Distinct Functions of Epidermal and Myeloid-Derived VEGF-A in Skin Tumorigenesis Mediated by HPV8

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Abstract

Beta human papillomaviruses (HPV) have been suspected to be carcinogenic in nonmelanoma skin cancers (NMSC), but the basis for potential viral contributions to these cancers is poorly understood. In particular, it is unresolved how HPV-infected keratinocytes escape cell-cycle control and whether their cross-talk with immune cells is critical for tumorogenesis. In nonviral preclinical models, the angiogenic cytokine VEGF-A has been identified as a critical regulator of NMSC. In this study, we dissected the contribution of epidermal versus myeloid cell–derived VEGF-A in HPV-mediated skin cancer by interbreeding an HPV8 transgenic mouse model with a conditional disruption of VEGF-A restricted to either epidermal or myeloid cells. Although only epidermal-derived VEGF-A was essential for initiation of skin tumor development, both spontaneously and UV-light triggered, both epidermal and myeloid cell–derived VEGF-A contributed to regeneration-induced tumorogenesis upon HPV8 overexpression, partly not only through a paracrine effect on endothelial cells, but also most probably through an additional autocrine effect on epidermal cells. Our findings offer new mechanistic insights into distinct functions of epidermal versus myeloid cell–derived VEGF-A during HPV-mediated tumorogenesis, with possible implications for preventing this disease.

Introduction

Human papillomaviruses (HPV) belong to a family of small DNA viruses that can infect the epithelia of skin and mucosa. Viral persistence in the epithelium can lead to the development of benign or malignant proliferative lesions. To date, over 170 different types of HPVs have been identified. The causal relationship between α-genus HPV infection of mucosa (e.g., HPV16) and the development of squamous cell carcinoma (SCC) of the genital tract is well established. HPV subtypes of the β-genus are suspected of playing a role in nonmelanoma skin cancer (NMSC), but their direct causal role in this process has been difficult to establish using epidemiologic approaches due to the ubiquitous prevalence of the viruses in the general population and their absence in some cancers. In 2009, the International Agency for Research on Cancer classified HPV5 and 8 as “possibly carcinogenic” in nonmelanoma skin cancer, but the basis for potential viral contributions to these cancers is poorly understood. In particular, it is unresolved how HPV-infected keratinocytes escape cell-cycle control and whether their cross-talk with immune cells is critical for tumorogenesis. In nonviral preclinical models, the angiogenic cytokine VEGF-A has been identified as a critical regulator of NMSC. In this study, we dissected the contribution of epidermal versus myeloid cell–derived VEGF-A in HPV-mediated skin cancer by interbreeding an HPV8 transgenic mouse model with a conditional disruption of VEGF-A restricted to either epidermal or myeloid cells. Although only epidermal-derived VEGF-A was essential for initiation of skin tumor development, both spontaneously and UV-light triggered, both epidermal and myeloid cell–derived VEGF-A contributed to regeneration-induced tumorogenesis upon HPV8 overexpression, partly not only through a paracrine effect on endothelial cells, but also most probably through an additional autocrine effect on epidermal cells. Our findings offer new mechanistic insights into distinct functions of epidermal versus myeloid cell–derived VEGF-A during HPV-mediated tumorogenesis, with possible implications for preventing this disease.

The mechanisms of HPV-mediated skin tumor development are still under debate. In contrast with mucosal high-risk HPV types (e.g., HPV16), the primary oncoproteins E6 and E7 of HPV5 and HPV8 do not or only weakly interfere directly with the central regulators of cell cycle and apoptosis p53 and pRB. Some activities of HPV5 and 8 E6 clearly act to enhance the carcinogenic potential of sun exposure. The E6 proteins of several beta papillomavirus types, including HPV5 and 8, target the proapoptotic protein Bak for degradation and thus prevent UV-induced apoptosis. They furthermore bind to p300, resulting in inhibition of p53-induced apoptosis. 9. They further enhance p300 degradation and thus prevent UV-induced apoptosis. 9, reduction of ATR mRNA and protein levels, and finally delayed repair of UV-damaged DNA. These activities may result in chromosomal instability particularly in the context of sun exposure and may eventually contribute to cancer development. Independent oncogenic effects may be expected from E6–MAML1 interactions, which repress the Notch signaling pathway, which has tumor suppressor function in the skin. HPV8 E7–induced upregulation of the MT1 matrix metalloproteinase may explain invasion of E7–positive keratinocytes into the dermis of organotypic keratinocyte cultures.

To gain insight into the molecular mechanisms underlying HPV8-mediated skin tumor development in vivo, we previously developed HPV8 transgenic mouse models that recapitulate the HPV8-induced SCC pathology and have been proven to be a valuable in vivo model to unravel the molecular pathology of HPV-induced skin cancer. Transgenic expression of the complete early genome region (CER) of HPV8 under the human keratin14 (K14) promoter in mice (FVB/N background) is sufficient to induce multifocal or single premalignant skin papillomas with varying degrees of epithelial dysplasia in over 90% of the mice within the first year of age. SCCs developed in...
approximately 6% of the transgenic mice. Early hyperproliferative skin lesions in HPV8 mice are characterized by a subepidermal immune cell infiltrate (14). Papillomatosis in HPV8 transgenic mice could be significantly accelerated by exposure to UV/A/UVB light or mechanical skin injury (15, 16).

The growth of new blood vessels is essential for the malignant phenotype in most human cancers (19), and most tumor cells induce the expression of angiogenic factors at an early point of development, such as the homodimeric glycoprotein VEGF-A (hereafter abbreviated with VEGF; ref. 20). In several carcinomas including SCCs, increased VEGF expression has been detected in epithelial tumor cells, and has been proposed to be linked to tumor progression and invasion (21–25). In addition, inflammatory cell types within the tumor stroma particularly cells of the myeloid lineage have been shown to be important sources of VEGF and to functionally affect tumorigenesis (26, 27). VEGF mediates its activities primarily through binding to two receptor tyrosine kinases, known as VEGFR1 and VEGFR2, as well as to coreceptors including neuropilins (Np; ref. 20). The classical concept that paracrine VEGF contributes to tumor growth through angiogenesis has been challenged by recent reports, proposing that VEGF may act in addition as an autocrine survival factor on tumor cells (24, 28, 29). Thus, the precise role of VEGF signaling and of neovascularization in HPV8-mediated skin development still remains elusive. Here, we investigated the in vivo function of epidermis- and myeloid cell–specific VEGF expression in HPV8-induced skin carcinogenesis by combining our established model of HPV8-induced skin cancer with cell type–restricted VEGF ablation. Our findings provide evidence for distinct functions of epidermis- and myeloid cell–specific VEGF in HPV8-mediated tumour formation.

Materials and Methods

Animals

Transgenic mice expressing CER of HPV8 (HPV8 mice) from the human keratin 14 (K14) promoter (14), VEGFβ/K14-Cre (VEGFβflata), and VEGFβ/Hytm-Cre (VEGFβflata) mice (30) were generated and genotyped as described. HPV8 mice were mated to VEGF mutants to obtain VEGFβflataHPV8 and VEGFβflataHPV8 mice, and additional genotypes as specified throughout the manuscript. Individual expression of Cre or the heterozygous/homozygous loxP-flanked alleles of VEGF did not affect the phenotype of HPV8 transgenic or wild-type mice. All mice were backcrossed to the FVB/N background for at least six generations. VEGF-lacZ knock-in reporter mice were mated with HPV8 mice to obtain VEGF-lacZHPV8 mice and genotyped as described (31). Mice were examined every 3 days to monitor the development of skin tumors. All animal experiments were approved by the national animal care committee and the University of Cologne (Cologne, Germany).

UV-light exposure and wounding

UV-light exposure, wounding, and tissue preparation for histology were performed as described previously (15). Briefly, for UV irradiation, mice were anesthetized, shaved, and an area of 2 cm² on the back was UV irradiated. UV radiation was generated by a UV device (UV801; Waldmann) equipped with 8 PUVA lamps (UV: 320–400 nm) and 4 U2V1 lamps (UVB: 280–360 nm). The energy output of the UV device as measured at the level of the skin surface using a radiometer was 1.41 mW/cm² for the UVB region and 6.34 mW/cm² for the UVA region (UV Radiometer Variocontrol; Waldmann). Animals received one single UV-light exposure (UVA 2.5 J/cm²; UVB 0.25 J/cm²) to induce tumor formation (15). For wounding, mice were anesthetized and shaved as described above, and four full-thickness punch biopsies were created on the back (30). For histologic analysis, tissues were either fixed in 4% formaldehyde or embedded in optimal cutting temperature compound (Tissue Tek).

Inhibition of VEGFR2

Inhibition of VEGFR2 was performed by intraperitoneal injections of anti-VEGFR2 blocking antibody (DC101, BioXcell) or the corresponding isotype-matched control antibody (IgG1, BioXcell; 40 mg/kg body weight in PBS). Injections started at day 5 after wounding and were continued for three times/week until day 26 after injury. This antibody has been shown to be a potent inhibitor of VEGFR2 (32, 33).

Immunohistochemistry

Immunohistochemistry (IHC) stainings were performed as described previously (30). Briefly, cryosections (10 μm) were fixed (4% PFA or in methanol), blocked (10% NG in PBS, 5% BSA, 0.2% Triton-X-100), and incubated (minimum 1 hour at room temperature – maximum overnight at 4°C) with the primary antibodies diluted in blocking buffer. Primary antibodies used were: CD31 (1:1000, BD Pharmingen); Ki-67 (1:50, Dako); active caspase-3 (1:100, Cell Signaling Technology); F4/80 (1:200, Dianova); Gr1 (1:100, BD Pharmingen); VEGF-A (1:100, Santa Cruz Biotechnology); VEGFR1 (1:200, Santa Cruz Biotechnology); VEGFR2 (1:50, BD Pharmingen); and Nrp1 (1:100, R&D). Bound primary antibody was detected by incubation with Alexa-Fluor 488- or Alexa Fluor 594-conjugated (Invitrogen) or peroxidase-conjugated (EnVision System, Dako) secondary antibodies (1 hour, room temperature), followed by counterstaining with DAPI (Invitrogen), propidium iodide, or hematoxylin. Specificity of primary antibodies was demonstrated by replacing them with irrelevant isotype-matched antibodies.

X-Gal staining

For X-Gal staining, cryosections (20 μm) were prepared from VEGF-lacZ wound tissues (fixed in 0.5% glutaraldehyde for 30 minutes at room temperature) and washed in washing buffer (PBS, 0.02% NP-40, and 0.2 mmol/L MgCl₂). Sections were incubated in X-Gal staining solution containing 0.5 mg/mL of X-Gal (Fermentas), 10 mmol/L K₃[Fe(CN)₆], 10 mmol/L K₃[Fe(CN)₆], and 0.2 mmol/L MgCl₂ for 6 hours at 37°C and counterstained with a nuclear fast red aluminum sulfate solution.

Morphometric analysis

Morphometric analysis was performed on hematoxylin and eosin (H&E) or Giemsa-stained paraffin tissue sections. Papilloma/tumor formation was quantified by measuring epidermal and dermal compartments in a defined area of the skin lesion. Immunofluorescence images were acquired using a fluorescence microscope (BX-9000 Fluorescence Microscope, KEYENCE). For investigation of the average blood vessel density, staining for CD31 and VEGFR2 was quantified in six representative high-power fields (HPF, 200 × 300 μm²)/section in at least two independent sections of one lesion using ImageJ software; the data are expressed as the percentage of CD31 or VEGFR2-positive areas within the dermal tumor stroma. Ki-67–positive keratinocytes were quantified as described previously (34, 35). Briefly,
Ki-67-positive cells were determined within the stratum basale of lesional skin over a distance of 100 μm in five representative fields/section in at least two independent sections of one lesion; the data are expressed as the percentage of Ki-67-positive basal cells. Numbers of F4/80⁺, Gr1⁺, and mast cells were determined by counting positively stained cells in six representative HPFs (200 × 300 μm²)/section of one lesion. Analyses were performed in a blinded manner by two independent investigators. Images were processed with Adobe Photoshop Version 7.0 software.

Cell culture

Keratinocytes were isolated from the skin of newborn mice and cultured using a mitomycin-C-treated 3T3-J2 feeder layer as previously described (36). Briefly, keratinocytes were expanded in growth medium [DMEM/Ham's F-12 (Biochrom), 10% chelex-treated FBS, 20 ng/mL EGF, 10 μg/mL insulin (R&D; 32°C, 5% CO₂)] and used for experiments at passage 3. For the cell proliferation assay, feeder cells were removed [0.1X Trypsin/EDTA (GIBCO)], keratinocytes were starved in starvation medium [DMEM/Ham's F-12 (Biochrom), 20 ng/mL EGF, 10 μg/mL Insulin (R&D), 4 hours] and subsequently stimulated in starvation medium complemented with increasing concentrations of serum, with or without recombinant murine VEGF-A [(rmVEGF) 100 ng/mL, R&D, 20 hours]. After bromodeoxyuridine (BrdUrd) pulse (10 μmol/L, 4 hours), BrdUrd incorporation was analyzed by FACS analysis (FACSCanto II flow cytometer) using the APC BrdU Flow Kit (BD).

Peritoneal cells were seeded in 6-well plates (0.5 × 10⁶ cells/cm²), macrophages enriched by plastic adhesion, cultured over night, and stimulated with lipopolysaccharide (LPS; 1 mg/mL; Sigma Aldrich) and recombinant IFNγ (0.1 mg/mL; R&D Systems) in DMEM (1% FCS) as previously described (30). RNA was isolated and used for qRT-PCR analysis as described below.
Flow-cytometric analysis and cell sorting

Keratinocytes were isolated from tumor tissues by a combination of enzymatic digestion (Liberase Blendzyme, Roche Applied Science) and mechanical disruption (Medimachine System, BD Biosciences) as previously described (30). For FACS sorting, keratinocytes were stained as described previously (37). Briefly, cells were passed through a 40-μm cell strainer, blocked for 15 minutes with BSA (2% in PBS), washed with PBS, and incubated with the following antibodies: FITC-conjugated anti-CD45 (clone 30-F11, eBiosciences), APC-conjugated anti-CD140a (clone APA5, eBiosciences) and anti-CD31 (clone MEC13.3, BD Pharmingen), and PE-Cy7-conjugated anti-Epcam (clone G8.8, Biolegend) in FACS buffer. Dead cells were excluded using 7-AAD (BD Biosciences). Epcam<sup>+</sup>Lin<sup>-</sup>/C0 (CD45/C0, CD31/C0, CD140a/C0) cells were sorted by FACSAria cell sorting system (BD Biosciences).

Real-time PCR analysis

RNA was isolated from tissues and reverse transcription PCR was performed as previously described (30). The hyperproliferative epidermis was separated from the dermis (0.5 mol/L ammonium thiocyanate solution) as described (38) and tissues were stored in RNA later (Ambion). Primer sequences for VEGF-A and Nrp1 were described previously (30) or will be available upon request; for HPV8-E6 and HPV8-E7 expression analysis, the following forward and reverse primers (5' to 3') were used:

- E6: GCGGCTTTAGGTATTCCATTGC, GCTACACAACAACAACACG
- E7: CCTGAAGTGTTACCAGTTGACCTGC, CAGTTGCGTTGACAAAAAGCG

Amplification reactions (triplicates) were set up using PowerSYBR Green PCR Master Mix (Applied Biosystems) and qRT-PCR was validated with the StepOnePlus Real-Time PCR system (Applied Biosystems). The comparative method of relative quantification (2<sup>-DeltaCt</sup>) was used to calculate expression level of the target gene normalized to S26.

Statistical analysis

Significance of difference was analyzed using ANOVA one-way test analysis with Bonferroni multiple comparison test. All data are presented as mean ± SD, a P value of ≤0.05 was considered significant. The Kaplan–Meier method and the log-rank test were used to compare tumor-free states between mouse genotypes.
Figure 3.
Epidermis-specific VEGF deletion prevents UV light-triggered skin tumor development in HPV8 transgenic mice. A, top, X-Gal staining of UV irradiated skin in VEGF-lacZ-HPV8 mice (n = 15); bottom, representative VEGF (red)/F4/80 (green) double immunofluorescent stainings of skin sections from HPV8 mice at day 10 (n = 5) and 34 (n = 5) after UV irradiation. B–D, macroscopic appearance (B), H&E staining (C), and morphometric quantification of skin lesions at different time points after UV irradiation (n = 8–14 mice/genotype; D). E, qRT-PCR analysis of E6 and E7 expression in epidermis of untreated skin and skin 10 and 34 days after UV irradiation in different mouse mutants; each dot represents one mouse. Data are expressed as means ± SD. ***, P < 0.001; **, P < 0.01; *, P < 0.05. e, epidermis; d, dermis; h, horn pearls outlined by hatched line; sf, subcutaneous fat tissue; sm, skeletal muscle; dotted line, the dermoepidermal junction. Genotypes used, control: n = 6 VEGFfl/fl, n = 4 LysMCre, n = 4 K14Cre; HPV8: n = 12 K14-HPV-CER/VEGFfl/fl, n = 4 K14-HPV-CER/LysMCre, n = 4 K14-HPV-CER/K14Cre.

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Results

Multistage squamous carcinogenesis in HPV8 transgenic mice is associated with increased angiogenesis

To examine whether tumor progression in HPV8 transgenic mice is associated with neoangiogenesis, IHC double stainings for CD31 and VEGFR2 were performed in tissue sections at various stages of tumor development. Although CD31 is constitutively detectable on the vascular endothelium, VEGFR2 expression is primarily upregulated in endothelial cells during neoangiogenesis. In macroscopically and histologically nonlesional back skin of HPV8 transgenic mice (8–10 weeks of age), few small CD31+ /VEGFR2− capillaries were detectable in the dermis comparable with wild-type mice (Fig. 1). At the age of 20 to 25 weeks, HPV8 transgenic mice developed hyperplastic lesions with acanthosis, hyperkeratosis, and increased density of CD31+/VEGFR2+ double positive, elongated vessels within papillomas (Fig. 1). Epithelial tumors characterized by epidermal dysplasia showed a high-density vessel network, with elongated and tortuous vessels in close apposition to the overlying neoplastic epithelium (Fig. 1). Infiltration of epidermal tumor cells into the dermal tissue in SSCs was associated with large vessel lumen spreading throughout the dermal tissue (Fig. 1). The remarkable alteration of the vasculature in vessel number, distribution, and morphology during tumor progression was confirmed by increased numbers of CD31+/VEGFR2+ double positive vascular structures particularly detected in close proximity to the neoplastic epidermis (Fig. 1). VEGFR2-positive staining was exclusively detected on vascular structures.

Epidermis-specific VEGF deletion prevents long-term spontaneous skin tumor development in HPV8 transgenic mice, whereas myeloid cell–derived VEGF is dispensable

To analyze the functional impact of epithelial-derived versus myeloid cell–derived VEGF synthesis in HPV8-mediated skin cell proliferation and angiogenesis in UV-triggered papilloma development in VEGFfl/fl−HPV8 mice. A and B, Ki-67 IHC (brown nuclear stain; hematoxylin counterstain) and CD31 immunofluorescence staining (red; DAPI counterstain, blue; A) and morphometric quantification of skin lesions at day 34 after UV irradiation (B); each dot represents the analysis of one lesion per mouse; data are represented as means ± SD; *** P < 0.001. e, epidermis; d, dermis; h, horn pearls outlined by hatched line; sf, subcutaneous fat tissue; dotted line, the dermoepidermal junction.

Genotypes used, control: n = 6 VEGFfl/fl; HPV8: n = 5 K14-HPV-CER/VEGFfl/fl.
tumor development, mice with epidermis-restricted VEGF deletion (VEGF EKO) and myeloid cell–restricted VEGF deletion (VEGFMKO) were mated with transgenic mice in which HPV8 early gene expression is driven by the human K14 promoter (HPV8 mice; refs. 14, 30; Fig. 2A and B). High efficiency of Cre-mediated vegf-a gene deletion in epidermis and macrophages in VEGF EKO and VEGF MKO mice, respectively, was verified by PCR and qRT-PCR analyses (Fig. 2C and D) and was consistent with earlier results in VEGF EKO and VEGF MKO mice (30). We monitored over a period of 52 weeks spontaneous tumor

Figure 5.
Epidermis- and myeloid cell–derived VEGFs are essential for mechanical injury-induced papilloma formation in HPV8 mice. A–C, macroscopic analysis (A), H&E staining (B), and morphometric quantification of papilloma formation in different mouse mutants (n = 6-10 mice/genotype; C). D, morphometric quantification of Ki-67–positive basal cells and CD31-positive area in skin lesions at day 34 after injury; each dot represents one wound. Data are presented as means ± SD; ***, P < 0.001. e, epidermis; d, dermis; st, scar; h, horn pearls outlined by hatched line; dotted line, the dermoeipidermal junction. Genotypes used, control: n = 4 wt, n = 6 VEGFfl/fl, n = 3 LysMCre, n = 4 K14Cre; HPV8: n = 5 K14-HPV8-CER/VEGFfl/fl, n = 4 K14-HPV8-CER/K14Cre, n = 3 K14-HPV8-CER/LysMCre.
development in cohorts of VEGF-EKO HPV8 (n = 10), VEGF-MKO HPV8 (n = 10), and HPV8 (n = 10) mice. The Kaplan–Meier plot illustrating the tumor-free state as a function of time showed striking differences between the three genotypes (Fig. 2E). In line with our previous observation, skin tumor development initiated in more than 50% of HPV8 mice at the age of 20 to 25 weeks and nearly all mice presented tumors at 52 weeks of age (Fig. 2E; ref. 14). The lesions were irregularly distributed and characterized by hair loss, ulcerations, and irregularly shaped papillomas; they occurred most often at the laterodorsal trunk (14). Notably, in VEGF-EKO HPV8 mice, spontaneous tumor development was entirely abrogated during 1 year of assessment (the oldest VEGF-EKO HPV8 mice are now 1.5 years old and are still tumor free; Fig. 2E). In contrast, no significant difference was observed in spontaneous tumor development in VEGF-MKO HPV8 mice when compared with HPV8 mice (Fig. 2E). The macroscopic appearance and the localization of skin tumors developing in VEGF-MKO HPV8 and HPV8 mice were similar.

Epidermis-specific VEGF deletion prevents UV-light triggered skin tumor development in HPV8 transgenic mice, whereas myeloid cell–derived VEGF is dispensable

We previously showed that a single UV-light exposure led to a synchronized induction of papilloma development in approximately 3 weeks in HPV8 transgenic mice (15). To study the role of VEGF in this rapid UV-triggered HPV8-mediated skin tumor development, VEGF-EKO HPV8, VEGF-MKO HPV8, HPV8, and control mice were subjected to UV irradiation. Yet, first we identified VEGF-expressing cells in tumors of UV-light exposed HPV8 transgenic mice, and we mated VEGF-lacZ reporter mice with HPV8 transgenic mice. As revealed by β-galactosidase staining, skin not exposed to UV-light showed...
whereas HPV8 and VEGFMKOHPV8 mice showed pronounced complete restoration of skin integrity over a time course of 34 days, VEGF signal was present in both nuclei and cytoplasm. keratinocytes and macrophages (Fig. 3A); in keratinocytes, the VEGFMKOHPV8 mice, respectively (Fig. 4). In addition, in HPV8 Ki-67 positive within the acanthotic epidermis in HPV8 and papillomas of UV-treated HPV8 mice identi
ing the macrophage antigen F4/80 in day 10 and day 34 stained for the presence of Ki-67 (Fig. 4). Although in control and exposed skin after 34 days of irradiation, tissue sections were data not shown). Expression of viral E6 and E7 in HPV8 and papilloma formation at the ulcer edges (Fig. 3B). In contrast, ulcers in VEGFMKOHPV8 mice showed a similar rate as in control mice, without developing any sign of papilloma. H&E-stained tissue sections at day 34 after UV irradiation confirmed the absence of papillomas and complete epithelialization of erosions in control and VEGFEGKOHPV8 mice (Fig. 3C and D). The irradiated area at day 34 was in both mouse strains characterized by a mild acanthosis. In contrast, UV-exposed skin in HPV8 and VEGFMKOHPV8 mice showed profound squamous papilloma development, including a significant increase in hyperplasia, hyperkeratosis, parakeratosis, and characteristic horn pearls (Fig. 3C and D). The papillary dermis in HPV8 and VEGFMKOHPV8 mice was characterized by a mixed inflammatory cell infiltrate dominated by mononuclear cells/macrophages and mast cells (Fig. 3C and data not shown). Expression of viral E6 and E7 in HPV8 and VEGFMKOHPV8 mice was confirmed by qRT-PCR analysis in epidermis separated from untreated and lesional skin (Fig. 3E).

To analyze the proliferation status of epidermal cells in UV exposed skin after 34 days of irradiation, tissue sections were stained for the presence of Ki-67 (Fig. 4). Although in control and VEGFEGKOHPV8 mice, about 29.81% ± 3.38 and 23.55% ± 2.32 of cells within the stratum basale stained positive for Ki-67, respectively, about 78.47% ± 1.41 and 79% ± 11.51 basal cells stained Ki-67 positive within the acanthotic epidermis in HPV8 and VEGFEGKOHPV8 mice, respectively (Fig. 4). In addition, in HPV8 and VEGFEGKOHPV8 mice Ki67+ cells scattered throughout supra-basal layers (Fig. 4). Furthermore, as shown by CD31 staining, the dermal compartment of papillomas in HPV8 and VEGFEGKOHPV8 mice showed a significant increase of elongated, enlarged, and tortuous blood vessels characteristic for tumor vascularization, when compared with few and small capillaries detected in the dermis of control and VEGFEGKOHPV8 mice (Fig. 4).

Epidermis- and myeloid cell–derived VEGF is critical for mechanical injury-induced papilloma formation in HPV8 transgenic mice

We next examined whether the opposing contribution of epidermal- and myeloid cell–derived VEGF in spontaneous or UV-light triggered tumor induction in HPV8 transgenic mice may be related to the nature of cell and/or tissue injury. We inflicted full-thickness excision skin wounds on the backs of control and mutant mice and monitored the wound-healing response over 34 days. After approximately 8 days, wound edges in HPV8 mice (here we included also K14-HPV8-CER/K14Cre and K14-HPV8- CER/LysMcCre mice as controls to exclude interference of Cre expression in K14Cre or LysMcCre mice with tumor formation) showed signs of developing papillomatosis, which were macroscopically clearly visible, and gave raise to full papillomas by day 34 after injury (Fig. 5A). Notably, wounds in control, VEGFMKOHPV8, and VEGFEGKOHPV8 mice did not develop signs of papillomatosis and healed completely by day 34 after injury (Fig. 5A). H&E-stained tissue sections of wounded skin at day 34 confirmed squamous papilloma formation in HPV8 mice (Fig. 5B and C). In contrast, tissue sections in control, VEGFMKOHPV8, and VEGFEGKOHPV8 mice all displayed a closed, slightly hyperproliferative epithelial and scar tissue, typical for late stage, closed excisional skin wounds in healthy mice (Fig. 5B). Papilloma development in wounds of HPV8 mice was paralleled by a significant increase in Ki67+ basal cells, which in addition scattered throughout the hyperplastic epidermis, and a highly vascularized tumor stroma characterized by increased numbers of F4/80+ cells (macrophages), Gr1+ cells (polymorphonuclear cells), and mast cells (Fig. 5D and Supplementary Fig. S1). To examine whether myeloid cell–derived VEGF controls tumor initiation by a direct effect on the inflammatory response (e.g., through chemotaxis), we analyzed the inflammatory cell infiltrate at the early stage of tumor development (day 8 after injury) in control, HPV8, and VEGFEGKOHPV8 mice. Although polymorphonuclear cells and mast cells were virtually absent in the papillary dermis of epitelialized wounds in all three genotypes, macrophages predominated the inflammatory cell infiltrate and their numbers were similar in different genotypes. Hence, based on these findings, it is unlikely that myeloid cell–derived VEGF-A promotes tumor initiation by a direct effect on the inflammatory response.

To investigate whether the vasculature is a target of VEGF in HPV8-mediated skin tumor development, we used an antibody-based strategy to inhibit angiogenesis by blocking VEGFR2 signaling in endothelial cells. As shown by IHC, VEGFR2 staining was exclusively detected on tumor vasculature in HPV8 transgenic mice, and not in the tumor epithelium (Fig. 1). Starting on day 5 until day 25 after wounding, anti-VEGFR2 antibody (DC101) or isotype control antibody was repetitively administered by intraperitoneal injections. In HPV8 mice receiving isotype control antibody, papilloma formation was macroscopically visible at wound edges at day 15 after injury and progressed in size by day 23 after injury (Fig. 6A). In contrast, in HPV8 mice receiving anti-VEGFR2 antibody (DC101), papilloma development was not detected until day 26 after injury (Fig. 6A). H&E-stained tissue sections of wounded skin in HPV8 mice receiving isotype control

**Figure 7.** Epidermal expression of Nrp1 and VEGFR2 in papillomas in HPV8 mice. A, keratinocytes isolated from newborn wild-type or HPV8 mice (K14-HPV8-CER) were cultured in different conditions as indicated, BrdUrd+ cells are expressed as fraction of all keratinocytes in the culture dish (n = 3 mice/genotype; two independent experiments were performed). B, qRT-PCR analysis for genes as indicated in epidermis isolated from skin of diverse mouse mutants at different time points after UV irradiation (control; n = 3 VEGFEGKOHPV8; HPV8; n = 4 K14-HPV8-CER/VEGFEGKOHPV8; VEGFEGKOHPV8; expression was normalized to gene expression in epidermis of untreated skin in control mice; data are presented as means ± SD. *P < 0.001; **P < 0.001; ***P < 0.05). C, qRT-PCR analysis for VEGF, Nrp1, VEGFR1, and VEGFR2 expression in Epcam+ Lin− keratinocytes isolated from untreated skin and day 30 UV-light-treated skin of HPV8 mice (n = 4 K14-HPV8-CER/VEGFEGKOHPV8). D, representative IHC staining for Nrp1 (brown; hematoxolin counterstain) and immunofluorescence double staining for VEGFR1 (green) and CD31 (red; counterstain DAPI, blue) in skin sections of HPV8 mice (n = 4 K14-HPV8-CER/VEGFEGKOHPV8) 34 days after irradiation.
cells and tumor vasculature were significantly increased in wounded skin in HPV8 mice receiving isotype control antibody, when compared with HPV8 mice receiving blocking antibody (Fig. 6D and E). Staining for Ki-67 and caspase-3 in nonlesional skin adjacent to tumors or wound tissue in IgG1 or αVEGFR2-treated mice was comparable, indicating that αVEGFR2 has no direct (neangiogenesis independent) effect on epidermal proliferation or apoptosis (Supplementary Fig. S2).

Epidermal expression of Nrp1 and VEGFR1 in papillomas in HPV8 mice

We next explored whether in addition to VEGF-mediated angiogenesis-dependent mechanisms of papilloma development in HPV8-transgenic mice also angiogenesis independent, autocrine signaling of VEGF in keratinocytes may contribute to rapid tumor development. Recent studies reported on unexpected and important autocrine functions of VEGF in epidermal tumor cells (24, 28, 33, 35). Therefore, we first addressed the question whether HPV8 transgenic keratinocytes acquire a cell-autonomous growth advantage when compared with wild-type keratinocytes, and whether external rmVEGF acts as possible mitogen on HPV8 transgenic keratinocytes. For this purpose, primary keratinocytes were isolated from newborn (nonlesional) wild-type and HPV8 transgenic mice and cell proliferation was analyzed by BrdUrd incorporation in the presence of growth medium (DMEM/Ham’s F-12, EGF 20 ng/mL, insulin 10 μg/mL) complemented with increasing serum concentrations and rmVEGF (100 ng/mL; Fig. 7A). Both wild-type and HPV8 transgenic cells showed robust BrdUrd incorporation in response to increasing serum concentrations (Fig. 7A), which, however, was independent of externally added rmVEGF (Fig. 7A). Thus, transgenic expression of CER of HPV8 in newborn (nonlesional) keratinocytes is not sufficient to convey a cell-autonomous growth advantage over wild-type keratinocytes, and neither renders the transgenic cells sensitive to external rmVEGF as mitogen. These findings suggest that additional factors are required to deregulate keratinocyte proliferation during carcinogenesis in HPV8 mice in vivo.

To gain further insight into potential autocrine VEGF effects in keratinocyte during tumorigenesis in HPV8 mice, next, we explored the expression status of epidermal VEGF receptors and VEGF during tumor development in HPV8 mice. For this purpose at days 10 and 34 after UV exposure, the epidermis was separated from dermis and epidermal expression for VEGF, VEGFR2, VEGFR1, and Nrp1 was determined by qRT-PCR analysis (Fig. 7B and C). Epidermis of untreated skin in control mice served as baseline for gene expression. Although papilloma formation in UV-light–exposed skin of HPV8 mice was associated with a robust induction of epidermal VEGF and Nrp1 expression, absence of tumor formation in VEGFR1−/−HPV mice was paralleled by absence of epidermal VEGF (as expected) and Nrp1 expression (Fig. 7B). In epidermal tissue, transcripts for VEGFR1 or VEGFR2 were hardly detectable in both UV-treated and untreated skin of the 3 mouse strains and we are reluctant to draw any conclusion on these findings with regards to expression of VEGFRs (ΔCt values for qRT-PCR analysis were in the range of 29–34). Therefore, to further refine the analysis of VEGF receptor expression in keratinocytes and to exclude contamination of epidermal tissue with dermal cells, Epcam−/−Lin−/−CD34+/CD140a+ cells were isolated from untreated skin and papilloma tissues of HPV8 mice (day 30 after UV-light–treated skin of HPV8 mice; Supplementary Fig. S3) and purified cells were subjected to qRT-PCR analysis. These studies confirmed strong expression of VEGF and Nrp1 in papilloma-derived keratinocytes and virtually no VEGFR2 (Fig. 7C). In contrast, enrichment of Epcam−/−Lin−/− cells revealed an upregulation of VEGFR1 in papilloma-derived keratinocytes although at low levels. Epidermal expression of Nrp1 and VEGFR1 in papillomas was confirmed by IHC staining (Fig. 7D). Staining for Nrp1 was shown in the cytoplasm (partially also the cell membrane), whereas VEGFR1 staining localized particularly (peri) nuclear. VEGFR2 staining was not detected in the epidermis (Fig. 1).

Discussion

Our study reveals a close functional interplay between the onset of HPV8-induced progressive squamous tumor formation and neovascularization in mice. Thus, our findings corroborate earlier studies reporting on vascular alterations during neoplastic progression in both human cervical carcinoma and the corresponding transgenic K14-HPV16 mouse model, speculating on a causal relationship of virus-induced carcinogenesis and neangiogenesis (39, 40). Although those early studies on HPV-induced mucosal carcinogenesis hypothesized that VEGF released from epidermal tumor cells and/or recruited inflammatory cells may contribute to vascular alterations critical for the essential vascular switch in tumor progression (19), here we provide clear evidence for a functional link between epidermal and myeloid cell–type-restricted VEGF-mediated angiogenesis and HPV8-induced skin tumor development.

However, emerging evidence in preclinical and clinical studies of various epithelial cancers suggests that epidermal synthesized VEGF in addition to regulating paracrine effects on the vasculature, mediates angiogenesis-independent, autocrine growth promoting, and survival effects on epidermal tumor cells (24, 28). In a transgenic mouse model of non-viral K5-SOS/EGFR-mediated epithelial tumorigenesis, Lichtenberger and colleagues reported that epithelium-derived VEGF induces cell-autonomous tumor cell proliferation via an autocrine mechanism, whereby VEGF could act intra- and/or extracellularly mediated by VEGFR1 and/or Nrp1 (35). Furthermore, recently, Beck and colleagues reported in the DMBA/TPA two-stage chemical skin carcinogenesis model, that CD34+/CD45− epithelial cancer stem cells express Nrp1 and that VEGF controls initiation and stemness through Nrp1 in an autocrine loop; in vitro studies showed that externally added VEGF controls proliferation of isolated CD34+/CD45− epithelial tumor cells (33). Yet, in this study, the question remained unclear through which additional VEGFRs, the coreceptor Nrp1 may direct VEGF-mediated signals in keratinocytes.

Here, we showed that the systemic inhibition of VEGFR2 by an antibody-based strategy effectively prevented wound-induced tumor formation in HPV8 mice. These findings strongly suggest an angiogenesis-mediated effect of paracrine/exogenous VEGF in HPV8-induced tumor development, because robust levels of VEGFR2 expression could only be detected in the tumor vasculature but not in keratinocytes. Currently, there is some controversy about the expression of VEGFR2 in keratinocytes (33, 35, 41–43), which may be explained by different
experimental conditions and/or keratinocyte sources. However, as shown by IHC and in part also gene expression analysis, Nrp1 and VEGFR1 were not only expressed in vascular structures, but also in the hyperplastic epidermis of tumor lesions, proposing in addition an angiogenesis-independent, potentially autocrine effect mediated by epidermal VEGF. Although Nrp1 staining revealed a cytoplasmatic pattern primarily in basal layer cells, VEGFR1 staining was characterized by a (peri)nuclear staining pattern predominantly in basal and few suprabasal cell layers. Interestingly, a (peri)nuclear staining pattern for VEGFR1 has been recently reported in breast cancer cells and has been shown to be critical for VEGF-mediated internal autocrine survival signals (28). Although at this stage, we are cautious to convey a mechanism of action based on our findings, we are tempted to speculate on an autocrine role of epidermal VEGF in our model. Furthermore, in context with our in vitro findings that externally added rmVEGF does not act as mitogen neither on wild-type keratinocytes nor on HPV8-transfected cells, we propose an intracrine mechanism of VEGF action in keratinocytes that promotes tumor development. In fact, intracellular activity of VEGF and unresponsiveness to external recombinant VEGF have been previously proposed in various cell types (35, 44–46), including carcinoma cells (24, 28, 29); however, up to date, underlying functional mechanisms are poorly investigated. Therefore, at this stage our findings in HPV8-mediated tumorigenesis support recent reports on potential intracrine, autocrine VEGF signaling in carcinomas and provide the basis for further studies examining the specific role of an intracrine, autocrine loop mechanism for VEGF signaling in epidermal carcinogenesis.

Tumor progression in HPV8 mice was paralleled by a strong inflammatory response within the tumor stroma, dominated by macrophages. In various tumor entities, infiltrating macrophages have been identified as critical cellular components promoting tumor initiation and progression (47). Specifically, in preclinical tumor models, a dual role of myeloid cell-derived VEGF has been described including both a tumor-promoting activity (48) and a negative function as tumor suppressor (26). So far, it is unclear whether HPV8-positive keratinocytes initiate a cross-talk with infiltrating immune cells that may promote epithelial tumorigenesis. Our findings of abrogation of tumor initiation in VEGF<sup>−/−</sup>HPV8 mice after excision skin injury corroborate a critical role of myeloid cell-restricted VEGF in HPV8-induced tumor formation, most likely a proangiogenic function. This concept of an angiogenic-dependent mechanism is consistent with a recent report of our group, demonstrating an essential role of myeloid cell–derived VEGF for the initiation of wound angiogenesis (30). In addition, beside its well-known proangiogenic potency, VEGF has also been described as monocyte chemotactant (49, 50), and someone may argue that myeloid cell–restricted VEGF acts as immunomodulator to promote carcinogenesis in HPV8 mice. Here, we could not detect any significant difference in the inflammatory cell response in HPV8 and VEGF<sup>−/−</sup>HPV8 mice few days after injury, the time point of tumor initiation. Thus, these findings argue against a major role of a direct action of myeloid cell–restricted VEGF on immune cells during HPV8-induced epidermal carcinogenesis. Our findings are consistent with earlier experimental in vivo studies that could also not detect a critical effect of myeloid cell–restricted VEGF on monocyte recruitment in skin inflammation (30, 51).

For UV-light–triggered tumor formation in HPV8 transgenic mice, myeloid cell–derived VEGF was dispensable, whereas epidermis-derived VEGF was essential. Different reasons may explain the prevailing role of epidermal VEGF under these conditions. UV-light has been identified as potent inducer of VEGF expression in keratinocytes (52), and in irradiated VEGF<sup>−/−</sup>HPV8 mice, increased epidermal VEGF synthesis may compensate for the lack of myeloid cell–derived VEGF. Furthermore, UV-light induces a strong cellular inflammatory response that may provide additional cellular sources for VEGF and/or other growth stimulating and/or proangiogenic factors (40). In addition, the UV-light–induced DNA damage response may increase expression of other proangiogenic factors that rescue the lack of myeloid cell–derived VEGF in VEGF<sup>−/−</sup>HPV8 mice (53). Finally, as discussed above, a potential autocrine–intracrine VEGF loop mechanism may direct growth promoting and survival effects on epidermal tumor cells, so that epidermal VEGF deletion protects effectively from tumorigenesis.

In conclusion, our analysis reveals a strong functional interaction between neoangiogenesis and the progression of squamous carcinogenesis in HPV8 transgenic mice. In addition, we show that VEGF serves cell-type specific functions in HPV8-mediated premalignant papilloma formation in context with the nature of the tumor-promoting condition. Furthermore, in addition to a paracrine effect on the vasculature, epidermal VEGF may control HPV8-induced tumor development through an autocrine–intracrine loop mechanism, independent from angiogenesis. Collectively, these findings propose that blocking VEGF signaling both externally and intracellularly, may represent a promising therapeutic target for the treatment of HPV8-mediated skin carcinogenesis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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