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c-Myc Interacts with Hypoxia to Induce Angiogenesis *In vivo* by a Vascular Endothelial Growth Factor-Dependent Mechanism

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ABSTRACT

The proto-oncogene *c-myc* is involved in the regulation of cell proliferation, differentiation, and apoptosis. In this study, we used an inducible transgenic mouse model in which c-Myc was targeted to the epidermis and, after activation, gave rise to hyperplastic and dysplastic skin lesions and to dermal angiogenesis, involving both vascular endothelial growth factor (VEGF) receptor-1 and VEGF receptor-2. After c-Myc activation, VEGF mRNA was expressed in postmitotic keratinocytes where it colocalized with transgene expression and areas of tissue hypoxia, suggesting a role of hypoxia in VEGF induction. *In vitro*, c-Myc activation alone was able to induce VEGF protein release and in conjunction with hypoxia, c-Myc activation further increased VEGF protein. Blocking VEGF signaling *in vivo* significantly reduced dermal angiogenesis, demonstrating the importance of VEGF as a mediating factor for the c-Myc–induced angiogenic phenotype.

INTRODUCTION

Angiogenesis, the sprouting of capillaries from pre-existing blood vessels, is an essential step for tumors to grow beyond 1 to 2 mm in diameter (1). The vasculature of most adult tissues is quiescent, due to the dominance of angiogenic inhibitors over stimulants (2). However, during embryogenesis or in pathological conditions such as tumor development, this equilibrium is changed in favor of a pro-angiogenic phenotype (2, 3). Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and one of the most potent mediators of physiologic and pathological angiogenesis. Its receptors are high-affinity receptor tyrosine kinases named VEGFR-1 (flt-1) and VEGFR-2 (flk-1), which in the adult, are expressed mainly on the endothelium (4, 5). A major physiologic stimulus for VEGF is hypoxia, which induces increased transcription of the gene and mRNA stability (6–8). The transcriptional regulation of VEGF under hypoxic conditions is mediated by hypoxia-inducible factor 1 (HIF-1), a transcription factor that regulates homeostatic responses to reduced oxygen availability by promoting erythropoiesis, angiogenesis, vasodilatation, and decreased oxygen use (9, 10).

Recently, it has emerged that tumor angiogenesis can be stimulated by oncogenes. A large body of evidence now links oncogenes such as *ras* (11), *v-src* (12, 13), *c-jun* (14), *c-fos* (15), and *HPV-16* (16) with the induction of VEGF mRNA and protein. Oncogene expression may exacerbate the effects of hypoxia by stimulating VEGF expression, as shown for Ha-ras (17–19). Oncogenes may also enhance angiogenesis by down-regulating inhibitors of angiogenesis. For example, the expression of thrombospondin-1 (tsp-1; an extracellular glycoprotein with antiangiogenic properties) is suppressed in ras-transformed cells (20, 21), and c-Myc is capable of down-regulating tsp-1 expression (22). c-Myc functions as a transcription factor and is involved in the regulation of cellular proliferation, differentiation, and apoptosis. De-regulated c-Myc expression occurs in one third of human cancers and is associated with a poor prognosis (23–25). To study the de-regulated activation of c-Myc in adult tissue, a switchable form of the c-Myc protein, c-MycER^{TAM}, was targeted to supra-basal keratinocytes in the mouse epidermis using the involucrin promoter (26). Activation of c-MycER^{TAM} with the ligand 4-hydroxytamoxifen led to an admixture of hyperplastic and dysplastic precancerous skin lesions (papillomatosis), which regressed after 4-hydroxytamoxifen withdrawal. Papillomatosis was accompanied by angiogenesis, with the newly formed vessels in close apposition to the overlying preneoplastic epidermis. We have analyzed the mechanism that leads to the angiogenic phenotype and provide evidence for a crucial role of VEGF in mediating this effect.

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MATERIALS AND METHODS

Transgenic Mice. Generation of the involucrin-c-MycER^{TAM} mice has been described previously (26). Adult heterozygous involucrin-c-MycER^{TAM} mice and wild-type littermates were treated daily with topical administration of 1 mg of 4-hydroxytamoxifen (Sigma-Aldrich, Poole, United Kingdom) to an area of shaved skin on the upper back of the animal. The treatment lasted for differing periods of time ranging from 2 to 21 days. Treatment with SU5416, SU6668 (gifts from Sugen, Inc., San Francisco, CA), or DMSO was started simultaneously. SU5416 was given twice weekly at 50 mg/kg body weight s.c. in a total volume of 50 μL of DMSO. SU6668 (in DMSO) was given daily i.p. at 100 mg/kg body weight in a total volume of 50 μL. Controls were treated with equivalent volumes of DMSO. 7-(4′-(2-Nitroimidazol-1-yl)-butyl)-theophilline (NITP) was injected i.p. at 0.45 μmol/g body weight (in 90% peanut oil, 10% DMSO) 2 hours before being sacrificed. NITP was a gift from Ian Stratford (The School of Pharmacy and Pharmaceutical Sciences, Manchester, United Kingdom). Animals were sacrificed by cervical dislocation, and skin samples were fixed in formal saline for 24 hours followed by 70% ethanol and embedded in paraffin.

Vascular Endothelial Growth Factor Detection from Tissue Extracts. Snap frozen tissues were homogenized under liquid nitrogen, and cold lysis buffer [20 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.5 mmol/L EDTA (pH 7.4), and Complete protease inhibitor tablets; Roche, Lewes, United Kingdom] was added in a ratio of 2 mL of buffer to 0.1 g of tissue. The suspension was further homogenized with an IKA Ultra Turrax T8 homogenizer (Janke & Kunkel, Staufen, Germany) using 3 × 10-second bursts at full speed. The homogenate was first sedimented at 4°C for 10 minutes at 3,000 × *g* and subsequently at 4°C for 40 minutes at 225,000 × *g*. The supernatant was analyzed using a mouse VEGF enzyme-linked immunosorbent assay (ELISA; R&D Systems, Abingdon, United Kingdom). The values were normalized to the total protein content of the sample as determined using the Bio-Rad DC protein assay (Bio-Rad, Hemel Hempstead, United Kingdom).

Immunohistochemistry. After a 10-minute proteolytic digest in 0.5 mg/mL Pronase (Roche) at 37°C, the antibody to von Willebrand Factor (rabbit anti-von Willebrand factor; DAKO, Glostrup, Denmark) was used 1:500 (in PBS). The antibody was detected using the ABC Vectastain kit (Novocastra Laboratories, Ltd., Newcastle upon Tyne, United Kingdom) according to the manufacturer's instructions. The tissue was counterstained with Mayer's Hemalum (Merck, Poole, United Kingdom). Dermal angiogenesis was evaluated according to Suri *et al*. (27).

For the staining of the estrogen receptor fusion protein, a rabbit antiserum [HL7; gratefully received from Hartmut Land (University of Rochester, Rochester, NY)] was used at a concentration of 1:500 (in PBS) after microwaving

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10 minutes in citrate buffer (pH 6.0). Detection of the antibody was performed as described for the von Willebrand factor staining.

For the staining of theophilline adducts, antigen retrieval was undertaken using pressure cooker treatment in combination with DAKO Target Retrieval Solution. The rabbit antitheophilline antiserum (Sigma) was used at a concentration of 1:100 (in PBS), and the detection of the antibody was performed using the CSA kit (DAKO) according to the manufacturer's instructions.

***In Situ* Hybridization.** *In situ* hybridization reactions were performed as described previously (28). Probes for murine VEGF (29), murine flt-1 (30) and flk-1 (31) were generated as described previously.

Cell Culture. DCHG2 cells (rat-1 fibroblasts stably transfected with an inducible c-MycER^{TAM} plasmid; ref. 32) and rat-1 cells were seeded out at a density of 1×10^6 cells per 100-mm^2 cell culture dish. For the experiment, cells were cultivated either in cell culture medium (Dulbecco's modified

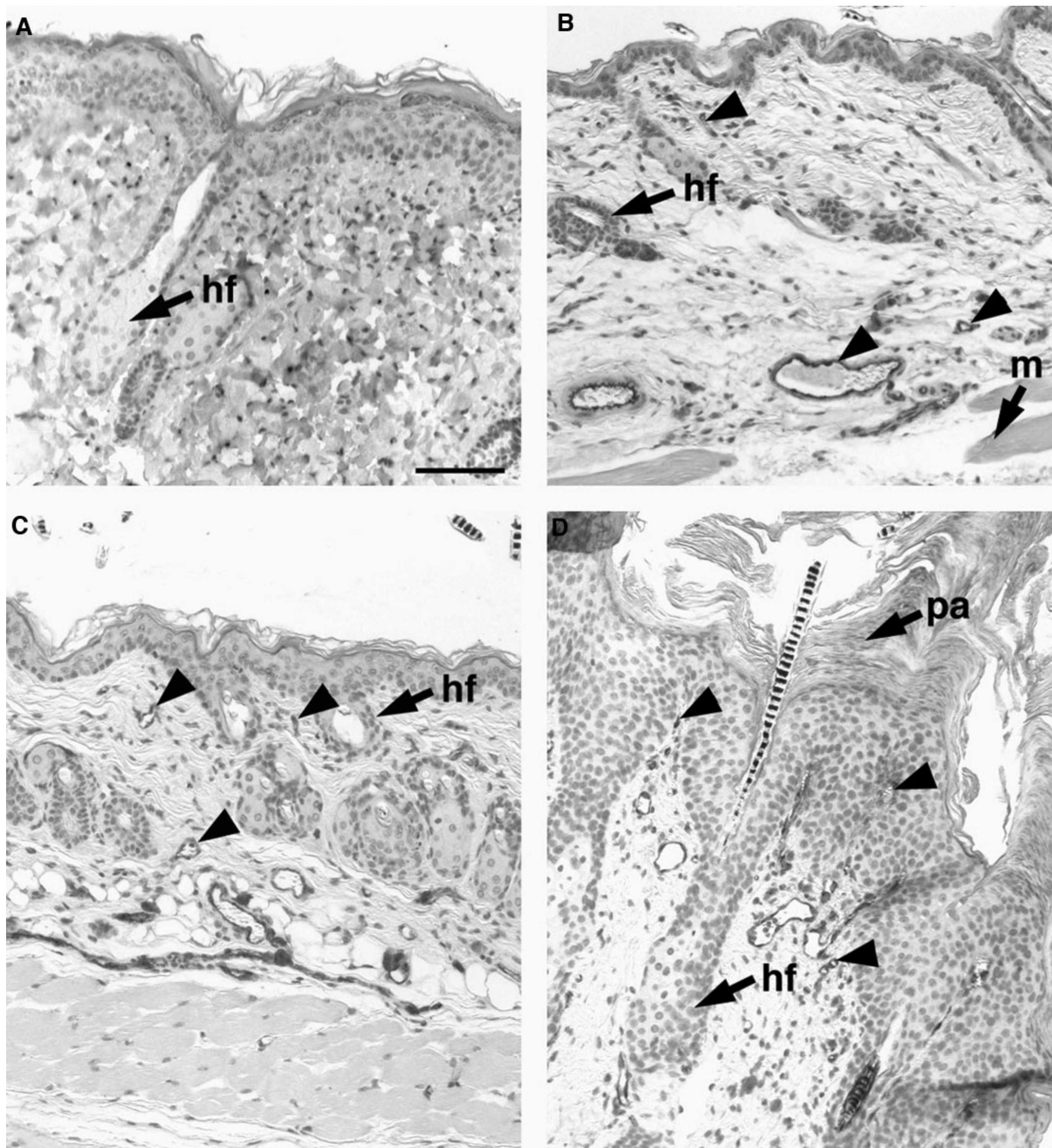

Fig. 1. von Willebrand factor staining of mouse skin. Transverse sections of mouse skin were stained with an antibody recognizing von Willebrand factor. *A*, control staining of transgenic mouse skin. *B*, staining of nontransgenic skin. *C*, increased vessel density in skin of transgenic mice after 7 days of c-Myc activation. *D*. After 21 days of c-Myc activation, many vessel profiles can be seen in the dermis of transgenic mice. Vessels are indicated by *arrowheads*. *hf*, hair follicle; *pa*, parakeratosis; *m*, muscle. *Scale bar* = 0.1 mm.

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Eagle's medium containing 4500 mg/L glucose, 10% fetal calf serum, and 4 mmol/L L-glutamine) or cell culture medium containing 100 nmol/L 4-hydroxytamoxifen. Hypoxia (0.1% oxygen, 5% CO_2, and balanced nitrogen) was generated using a Heto Cellhouse 170 HI incubator (Heto Holten, Camberley, United Kingdom). After 16 hours, cell-free culture supernatants were harvested, and VEGF content was analyzed by ELISA. The results of the VEGF ELISA were normalized to the cell number at the time of harvesting. For each experimental condition, the assay was performed in triplicate.

The preparation of protein lysates and the detection of HIF-1α protein were performed as described previously (19). Forty μg of protein per lane were analyzed using the HIF-1α antibody NB100-105H4 by NOVUS (Littleton, CO). Western Blots were quantified by densitometry of ECL films (Amersham Biosciences, Little Chalfont, United Kingdom) using an Epson scanner.

Statistical Analysis. For all experiments, *P* values were calculated using the unpaired, two-tailed Student's *t* test.

RESULTS

Activation of c-Myc Leads to Angiogenesis *In vivo*. Involucrin-c-MycER^{TAM} mice and wild-type littermates were treated with 4-hydroxytamoxifen for different periods of time. To visualize vessel profiles after c-Myc activation, skin sections were immunostained with an anti-von Willebrand factor antibody. von Willebrand factor is a protein characteristically expressed in Weibel Pallade Bodies of endothelial cells (33). Staining with rabbit immunoglobulins was negative (Fig. 1*A*). In normal skin ($n = 4$), the blood vessels (12.4 ± 0.4 SD vessels/mm^2) were restricted to areas in the dermis adjacent to the underlying *panniculus carnosus* muscle layer (Fig. 1*B*). After 6 to 7 days of c-Myc activation ($n = 4$), a significant increase ($P < 0.01$) in vascular density was detectable (18.1 ± 0.7 SD vessels/mm^2), accompanied by mild hyperplasia of the epidermis (Fig. 1*C*). After 21 days ($n = 4$), a pronounced hyperplasia of the epidermis was visible, which was accompanied by parakeratosis and extensive angiogenesis (41.2 ± 5.7 SD vessels/mm^2) with vessels closely associated with the hyperplastic epidermis (Fig. 1*D*). These vessels were never observed to penetrate the dermo-epidermal barrier, consistent with the hypothesis that c-Myc is capable of inducing angiogenesis *in vivo* via a diffusible factor.

Vascular Endothelial Growth Factor Levels Are Elevated in Skin after c-Myc Activation. To investigate whether VEGF was involved in c-Myc–induced angiogenesis, we measured VEGF protein in skin from transgenic mice by ELISA. Involucrin-c-MycER^{TAM} mice were treated with 4-hydroxytamoxifen for 10 ($n = 3$) and 21 ($n = 5$) days, and samples of nontreated involucrin-c-MycER^{TAM} mice ($n = 5$) and wild-type littermates ($n = 5$) served as controls. In wild-type skin, an average of 17.6 pg (± 10.3 SD) of VEGF per microgram of total protein were detected (Fig. 2). Nontreated transgenic mice showed comparable levels of VEGF (15.8 pg ± 12.5 SD of VEGF per microgram of total protein). After 10 days of c-Myc activation, VEGF levels in transgenic mice averaged 68.1 pg (± 39.5 SD); after 21 days, they were significantly elevated by 36-fold [620 pg ± 277 SD of VEGF per microgram of total protein ($P < 0.01$)].

Expression of Vascular Endothelial Growth Factor in Transgenic Keratinocytes. To localize areas of VEGF expression, a time course of c-Myc induction was analyzed by *in situ* hybridization. No VEGF mRNA was detectable in normal skin (Fig. 3*A* and *B*) or skin of wild-type littermates treated with 4-hydroxytamoxifen (data not shown). The earliest hybridization signal for VEGF mRNA was detected in sebocytes and cells of the inner root sheath of the hair follicle 2 days after c-Myc activation (Fig. 3*C* and *D*), which could be attributed to a high local concentration of 4-hydroxytamoxifen. VEGF was first detected in post-mitotic keratinocytes 6 days after the start of c-Myc activation (data not shown). After 10 days of treatment, the majority of post-mitotic keratinocytes in the hyperplastic epidermis were positive for VEGF (Fig. 3*E* and *F*), and prominent signals were detected in areas adjacent to hair follicles. After 21 days of c-Myc activation, VEGF was localized to the tips of papillomatous lesions (Fig. 3*G* and *H*). These results indicated that post-mitotic keratinocytes were the source of the high levels of VEGF detected by ELISA.

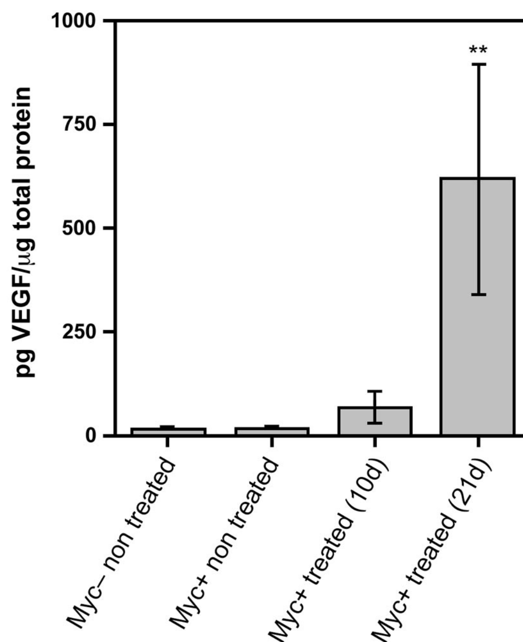

Fig. 2. VEGF protein content of mouse skin. Skin samples of transgenic mice ($n = 5$) treated with 4-hydroxytamoxifen for 10 and 21 days were analyzed for their VEGF content. Nontreated transgenics ($n = 5$) and wild-type littermates ($n = 5$) served as controls. The mean ± SD values are represented. Levels of VEGF were significantly increased after 21 days of 4-hydroxytamoxifen treatment. **, $P < 0.01$ *versus* either control.

Expression of Vascular Endothelial Growth Factor Receptors on Dermal Vessels. We further analyzed the expression of both VEGF receptors by performing *in situ* hybridization with probes specific for mouse flt-1 (Fig. 4*A* and *B*) or mouse flk-1 (Fig. 4*C* and *D*). Nontreated transgenic mice and wild-type littermates treated with 4-hydroxytamoxifen showed no hybridization signal for flk-1 but individual flt-1–positive endothelial cells were detected in vessels in the subcutaneous muscle. Throughout the time course of c-Myc activation (data not shown), the most prominent hybridization signals for both receptors were seen at the tips of the growing vessels in close apposition to the overlying hyperplastic epidermis. This demonstrated the potential for an induction of the VEGF signaling pathway in the dermis of c-MycER^{TAM} transgenic mice.

Transgene and Vascular Endothelial Growth Factor mRNA Colocalize in Areas of Tissue Hypoxia. To correlate the VEGF expression pattern with transgene expression, we stained sections for the estrogen receptor portion of the c-MycER^{TAM} fusion protein. Transgene-negative mice were estrogen receptor–negative, and nontreated involucrin-c-MycER^{TAM} mice showed individual positive postmitotic keratinocytes and cells of the inner root sheath of the hair follicle (data not shown). After 10 days, estrogen receptor–positive cells were found in all postmitotic keratinocyte layers but mainly in the upper part of the hyperplastic epidermis (Fig. 5*A*). After 21 days, estrogen receptor staining was detectable only in the very top layers of the epidermis (Fig. 5*C*). Immunohistochemical analysis of involucrin showed a similar expression pattern (data not shown). Thus VEGF mRNA colocalized with areas of transgene expression in involucrin-c-MycER^{TAM} transgenic mice.

The most potent physiologic stimulus for VEGF *in vivo* is hypoxia.

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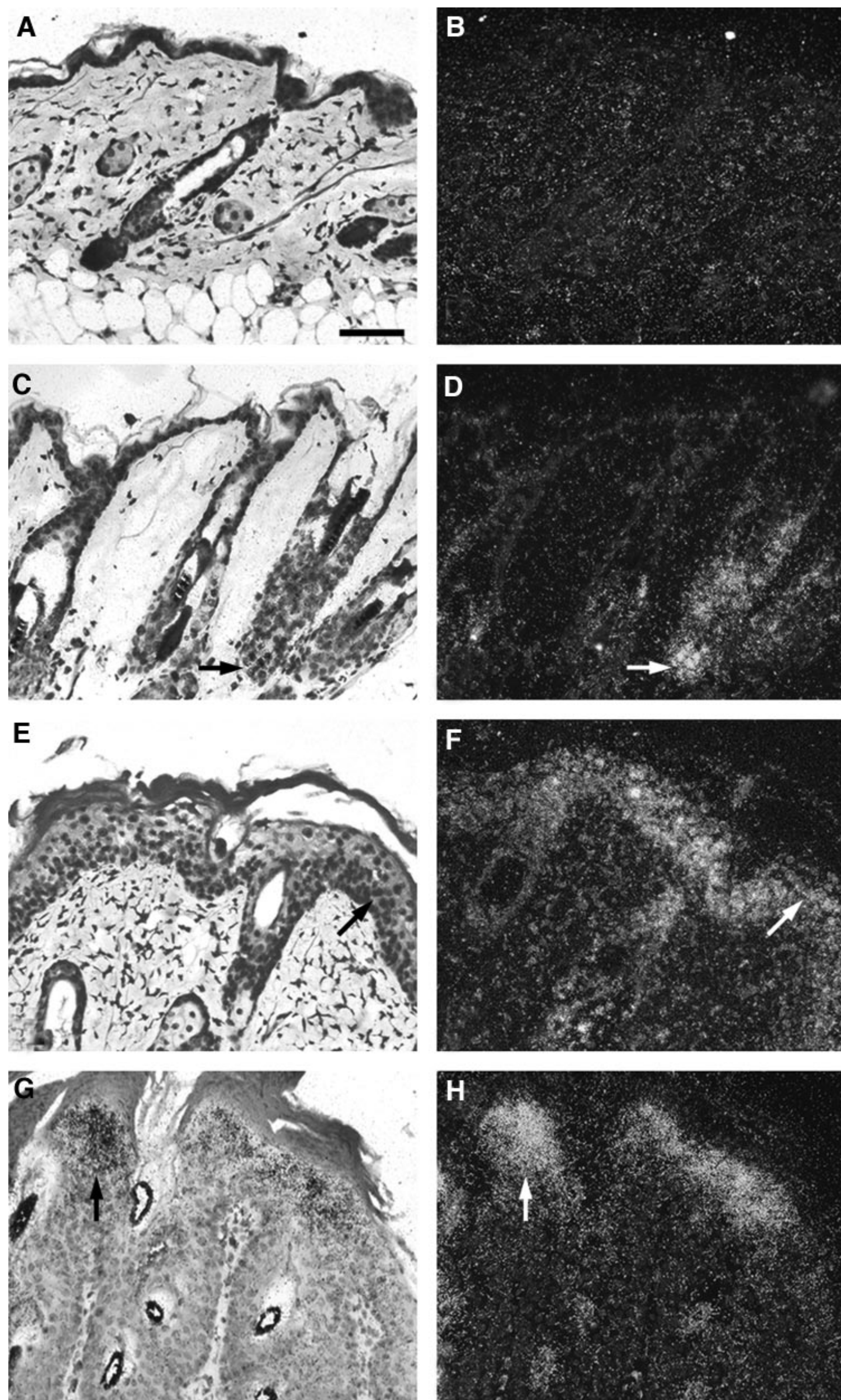

Fig. 3. Expression of VEGF mRNA after c-Myc activation. *A* and *B*. *In situ* hybridization of normal skin using a mouse VEGF-specific probe revealed no hybridization signal. *C* and *D*. After 2 days of c-Myc activation, VEGF mRNA was detected in the inner root sheath of hair follicles (*arrow*). *E* and *F*. After 10 days of c-Myc activation, signals for VEGF were visible in post-mitotic keratinocytes (*arrow*). *G* and *H*. Focal hybridization pattern (*arrow*) for VEGF mRNA after 21 days of c-Myc activation. *A*, *C*, *E*, and *G*, bright-field photographs; *B*, *D*, *F*, and *H*, dark-field photographs. *Scale bar* = 0.1 mm.

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We therefore assessed the contribution of tissue hypoxia to the induction of VEGF expression in our transgenic tumor model by labeling hypoxic areas *in vivo* using NITP (34). Immunohistological staining of wild-type littermates showed suprabasal keratinocytes to be hypoxic (data not shown). After 10 days (Fig. 5*B*) and 21 days of c-Myc induction (Fig. 5*D*), the upper layers of the hyperplastic epidermis stained positive for NITP with the staining being most focal at the 21-day time point. Overall, we observed a close local correlation between NITP antibody staining and areas of transgene expression, which in turn were positive for VEGF mRNA.

Activation of c-Myc Synergized with Hypoxia *In vitro*. To assess the individual contributions of c-Myc and hypoxia to the angiogenic

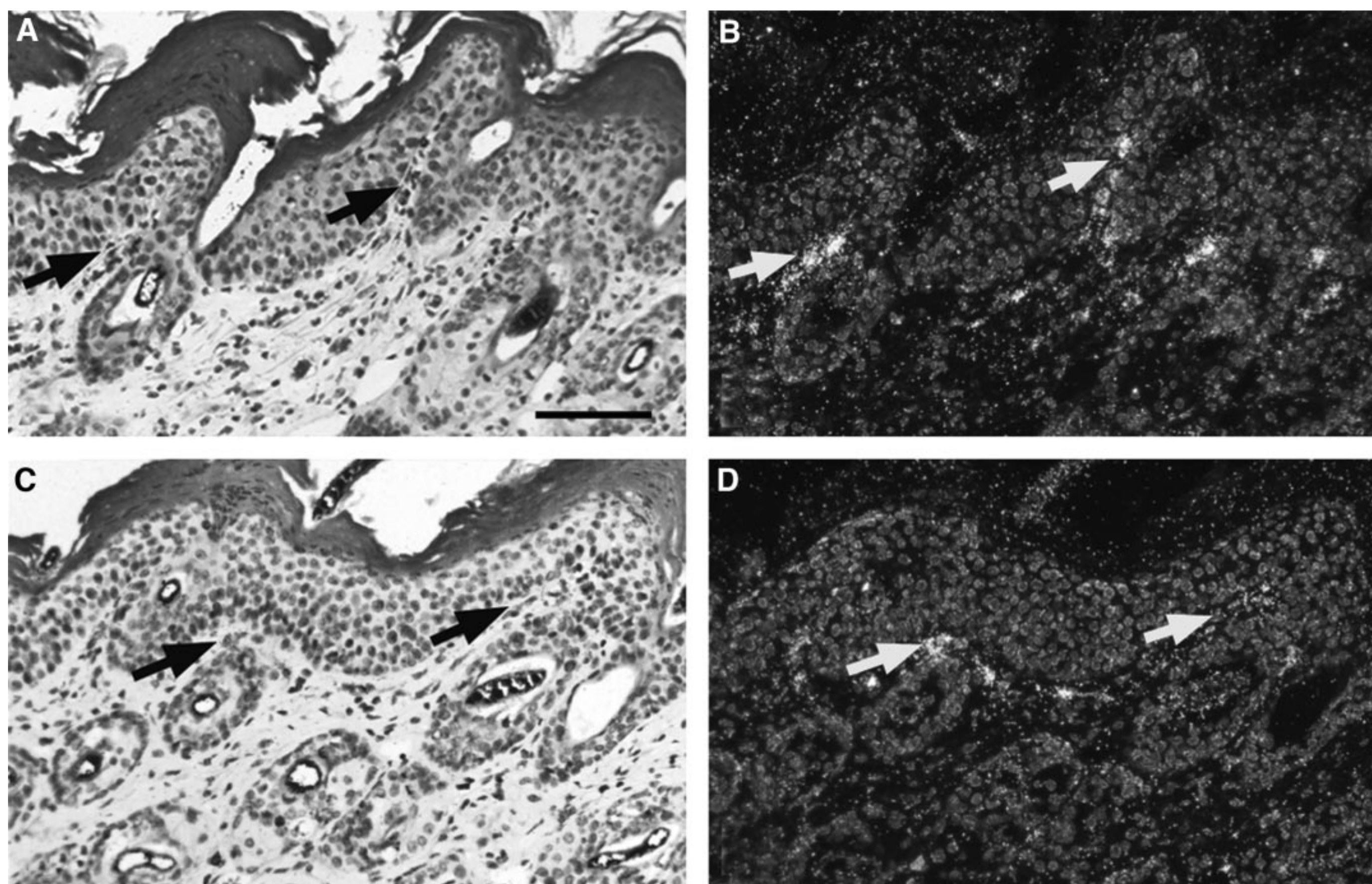

Fig. 4. Expression of VEGF receptors after c-Myc activation. *In situ* hybridization analysis of transgenic mouse skin after 21 days of c-Myc activation using mflt-1– or mflk-1–specific probes. *A* and *B*. flt-1 mRNA was detected on dermal vessels (*arrows*). *C* and *D*. Hybridization signals for flk-1 were detected on dermal vessels close to the hyperplastic epidermis (*arrows*). *A* and *C*, bright-field photographs. *B* and *D*, dark-field photographs of the same sections. *Scale bar* = 0.1 mm.

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phenotype, DCHG2 cells and mock-transfected rat-1 fibroblasts were grown under conditions of normal (control) or low oxygen (hypoxia), with or without activation of c-Myc using 4-hydroxytamoxifen and subsequently analyzed for VEGF. Rat-1 fibroblasts and DCHG2 cells showed a comparable baseline release of VEGF under control conditions (Fig. 6). After 16 hours of hypoxia, the total amount of VEGF increased 2-fold in both cell lines. When c-Myc was activated in DCHG2 cells under normoxia, VEGF release into the culture supernatant increased by 1.6-fold in comparison with the control ($P < 0.01$). Furthermore, the activation of c-Myc under hypoxia resulted in a significant increase of VEGF release when compared with c-Myc activation (2.5-fold; $P < 0.01$) or hypoxia alone (1.9-fold; $P < 0.01$). In the control rat-1 cells, not transfected with myc, the addition of 4-hydroxytamoxifen in did not alter VEGF levels. We therefore concluded that *in vitro*, c-Myc activation cooperated with the effects of hypoxia to further induce VEGF protein production and secretion.

Effect of c-Myc Activation on Hypoxia-Inducible Factor 1α. To study a possible involvement of HIF-1α in the c-Myc–induced VEGF release, lysates of DCHG2 cells were analyzed for HIF-1α by Western blot (Fig. 7). We found that after 4 hours of c-Myc activation, HIF-1α protein levels were increased 1.7-fold in DCHG2 cells ($P = 0.01$). However, this induction was not maintained at 16 hours. After 4 hours, cells grown under hypoxic conditions showed stabilization of HIF-1α with protein levels increased 3.7-fold ($P < 0.01$). A similar induction was seen after 16 hours. After 4 or 16 hours, HIF-1α protein levels under hypoxia were not further increased by activation of c-Myc.

Vascular Endothelial Growth Factor Inhibition *In vivo* Results in Suppression of c-Myc–Induced Angiogenesis. The VEGF inhibitor SU5416, which blocks VEGF signaling via VEGFR-2 (35), was used to assess the role of VEGF *in vivo*. We also used SU6668, a broad-spectrum angiogenesis inhibitor targeting the VEGF, fibroblast growth factor, and platelet-derived growth factor pathways (36). To assess the efficiency of the treatment, we used vessel count. After a 21-day treatment course, mice treated with 4-hydroxytamoxifen ($n = 3$) showed an average vessel count of 53.5 (± 1.7 SD) vessels/mm^2, which was a significant increase ($P < 0.01$) when compared with nontreated controls (average vessel count, 17.2 vessels/mm^2; Fig. 8). SU5416 ($n = 5$) caused a reduction in the vessel count to 39.2 (± 7.3 SD) vessels/mm^2, which was statistically significant ($P < 0.01$). SU6668 reduced the average vessel count to 32.8 (± 11.3 SD) vessels/mm^2 ($n = 5$; $P < 0.01$). These results demonstrated the critical importance of VEGF as a mediator for c-Myc–induced angiogenesis.

DISCUSSION

Activation of the c-MycER^{TAM} transgene in the mouse epidermis resulted in profound hyperplasia accompanied by dermal angiogenesis underneath the papillomatous lesions. In this study, we have investigated the mechanisms by which c-Myc induced angiogenesis, which appeared to be mediated by VEGF.

After c-Myc activation, the levels of VEGF protein were dramatically elevated in skin extracts from transgenic mice compared with either of the control groups. Post-mitotic keratinocytes were identified

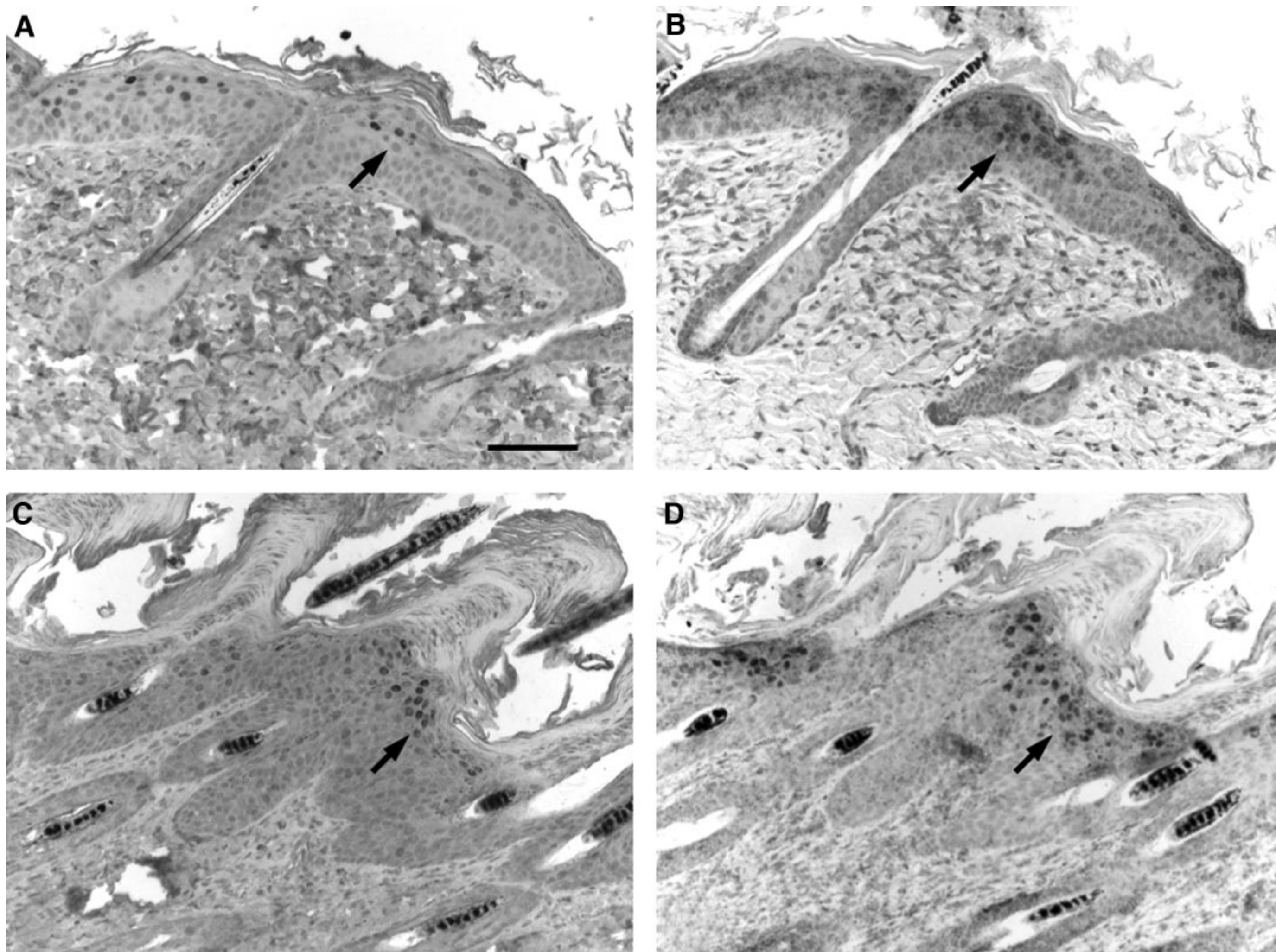

Fig. 5. Areas of transgene expression colocalize with tissue hypoxia. Consecutive sections of mouse skin were stained with the antibody HL7 (*A* and *C*), against the estrogen receptor portion of the c-MycER^{TAM} fusion protein, or with an antitheophilline antibody (*B* and *D*) detecting tissue hypoxia. After 10 days (*A* and *B*) and after 21 days (*C* and *D*) of c-Myc activation, areas of transgene expression colocalized with areas positive for tissue hypoxia. *Arrows*, areas of colocalized staining. *Scale bar* = 0.1 mm.

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as being the major producers of VEGF. It is known that VEGF is up-regulated in several examples of skin pathology such as wound healing, psoriasis, and squamous cell carcinoma (37, 38). In psoriatic skin, VEGF is mainly expressed in post-mitotic keratinocytes, and when taken into culture, keratinocytes can be induced to express the VEGF isoforms VEGF121 and VEGF165 (39, 40). These diffusible VEGF isoforms are capable of crossing the epidermis and the basal lamina to reach their receptors on the dermal blood vessels. We found expression of the VEGF receptors flt-1 and flk-1 on vessels in the 4-hydroxytamoxifen–treated transgenic mice, indicating the potential for active VEGF signaling.

The crucial importance of VEGF for the development of the angiogenic phenotype was underlined using the VEGF inhibitors SU5416 and SU6668 *in vivo*. We demonstrated that after administration of either SU5416 or SU6668, the vessel count was significantly reduced, indicating VEGF to be a major mediator of the angiogenic property of c-Myc. SU5416 was unable to completely reverse the angiogenic phenotype, possibly because only blocking flk-1 was not sufficient to completely abrogate VEGF signaling. This would be necessary because we detected both flt-1 and flk-1 on the newly formed vessels in the dermis. Recent data has indicated that the importance of flt-1 may have been underestimated as PlGF signaling via flt-1 was shown to stimulate angiogenesis *in vivo* (41). Another reason for an incomplete block of angiogenesis using SU5416 might be that other pathways are involved. The fact that SU6668, which also blocks the fibroblast growth factor and platelet-derived growth factor pathway, performs better than SU5416 with regard to the inhibition of the phenotype is suggestive of this.

In the involucrin-c-MycER^{TAM} mice, hypoxia seemed to play an important role in the up-regulation of VEGF, because areas of transgene expression in the epidermis were always found to be hypoxic, indicating that the pathways of transgenic c-Myc activation and hypoxia signaling overlap with regard to the induction of VEGF. It is possible that hypoxia regulates involucrin, the promoter driving the transgene, particularly as Raleigh *et al*. (42) recently reported a colocalization of involucrin with areas of hypoxia in a human squamous cell carcinoma. However, there is no evidence for a regulation of involucrin by hypoxia, and no hypoxia response element in the involucrin promoter has been identified to date.

An alternative, and more likely, possibility is that the hypoxia detected was due to an excess consumption of oxygen by the hyperproliferating epidermis as well as cellular distance from the dermal vessels increasing with progressing hyperplasia. The importance of hypoxia for the development of the angiogenic phenotype was supported by the observation that the VEGF mRNA signal peaked late during c-Myc activation in areas of acute hypoxia at the tips of the

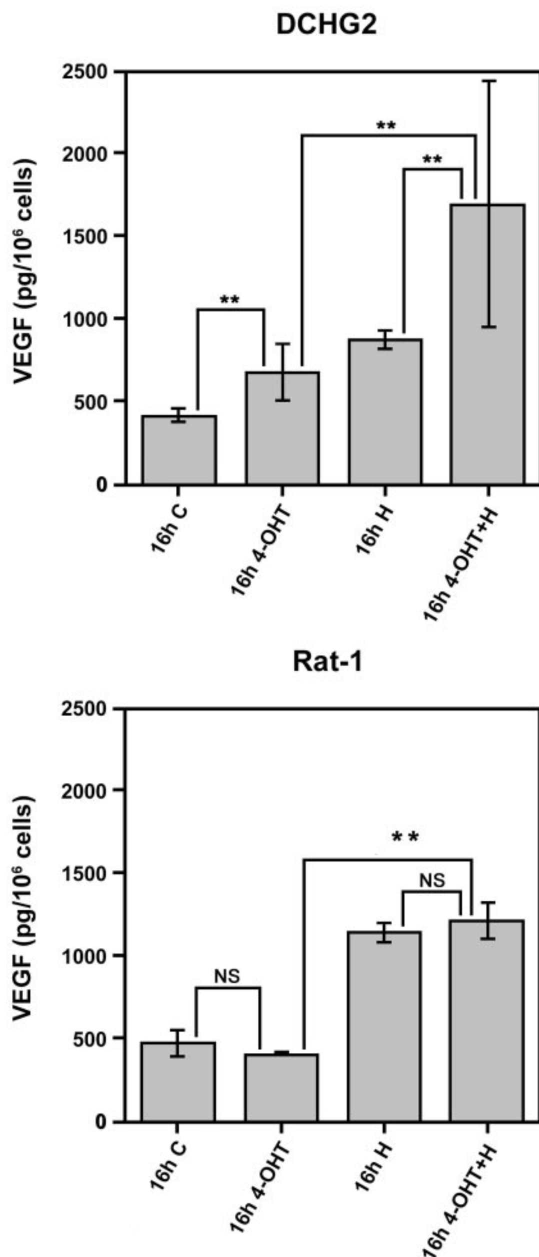

Fig. 6. c-Myc cooperates with hypoxia *in vitro*. Rat-1 and DCHG2 fibroblasts were incubated under normal or hypoxic conditions with or without addition of 4-hydroxytamoxifen (*4-OHT*). Cell culture supernatants were analyzed for VEGF. Each *bar* represents the mean ± SD of three independent experiments each performed in triplicate. **, $P < 0.01$; *NS*, not significant.

hyperplastic epidermis. Moreover, we were able to demonstrate *in vitro* that hypoxia and c-Myc cooperate to induce VEGF protein release.

Although *in vitro* we found a transient effect of c-Myc on HIF-1α expression in normoxia, this direct effect is unlikely to contribute *in vivo* because of its short duration and extent. However, other mechanisms could play a role in the cooperation between c-Myc and hypoxia. One is the effect of c-Myc on ribosomal entry, with enhancement of translation via eIF4E, the mRNA cap binding protein that is a target for c-Myc. Thus up-regulation of VEGF mRNA by hypoxia would be enhanced by the effects of c-Myc on translation (43). Under severe hypoxia, cap-independent translation is maintained and both c-Myc and VEGF protein synthesis can be initiated by cap-dependent and -independent mechanisms (44). Thus, in a novel mechanism of synergy, c-Myc might induce hypoxia by driving proliferation and increasing oxygen consumption in the involucrin-c-MycER^{TAM} mouse, and the subsequently elevated VEGF mRNA would be more efficiently transcribed.

It is conceivable that VEGF is a direct transcriptional target of c-Myc as Myc/Max dimers (consensus binding site sequence, 5′-RCGTG-3′) and HIF-1 (consensus binding site sequence, 5′-CACGTG-3′) recognize overlapping DNA binding sites. However, in the involucrin-c-MycER^{TAM} mice, the time course of c-Myc–induced VEGF expression was different to that of ornithine decarboxylase, a downstream target of c-Myc (26). This indicates that the mechanism of c-Myc–induced VEGF expression is likely to be different from the one affecting ornithine decarboxylase.

Myc has joined the growing number of oncogenes implicated in the induction of an angiogenic response. Previously, the potent angiogenic capacity of Myc had been observed in experimental tumors of the skin, lymphoma, neuroblastoma, and a fibroblast xenograft model,

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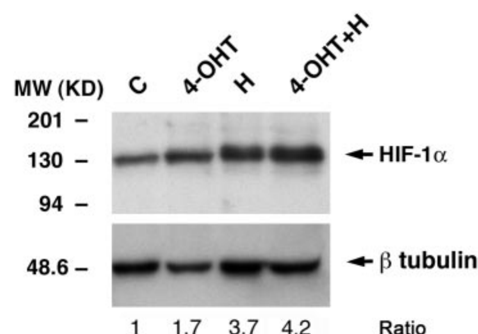

Fig. 7. HIF-1α expression after activation of c-Myc *in vitro*. Rat-1 and DCHG2 fibroblasts were incubated under normal or hypoxic conditions with or without addition of 4-hydroxytamoxifen (*4-OHT*). After 4 hours, cell lysates were analyzed for HIF-1α. A representative Western blot is shown. The ratio of HIF-1α to β tubulin signal is a mean of three independent experiments.

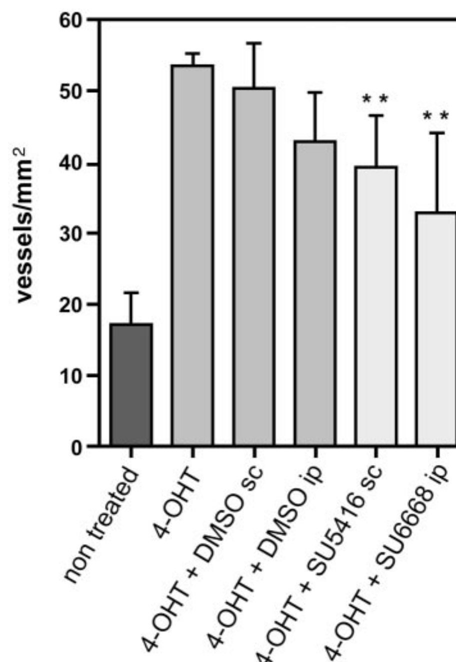

Fig. 8. Effect of SU5416 and SU6668 on c-Myc–induced angiogenesis. Involucrin-c-MycER^{TAM} transgenic mice were treated with 4-hydroxytamoxifen (*4-OHT*) for 21 days, and SU5416 and SU6668 were simultaneously administered. The effect of both drugs on c-Myc-induced angiogenesis was quantified by microvessel count as described in Materials and Methods. Values plotted are mean number of vessels/mm^2± SD; **, $P < 0.01$.

although no mechanism was confirmed (26, 45–47). Tikhonenko *et al*. (22) reported the down-regulation of tsp-1 by c-Myc, which was shown to be due to an increased turnover of the tsp-1 mRNA (48). Recently, Baudino *et al*. (49) showed that the embryonic lethality of c-Myc$^{-/-}$ mice was in part due to defects in vasculogenesis and erythropoiesis, which was associated with a failure in VEGF expression. Our study confirms the angiogenic properties of c-Myc, and we found that active c-Myc cooperated with hypoxia to induce VEGF. Our data emphasize the importance of the microenvironment during the early stages of tumor growth.

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