sub> crosslinking to NRP1 on Hacat cells was competed in the presence of excess SEMA3A protein (right panel). Arrowheads point to the crosslinked complex of NRP1/VEGF.

Table 1

Neuropilin expression correlates with differentiation status in human squamous cell carcinoma samples in a tissue microarray.

Differentiation Status	NRPI Staining		Sex	Age	TNM	Stage	Size (cm)	
	Area	Intensity Index						
PD <sup>#</sup>	2	1	2	M	78	T1N0M0	I	1.8*1.7*1.5
PD	2	1	2	M	78	T2N0M0	II	2.5*1.5
PD	3	1	3	M	48	T2N0M0	II	3.5*3.0*1.7
PD	3	1	3	F	63	T2N1M1	IV	5.0*3.8*1.8
MD	3	1	3	M	67	T1N0M0	I	2.0*1.6*1.6
MD	3	1	3	F	66	T1N0M0	I	1.8*0.8
MD	3	1	3	F	71	T1N0M0	I	0.7
MD	3	1	3	F	74	T1N0M0	I	0.7*0.2
MD	3	1	3	M	29	T3N0M0	II	6.0*6.0*3.0
MD	3	1	3	F	78	T N&M1	IV	4.5*3.5
MD	3	2	6	F	85	T1N0M0	I	0.7
MD	3	2	6	M	44	T4N2bM1	IV	1.5
MD	3	2	6	M	74	T2N0M0	II	4.0*3.0*1.0
MD-HD	3	2	6	M	66	T2N0M0	II	3.5*2.5*0.8
HD	3	2	6	M	74	T1N0M0	I	2.2*1.2
HD	3	2	6	M	72	T3N0M0	II	5.5*5.5
HD	3	3	9	M	53	T2N0M0	II	5.0*3.0
HD	3	3	9	M	53	T3N0M0	II	8.0*7.5
NR	3	2	6	M	45			
NR	3	2	6	M	70			
NR	3	3	9	M	45			
NR	3	3	9	F	75			

<sup>#</sup> poorly differentiated (PD), moderately differentiated (MD), highly differentiated (HD), normal skin (NR)