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A prolonged and exaggerated wound response with elevated ODC activity mimics early tumor development

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Induction of ornithine decarboxylase (ODC), a key enzyme in polyamine biosynthesis, in ODC transgenic skin stimulates epidermal proliferation but not hyperplasia, activates underlying stromal cells and promotes skin tumorigenesis following a single subthreshold dose of a carcinogen. Because chronic wounds are a well-recognized risk factor for skin cancer, we investigated the response to a tissue remodeling event in normal skin that is abraded to remove only the epidermal layer in K6/ODC transgenic (follicular ODC expression) and in inducible ODCER transgenic mice (suprabasal ODC expression). When regenerative epidermal hyperplasia was resolved in normal littermates following abrasion, ODC transgenic mice exhibited progressive epidermal hyperplasia with formation of benign tumor growths and maintained an increased epidermal proliferation index and activation of translation-associated proteins at abrasion sites. The epidermal hyperplasia and tumor-like growth was accompanied by activation of underlying stromal cells and prolonged infiltration of inflammatory cells. Treatment with the anti-inflammatory agent dexamethasone did not reduce the high proliferative index in the regenerated epidermis but dramatically reduced the epidermal hyperplasia and prevented the wound-induced tumor growths in abraded ODCER skin. Treatment with α-difluoromethylornithine, a specific inhibitor of ODC activity, normalized the wound response in transgenic mice and decreased wound-induced inflammation if administered from the time of abrasion but not if initiated 4 days following abrasion. These results suggest a role for polyamines in prolonging wound-associated inflammation in addition to stimulating proliferation both of which are sufficient to sustain epidermal hyperplasia and benign tumor growth even in the absence of genetic damage.

Introduction

Polyamines have long been known to be associated with cell proliferation in both normal and neoplastic tissues (1). Tightly regulated metabolism of polyamines is critical for cell survival and normal skin homeostasis, and these controls are dysregulated in skin tumorigenesis. A key enzyme in polyamine biosynthesis, ornithine decarboxylase (ODC), is upregulated in both human and animal skin tumors compared with normal skin (2–4). ODC is responsible for the biosynthesis of the diamine putrescine that is subsequently converted to the polyamines spermidine and spermine. ODC expression is upregulated in tumor epithelial cells by a variety of oncogenes such as c-*myc* (5, 6), v-*src* (7), v-*raf* (8) or an activated Ras or RhoA (8), all of which also play essential roles in normal tissue remodeling events such as wound healing. Use of transgenic mouse models has demonstrated that polyamines play an essential role in the early promotional phase of skin tumorigenesis since elevated epidermal ODC activity is sufficient to promote skin tumorigenesis following a single subthreshold dose of a carcinogen (9–11). The formation of skin tumors in these transgenic mice is dependent upon polyamine biosynthesis, especially putrescine, since treatment with inhibitors of ODC activity blocks the formation of skin tumors and causes the rapid regression of existing tumors (10–13). Although the mechanisms by which polyamines promote skin tumorigenesis are not well understood, *de novo* induction of epidermal ODC activity in ODCER transgenic mice has been shown to stimulate epidermal proliferation, alter keratinocyte differentiation status, increase neovascularization but is not sufficient in itself to lead to epidermal hyperplasia or skin tumor formation (11,14). This polyamine-activation of keratinocytes and underlying stromal cells is most probably an early event in the tumor process that creates a more permissive microenvironment for tumor development.

Wounding is known to promote the development of tumors (15), and chronic wounds and acute trauma are well-recognized risk factors for skin cancer (16–18). Cutaneous injury initiates an intricately regulated sequence of processes that involve cellular and biochemical events orchestrated to repair the wound (19). Wound healing processes involve cell migration, infiltration of inflammatory cells, proliferation, neoangiogenesis and extracellular matrix (ECM) degradation and resynthesis. Although polyamines are essential for cell proliferation and ODC and polyamine levels increase within 12 h after wounding (20–22), the role of polyamines during wound repair remains unclear. We describe skin abrasion studies using K6/ODC and ODCER transgenic mice to investigate the wound healing response in a skin microenvironment that is activated as the result of elevated epidermal polyamine biosynthesis.

Abbreviations: BrdU, bromodeoxyuridine; DFMO, α-difluoromethylornithine; ECM, extracellular matrix; eIF5A, eukaryotic initiation factor 5A; IL, interleukin; KC/CXCL1, keratinocyte-derived chemoattractant; MCP-1, monocyte chemotactic protein-1; MPO, myeloperoxidase; mTOR, mammalian target of rapamycin; ODC, ornithine decarboxylase; 4OHT, 4-hydroxytamoxifen; TNF, tumor necrosis factor.

Materials and methods

Animals

Mice used in wound healing experiments included K6/ODC transgenic mice, ODCER transgenic mice and their normal littermates, all backcrossed into either the FVB or C57BL/6 background for at least 10 generations. A keratin 6 promoter constitutively directs *ODC* transgene expression to the outer root sheath cells of hair follicles in K6/ODC transgenic mice (9), and an involucrin promoter directs the expression of the inducible *ODC* complementary DNA, fused in frame to a 4-hydroxytamoxifen (4OHT)-responsive mutant estrogen receptor ligand-binding domain, to the suprabasal epidermis in ODCER transgenic mice (11). K6/ODC transgenic mice exhibit loss of hair accompanied by the development of dermal cysts from the degenerating hair follicles (9, 23). Without treatment with the inducing agent, 4OHT, ODCER transgenic mice demonstrate a low basal ODC activity similar to wild-type mice and have a normal skin phenotype with no hair loss (11). ODC activity was induced in ODCER transgenic mice beginning 1 week prior to abrasion by topical application of 4OHT dissolved in ethanol (1.0 mg/0.1 ml) applied each day to a shaved area of the dorsal skin just behind the neck and distant to the wound area (closer to the tail). To inhibit ODC enzyme activity, mice were given 1% (wt/vol) α-difluoromethylornithine (DFMO) in their drinking water.

Skin abrasion

Regenerative epidermal growth was induced by abrasion of the dorsal skin of 7-week-old K6/ODC or ODCER transgenic mice and their normal littermates. Briefly, mice were anesthetized with an intraperitoneal injection of Avertin and then the dorsal back fur of the non-transgenic haired mice shaved with electric clippers and depilated with Nair for 2 min. Following careful removal of the Nair, a 2 cm^2 area of the dry dorsum was gently abraded with a felt wheel on a motor tool leaving the abraded area shiny and pink and bloodless. Some mice were subcutaneously injected with dexamethasone [100 μg in 100 μl phosphate-buffered saline; Sigma/Aldrich, St. Louis, MO] near the wound site daily starting the

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day after wounding until killing. Two hours before killing, some mice were injected intraperitoneal with bromodeoxyuridine (BrdU) (Sigma, St Louis, MO) at a dose of 100 μg/g body wt. Mice were killed 1, 2, 3, 5, 7, 14, 21, 28 and 56 days following the abrasion, and the dorsal skin removed for analyses.

Autoradiography

Proliferating cells that incorporate [^{3}H]thymidine were detected using autoradiography as described previously (24). To evaluate the epidermal transit distance or the distance that a basal cell pulse labeled with [^{3}H]thymidine travels toward the uppermost layer of the epidermis in a given time, mice were injected intraperitoneally with [methyl-^{3}H] thymidine (specific activity, 45–61 Ci/mmol; Amersham/Searie Corp., Arlington Heights, IL) at a concentration of 30 μCi per mouse on the third day following abrasion and killed 9 h later. Slides containing 10–12 serial 7 μm sections were deparaffinized, rinsed in absolute ethanol and dipped in 40°C NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, NY) diluted 1:1 with distilled water. The coated slides were dried in darkness and exposed for 14 days at 4°C in light-tight boxes containing silica gel desiccant. The autoradiographs were developed in Kodak D-19 for 3 min at 18°C, fixed in Kodak fixer, washed in cold running tap water for 1 h and then lightly stained in hematoxylin and eosin. Nuclei with a minimum of three to five silver grains were scored as labeled.

Immunohistochemistry

Mouse skin tissues were fixed in 4% *p*-formaldehyde in phosphate-buffered saline overnight and embedded in paraffin. Skin sections were deparaffinized, hydrated and then heated in 0.01 M sodium citrate buffer (pH 6.0) in a steamer for 8 min. Sections were incubated with primary antibodies followed by biotinylated secondary antibody and then an avidin horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). Primary antibodies used were monoclonal antibody against alpha-smooth muscle actin (Biomedia, Foster City, CA); rat anti-mouse neutrophil antibody recognizing the 7/4 antigen (Clone 7/4; Cedarlane, Burlington, NC); rat monoclonal anti-mouse F4-80 antigen (AbD Serotec, Raleigh, NC); rabbit polyclonal antibody against fibroblast-specific protein-1 or S100A4 (LabVision Corp, Fremont, CA); rabbit polyclonal antibody against tenascin-C (Chemicon, Ternecula, CA); rabbit polyclonal anti-spermine oxidase (kind gift from R. Casero, Johns Hopkins University, Baltimore, MD); rabbit polyclonal antibody specific for hypusine-containing eukaryotic initiation factor 5A (eIF5A) (kind gift from M. Park, NIH, Bethesda, MD) and rabbit polyclonal antibodies against phosphorylated Akt (Ser473) or phosphorylated S6 ribosomal protein (Ser235/236) both from Cell Signaling Technology (Beverly, MA). Immunoreactive cells were localized by incubating the sections with a chromagen solution containing diaminobenzidine and peroxide and then counterstaining with hematoxylin. BrdU incorporation in cells undergoing DNA synthesis was detected in skin sections using a rat monoclonal anti-BrdU antibody (Zymed Laboratories, San Francisco, CA). Pictures were acquired using a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany), with a digital color camera and corresponding software (Axiocam; Zeiss). All images were processed for printing using Adobe Photoshop software.

Measure of myeloperoxidase enzyme

Levels of myeloperoxidase (MPO) enzyme were used as an indirect measure of neutrophil content in abraded tissue. Frozen abraded skin tissue was homogenized in sample buffer provided in the MPO Assay Kit (Cytostore, Calgary, Canada). The MPO activity in tissue lysates was measured by the enzymatic degradation of H_2O_2 to an oxygen radical that was then converted to a colored compound upon binding to a hydrogen-donating chromagen supplied in the kit. The presence of MPO was measured colorimetrically over time using a KC4 3.03 spectrophotometer (Bio-Tek Instruments, Winooski, VT).

Cytokine bead array analysis.

Two weeks following skin abrasion, ODCER transgenic and normal littermate mice were killed and both abraded skin and distant non-abraded skin excised, immediately frozen in liquid nitrogen, ground to a fine powder and then homogenized in phosphate-buffered saline with protease inhibitors and dithiothreitol. The debris was cleared by centrifugation and the supernatants were analyzed using Cytometric Bead Array reagents (BD Biosciences, San Jose, CA) for tumor necrosis factor (TNF)-α, granulocyte-macrophage colony stimulating factor, interleukin (IL)-10, IL-2, interferon-γ, monocyte chemotactic protein-1 (MCP-1), IL-6, IL-1 and keratinocyte-derived chemoattractant (KC/CXCL1) using a BD FACSCanto II cytometer with FACSDiva software (BD Biosciences) as per the manufacturer's protocol.

Results

Wounding induces a prolonged hyperplastic response and tumors in ODC transgenic mouse skin

To study the effect of elevated polyamine biosynthesis on wound healing in skin, we used a motorized felt wheel to abrade the skin of K6/ODC transgenic mice and their normal littermates. Abrasion removes only the epidermal layer which is subsequently regenerated from remaining follicular stem cells in the underlying dermis with a dramatic epidermal hyperplastic response which peaks at 5–7 days following abrasion (Figure 1A) (15, 24). Similar to wild-type mice (15, 25), regenerative epidermal hyperplasia peaked at 5 days following abrasion in K6/ODC transgenic skin. However, the degree of epidermal hyperplasia was dramatically accentuated in K6/ODC transgenic skin and was characterized by increased proliferation of the epithelial cells lining the follicular cysts in the dermis (Figure 1B and E). These follicular cysts develop in the dermal layer of non-wounded K6/ODC transgenic mice due to degeneration of the hair follicles at 2 weeks of age resulting from constitutively high levels of ODC activity (9, 23). We have previously reported that epithelial cells lining these dermal cysts express increased ODC protein and are proliferating at a higher rate compared with interfollicular, quiescent epidermis (26). Although epidermal hyperplasia was dramatically increased in K6/ODC transgenic skin 5 days following abrasion, the epidermal proliferation index, as determined by BrdU incorporation, was increased to a similar extent in both abraded K6/ODC transgenic and normal littermate skin (Figure 1D–F). The exaggerated epidermal hyperplasia in K6/ODC transgenic skin at 5 days following abrasion was accompanied by a visible increased vascularization of the underlying dermis (Figure 1G). The increased epidermal hyperplastic response was prevented in K6/ODC transgenic mice by the administration of a specific inhibitor of ODC activity, 1% DFMO in their drinking water (Figure 1C). DFMO treatment significantly attenuated the early increase in the proliferation index and reduced the degree of epidermal hyperplasia in not only K6/ODC transgenic but also normal littermate mice at 5 days after abrasion (Figure 1F and data not shown).

By 3 weeks following abrasion, individual well-differentiated papillomatous growths developed in all K6/ODC transgenic mice, although epidermal hyperplasia was no longer observed in abraded normal littermate skin (Figure 1H–J). These papillomas were numerous, appeared in only previously abraded K6/ODC transgenic and never normal littermate skin and appeared to develop from the cells lining the follicular cysts that had developed in K6/ODC dermis. By 5 weeks following abrasion, the majority of wound-induced tumors had regressed in size but small tumor-like growths were still observed at 10 weeks following abrasion (Figure 1H–J).

Use of [^{3}H]thymidine autoradiography has been used to show that the transit time (amount of time for a keratinocyte in the basal layer of the epidermis to reach the topmost layer of the epidermis) is dramatically reduced to ∼24 h at 3 days following abrasion (24). Since the transit time is the lowest at 3 days past abrasion, we looked at the distance traveled over a 9 h time period by labeled basal epidermal cells when pulsed with [^{3}H]thymidine at 3 days past abrasion. Labeled nuclei were detected in higher layers of the hyperplastic, newly regenerated epidermis of abraded K6/ODC transgenic skin (Figure 2D–F) compared with that in abraded normal littermate skin (Figure 2A–C), suggesting that epidermal cells in abraded K6/ODC transgenic skin have a shorter transit time at 3 days following abrasion compared with that in abraded normal littermate skin.

Since K6/ODC transgenic mice develop morphologically abnormal skin with alopecia and dermal cysts resulting from degenerating hair follicles, abrasion studies were also performed using inducible ODCER transgenic mice in which the skin retains its normal morphology with no hair loss (11). ODC activity was induced *de novo* in the suprabasal epidermis of ODCER transgenic skin with 1 week of topical application of 4OHT prior to abrasion. With elevated ODC activity directed to the suprabasal epidermis in ODCER transgenic skin, we have shown that the proliferative index is increased to 7.5% in quiescent, non-wounded ODCER skin with BrdU-labeled nuclei detected in the basal epidermal layer and with no resulting hyperplasia (11). Abrasion increased the proliferative index in ODCER transgenic skin (22.3% BrdU-labeled

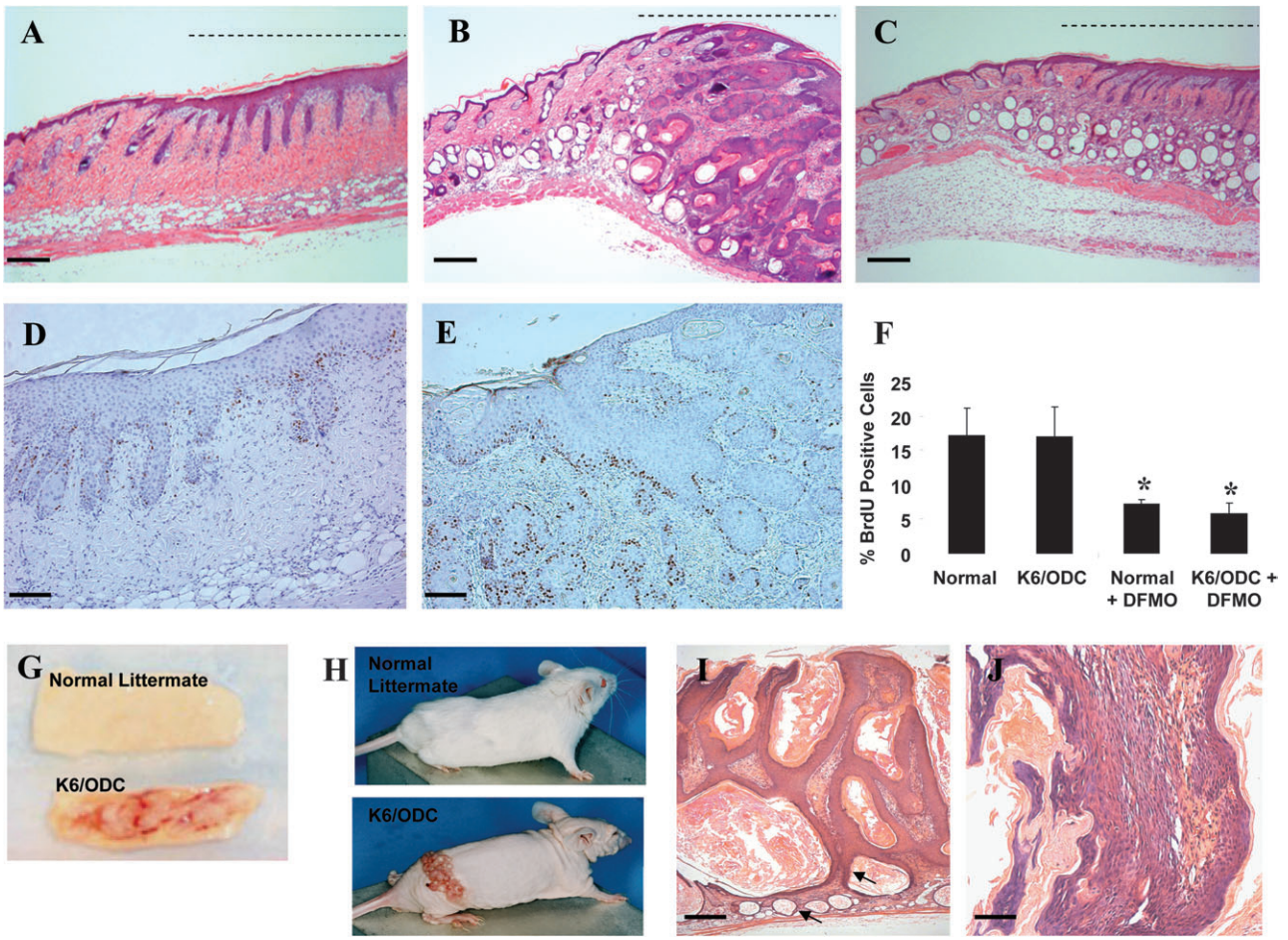

Fig. 1. Wounding induces an exaggerated epidermal hyperplastic response and benign tumor formation in K6/ODC transgenic mouse skin. Normal littermates (**A**, **D** and **H**) and K6/ODC transgenic mice (**B**, **C**, **E**, **F** and **I**) were abraded at 7 weeks of age. (**C** and **F**) Some K6/ODC transgenic mice were given 1% DFMO in their drinking water for 3 days before abrasion and until killed. All mice were injected intraperitoneally with BrdU (100 μg/g body wt) 2 h before killing. (**A**–**C**) Hematoxylin- and eosin-stained skin sections of abraded skin 5 days post abrasion with a dashed line over the abraded sites. (**D** and **E**) Skin sections were stained for cells that incorporated BrdU by immunohistochemical staining and counterstained with hematoxylin. (**F**) BrdU-positive cells/1000 cells in the basal layer were counted in three to five sections for each treatment group. The percentage of BrdU-positive cells expressed as the mean ± SEM. An asterisk denotes significant differences compared with non-treated mice with *P*-values $\leq$0.05. (**G**) Abraded skin was excised from K6/ODC transgenic mice and their normal littermates 5 days post abrasion, and the dermal side of the skin photographed. (**H**) At 5 weeks following abrasion, multiple benign tumors remained in the K6/ODC transgenic skin, whereas no tumors developed in the fully resolved wound site in the normal littermates. (**I** and **J**) Hematoxylin- and eosin-stained sections of wound-induced tumors in K6/ODC transgenic mice at 5 weeks following abrasion at (**I**) low magnification and (**J**) higher magnification. Arrows point to the follicular cysts that form in the dermis of K6/ODC transgenic skin. Note that the tumors grow from the cells that line the follicular cysts. Scale bars: (**A** and **C**) 150 μm; (**B**) 250 μm; (**D** and **E**) 50 μm; (**I**) 250 μm and (**J**) 50 μm.

epidermal cells) to the same extent as that in abraded normal littermates (22.5%) at 7 days following abrasion, but with no difference in the degree of epidermal hyperplasia as was seen in abraded K6/ODC transgenic skin (data not shown). At 14 days after abrasion, epidermal hyperplasia and the epidermal proliferative index (4.6%) was greatly reduced or absent in normal littermates, whereas 4OHT-treated ODCER mice maintained a significantly elevated epidermal proliferative index (20.3%) and a progressive epidermal hyperplasia with papillomatous growths at the wound site (Figure 3B). As with K6/ODC transgenic mice, the regenerative hyperplastic wound response following abrasion persisted in ODCER transgenic skin with increased numbers of basal and suprabasal BrdU-labeled nuclei and tumor-like growths at 8 weeks past abrasion unlike that seen in abraded normal littermate skin where epidermal hyperplasia was no longer observed and scarce BrdU-labeled nuclei were confined to the basal cells of the epidermis (Figure 3D).

Sustained activation of stromal cells following abrasion in ODCER transgenic skin

Underlying stromal cells are activated in response to angiogenic factors and cytokines released by activated keratinocytes in a hyperproliferative epidermis in wound healing (27). Fibroblasts play important roles in wound healing, including deposition of ECM as well as synthesis of ECM-degrading proteases, regulation of inflammation and facilitation of wound contraction. Dermal tenascin-C expression has been shown to be strongly induced in dermal tissue adjacent to hyperproliferative epidermis during wound healing (28) and has been implicated in angiogenesis (29) and in modulating cell adhesion and cell motility (30). Along with the exaggerated epidermal hyperplasia at 2 weeks following abrasion, ODCER mice demonstrated sustained and increased dermal expression of alpha-smooth muscle actin and tenascin-C, both markers of activated stromal cells (Figure 3E–H). In addition to indicating the presence of activated fibroblasts, alpha-smooth muscle actin-positive pericytes also stained many of the dermal blood vessels showing the increased vascularization of the abraded ODCER skin compared with that in normal littermate skin 2 weeks after abrasion (Figure 3F). In marked contrast, 4OHT-treated normal littermates demonstrated minimal epidermal hyperplasia and no reactive stromal cells by 14 days following abrasion (Figure 3E and G).

Metabolic activation in abraded ODCER transgenic skin

In an attempt to identify biological processes that are enriched in ODC transgene-specific differentially expressed genes following

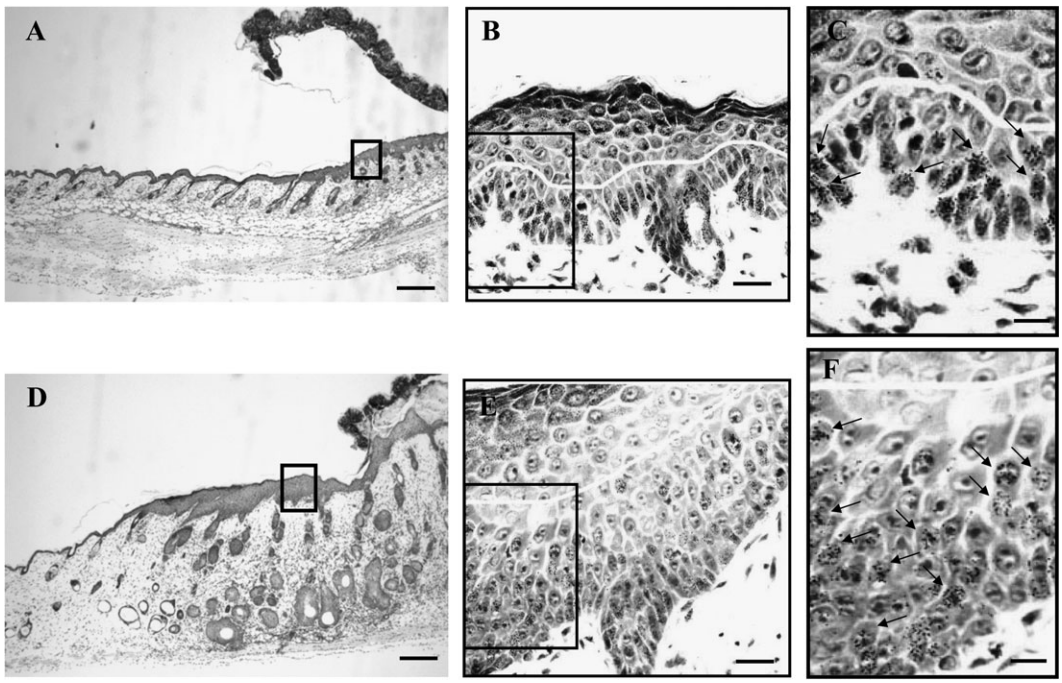

Fig. 2. Reduced epidermal transit time in K6/ODC transgenic skin following abrasion. Three days following abrasion, normal littermates (**A**–**C**) and K6/ODC transgenic mice (**D**–**F**) were injected intraperitoneally with 30 μCi [^{3}H]thymidine and killed 9 h later. (**B** and **E**) Higher magnification views of boxed regions in **A** and **D** and (**C** and **F**) higher magnification views of boxed regions in **B** and **F** show silver grain deposits over proliferating cells that have incorporated [^{3}H]thymidine (arrows pointing at representative labeled cells). A white line marks the top epidermal layer reached by labeled cells. Scale bars: (**A** and **D**) 300 μm; (**B** and **E**) 40 μm and (**C** and **F**) 10 μm.

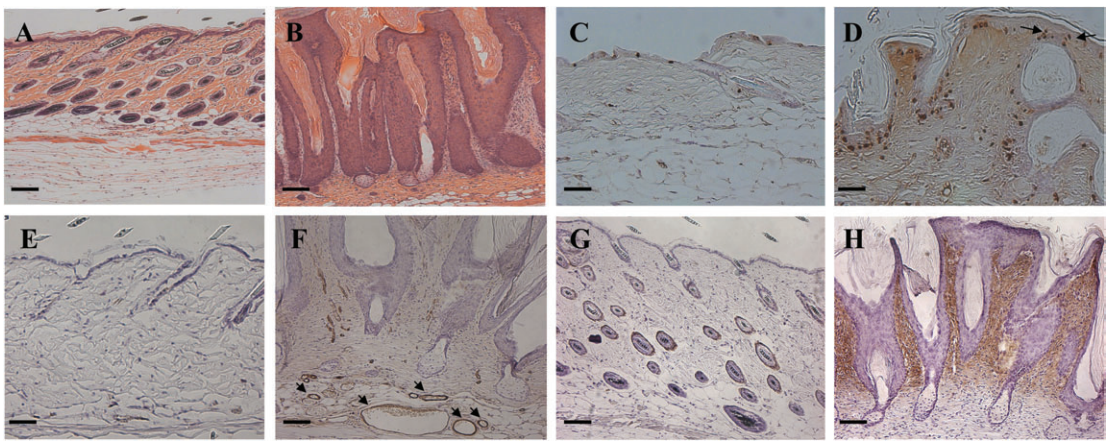

Fig. 3. Chronic progressive epidermal hyperplasia and stromal cell activation in abraded ODCER transgenic skin. Normal littermates (**A**, **C**, **E** and **G**) and ODCER transgenic mice (**B**, **D**, **F** and **H**) were topically treated with 4OHT for 1 week before abrasion until killed 14 days (**A**, **B**, **E**–**H**) or 8 weeks (**C** and **D**) after abrasion. All mice were injected intraperitoneally with BrdU (100 μg/g body wt) 2 h before killing. (**A** and **B**) Skin sections from mice abraded 14 days previously were stained with hematoxylin and eosin. (**C** and **D**) Skin sections from mice abraded 8 weeks previously were stained for proliferating cells that incorporated BrdU by immunohistochemical staining. (**E**–**H**) Skin sections from mice abraded 14 days previously were immunohistochemically stained for (**E** and **F**) alpha-smooth muscle actin or (**G** and **H**) tenascin-C protein and counterstained with hematoxylin. Arrows point to blood vessels. Scale bars: (**A**–**F**) 150 μm.

wounding, microarray analyses of RNA isolated from the abraded skin of K6/ODC transgenic mice and their normal littermates at 3 and 5 days following abrasion was performed. Biological processes enriched in genes that were increased in K6/ODC transgenic skin at 3 and 5 days post abrasion compared with the response in normal littermate skin included protein synthesis, vascularization and some metabolism genes (Supplementary data and Figure S1 are available at *Carcinogenesis* Online).

We used immunohistochemical analyses to look for activation of proteins associated with protein translation in hyperplastic ODCER epidermis at 14 days following abrasion. Activation of Akt signaling has been shown to mediate cell proliferation and the initiation of protein synthesis via phosphorylation and activation of mammalian target of rapamycin (mTOR) and its downstream target S6 ribosomal protein (S6rp) (31). Phosphorylation of S6rp correlates with increased translation, particularly of messenger RNAs (5′ terminal oligopyrimidine tract) encoding for proteins involved in cell cycle progression and the translational machinery. Previously we reported that increased ODC expression in primary keratinocytes led to up-regulation of Akt/mTOR signaling (32). Phosphorylated Akt was detected in suprabasal epidermal cells of abraded ODCER skin, whereas no evidence of activated Akt was detected in normal littermate skin at 14 days following abrasion (Figure 4). Similar to increased expression of phosphorylated Akt, phosphorylated S6rp was differentially increased in

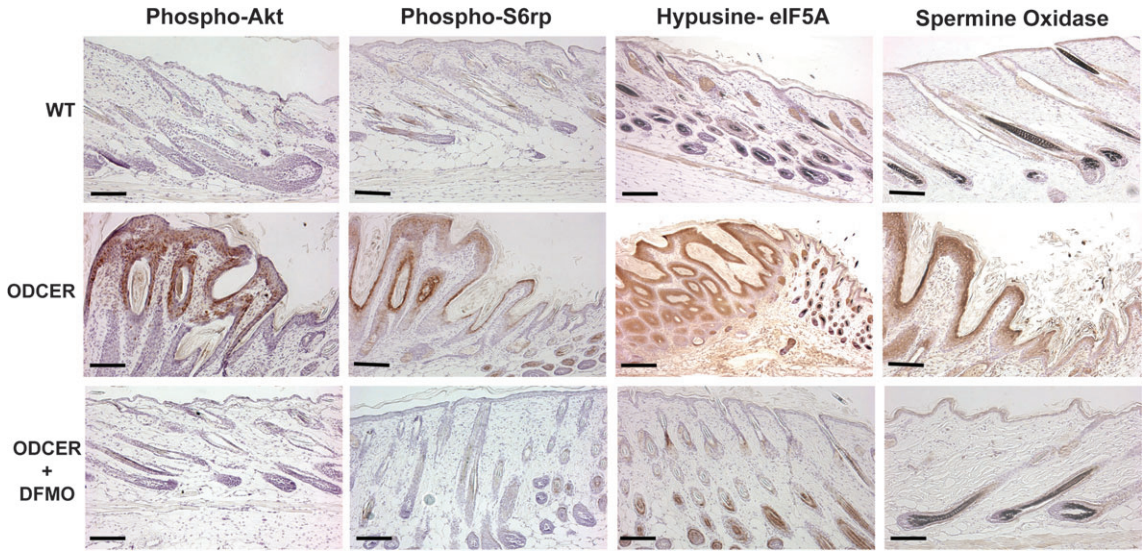

Fig. 4. Metabolic activation in abraded ODCER transgenic skin. ODCER transgenic mice and their normal littermates were topically treated with 4OHT for 1 week before abrasion and 2 weeks after abrasion. Some mice were given 1% DFMO in their drinking water beginning 3 days before abrasion and until killed 14 days later. All mice were killed 14 days after abrasion, and skin sections were immunohistochemically stained for phosphorylated Akt, phosphorylated S6rp, hypusine-containing eIF5A and spermine oxidase protein and counterstained with hematoxylin. Scale bars for all panels: 150 μm.

ODCER transgenic epidermis but not in normal littermate skin at 14 days following abrasion (Figure 4).

Specific to polyamine metabolism, the synthesis of the mature form of eIF5A requires incorporation of hypusine, which is derived from the polyamine spermidine (33, 34). Unlike that seen in normal littermate abraded skin, levels of hypusine-containing eIF5A were elevated in the suprabasal epidermis of ODCER transgenic skin at 14 days following abrasion (Figure 4). The catabolic enzyme spermine oxidase was elevated in abraded ODCER transgenic skin as well (Figure 4). Although DFMO treatment prevented the epidermal hyperplastic response and expression of these translation-associated proteins in ODCER transgenic skin at 2 weeks following abrasion (Figure 4), treatment with the mTOR inhibitor, rapamycin, did not reverse the exaggerated epidermal hyperplasia in abraded ODCER transgenic skin (data not shown).

Wound-induced tumor formation in ODC transgenic mouse skin dependent on recruitment of inflammatory cells

Activated keratinocytes play an important role as modulators of the immune response following tissue injury via the secretion of cytokines such as IL-1 and chemokines such as MCP-1 (35, 36). We assayed abraded ODCER transgenic and normal skin for protein levels of cytokines including TNF-α, granulocyte-macrophage colony stimulating factor, IL-10, IL-2, interferon-γ, MCP-1, IL-6, IL-1 and KC at a time of early (3 days post abrasion) and late (14 days post abrasion) wound healing. TNF-α, MCP-1, IL-6, IL-1 and KC were all elevated at early stages of wound healing (data not shown) with no significant differences between ODC transgenic and normal littermate wound levels. By 14 days following abrasion, only MCP-1 protein levels remained significantly elevated and only in abraded ODCER skin compared with abraded normal littermate skin or non-abraded skin (Figure 5A). Protein levels of TNF-α, IL-6, IL-1 and KC were no longer detected by 14 days after abrasion in either ODCER transgenic mice or normal littermates. The elevated levels of MCP-1 at 14 days following abrasion in ODCER abraded skin was associated with a persistent infiltration of inflammatory cells including macrophages, neutrophils and mast cells as detected by immunohistochemical staining and increased MPO activity in ODCER abraded skin even at 8 weeks following abrasion (Figure 5B and D; (Supplementary data and Figure S2 are available at *Carcinogenesis* Online). In contrast, inflammatory cell infiltrates were considerably decreased in normal littermate skin by 14 days after wounding (Figure 5C).

To investigate whether inflammation contributed to the wound-induced papillomatous growth in ODCER transgenic mice following abrasion, we treated mice following abrasion with dexamethasone, a glucocorticoid anti-inflammatory drug. As expected, dexamethasone treatment greatly diminished infiltrating macrophages and MCP-1 cytokine levels in abraded ODCER skin (Figure 5A and E). The proliferation rate in dexamethasone-treated ODCER transgenic mice (13.56%), as determined by the numerous BrdU-labeled basal epidermal cells, was not significantly different from that seen in non-treated ODCER transgenic mice (20.3%), both of which remained significantly elevated compared with that in normal littermate mice (4.6%) at 14 days past abrasion (Figure 5F–H). However, the exaggerated epidermal hyperplasia was greatly reduced and no tumor-like growths developed in any dexamethasone-treated ODCER transgenic mice (Figure 5E and H).

To further look at the effect of elevated epidermal polyamine levels on recruitment of inflammatory cells, ODCER transgenic mice were administered DFMO to inhibit ODC activity during wound healing. Not only did DFMO treatment completely prevent the exaggerated, prolonged epidermal hyperplasia in ODCER abraded skin, but it also significantly decreased MPO activity in both ODCER transgenic and normal littermate skin at 14 days following abrasion (Figure 5B). Interestingly, whereas DFMO treatment administered throughout wound healing normalized the wound response in ODCER transgenic mice (Figure 6A), DFMO treatment initiated at 4 days post wounding did not normalize the wound healing response in ODCER transgenic mice (Figure 6). Since the early inflammatory stage of wound healing is characterized by infiltration of the wound site with neutrophils, we looked at the effect of DFMO treatment on neutrophil infiltration at 3 days following abrasion. As expected, immunohistochemical staining revealed large infiltrates of neutrophils in both transgenic and normal littermate skin at 3 days following wounding. However, the 3 days neutrophil infiltration was greatly diminished in both transgenic and normal littermate mice treated with DFMO (Figure 6). These data indicate that inflammation contributes to the wound-initiated tumor formation that is dependent on elevated epidermal polyamine biosynthesis.

Discussion

Normal wound healing is characterized by an initial inflammatory response followed by reformation of the epithelial barrier and ECM deposition. Ideally, inflammation quickly resolves and tissue repair occurs in a regulated fashion. In contrast, we have found that wound

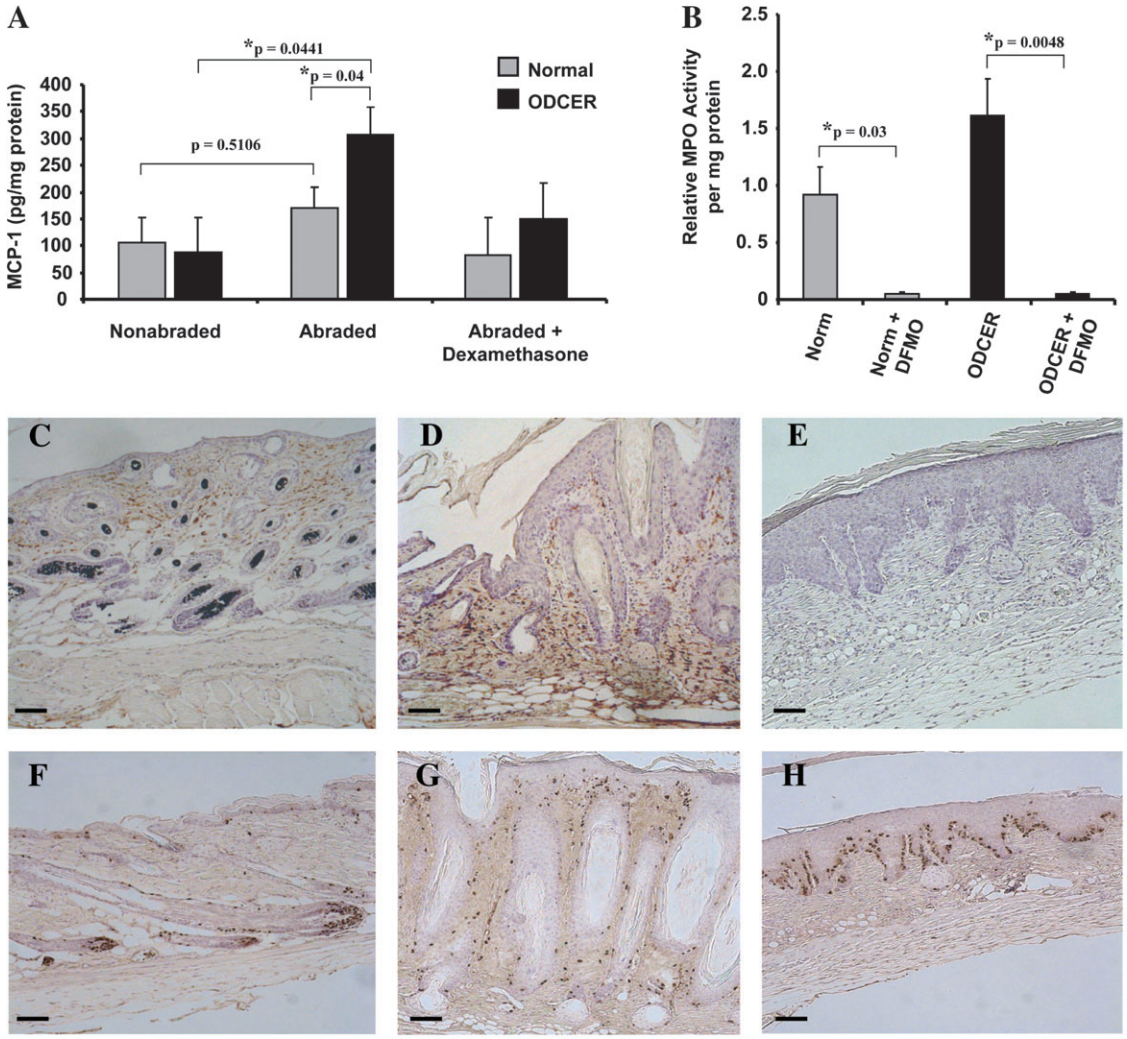

Fig. 5. Persistent inflammatory cell infiltrates in ODCER transgenic skin following wounding. ODCER transgenic mice and normal littermate mice were killed 14 days following abrasion. Some mice were subcutaneously injected with dexamethasone (100 μg in 100 μl phosphate-buffered saline) near the wound site every day starting the day after abrasion. Another group of mice was given 1% DFMO in their drinking water beginning 3 days before abrasion and until killed 14 days after abrasion. The abraded skin was assayed for (**A**) MCP-1 cytokine levels by flow cytometry using Cytokine beads and (**B**) for MPO activity as an indicator of neutrophil infiltrates. Values for MCP-1 protein levels and MPO activity are given as the mean ± SEM with an asterisk marking significant differences between groups with *P*-values ≤ 0.05. (**C**–**E**) Skin sections from normal littermate mice (**C**), ODCER transgenic mice (**D**) and dexamethasone-treated ODCER transgenic mice (**E**) at 14 days following abrasion were stained for macrophage infiltrates by immunohistochemical staining for F4/80 positive macrophages. (**F**–**H**) Skin sections from normal littermate mice (**F**), ODCER transgenic mice (**G**) and dexamethasone-treated ODCER transgenic mice (**H**) at 14 days following abrasion were stained for cells that incorporated BrdU by immunohistochemical staining. Scale bars: (**C**–**H**) 100 μm.

repair in skin with elevated epidermal polyamine biosynthesis exhibits exaggerated epidermal hyperproliferation and persistent inflammation leading to the development of benign tumors. This wound-induced tumor formation was not observed in normal littermate mice and was dependent on ODC activity and polyamine biosynthesis since DFMO treatment normalizes the wound response and prevents tumor formation in transgenic mice. Remarkably, tumors developed in these animals in the absence of chemical carcinogens or the initiation of genetic lesions but were dependent on inflammatory cell recruitment resulting from the wounding and the elevated epidermal ODC activity.

Previously, we reported that elevated epidermal ODC activity in both K6/ODC and ODCER transgenic skin increases the proliferation index in the basal layer of non-wounded epidermis but does not stimulate inflammation nor result in epidermal hyperplasia or tumor formation (11). Here, we show that wounding induces exaggerated epidermal hyperplasia and tumor growth in both K6/ODC and ODCER transgenic mice. Re-epithelialization following wounding involves recruitment of stem cells from the bulge area of the remaining hair follicles (37). Because ODC expression was targeted to the epidermal compartment, we chose to abrade ODC transgenic mouse skin which only removes the epidermal layer. Abrasion precipitates a robust predictable epidermal hyperplastic response with relative absence of granulation tissue. In contrast, full thickness wounds result in both epidermal hyperplasia and extensive proliferation of the underlying connective tissue. Abrasion triggered a prolonged epidermal hyperplastic response in both K6/ODC and ODCER transgenic mice. Unlike the early hyperproliferative overshoot in K6/ODC abraded skin which was marked by hyperplasia of cells lining follicular-derived cysts in the dermis, the early wound healing response in inducible ODCER skin was similar to that in normal littermate skin at 7 days following abrasion. The proliferation rate in newly regenerated epidermis was similarly elevated in K6/ODC, ODCER and normal mice at 1 week following abrasion. However, papillomatous growths and increasing epidermal hyperplasia developed in both K6/ODC and ODCER transgenic skin by 14–21 days following abrasion when both the epidermal proliferation index and hyperplasia had significantly regressed in normal littermate skin. The epidermal proliferation rate remained significantly elevated in both K6/ODC and ODCER

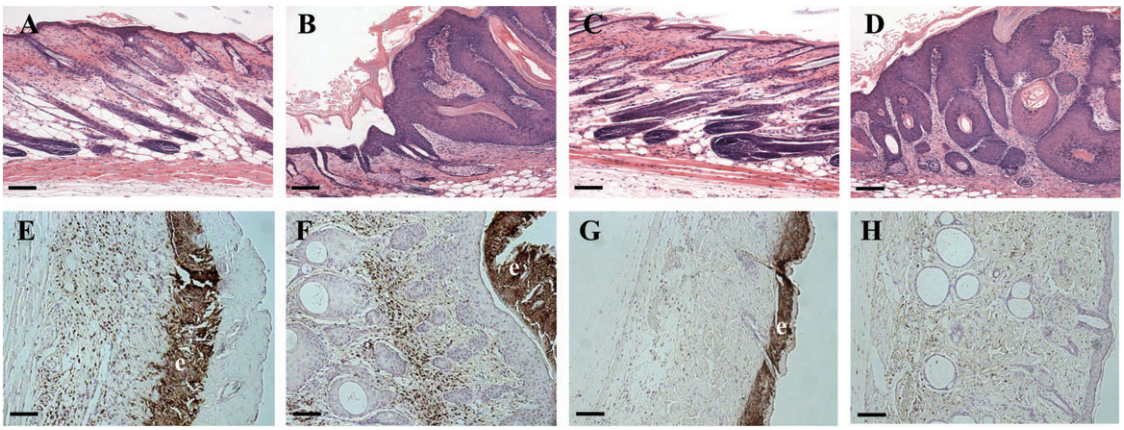

Fig. 6. DFMO prevents wound-induced hyperproliferative response and tumor growth in ODC transgenic mice only if given before wounding. Normal littermates (**A**) and ODCER transgenic mice (**B**–**D**) were topically treated with 4OHT for 1 week before abrasion at 7 weeks of age. (**A**–**D**) ODCER transgenic mice and their normal littermates were killed 14 days after abrasion, and skin sections were stained with hematoxylin and eosin. (**C**) ODCER transgenic mice were administered 1% DFMO in their drinking water for 3 days before abrasion and until killed at 14 days after wounding. (**D**) ODCER transgenic mice were administered 1% DFMO in their drinking water at 4 days following abrasion and until killed at 14 days after wounding. (**E**–**H**) Normal littermates (**E** and **G**) and ODC transgenic mice (**F** and **H**) were killed 3 days after abrasion, and skin sections immunohistochemically stained for neutrophils. (**G** and **H**) Mice were administered 1% DFMO in their drinking water for 3 days before abrasion and until killed at 3 days after wounding. e, eschar formed at the site of abrasion; scale bars: (**A**–**H**) 150 μm.

transgenic skin at 14 days following abrasion compared with that in normal littermates. Since the wound-induced tumor growth in K6/ODC and ODCER transgenic mice slowly regressed, the majority of wound-induced tumors in transgenic mice were most probably low risk, terminally benign papillomas (38). Additional studies are needed to determine whether a small subgroup of these tumors can progress to a malignant phenotype.

Consistent with the exaggerated epidermal hyperplasia in abraded transgenic skin, Akt and mTOR signaling remains activated in ODCER and K6/ODC transgenic mice compared with that in normal littermates. In addition, polyamines are known to bind to RNA (39) and modulate both translation initiation (40, 41) and elongation (42). Specifically spermidine is required for the formation of hypusine which is incorporated in the posttranslational modification and activation of the translational elongation factor, eIF5A (33, 34). Up-regulation of these polyamine-modulated effectors of protein synthesis most probably plays an important role in sustaining the high proliferation rate and epidermal hyperplasia in wounded ODC transgenic skin. Because treatment with rapamycin did not reverse the exaggerated epidermal hyperplasia in abraded ODCER transgenic skin, it is probably that polyamine-stimulation of protein synthesis via activation of mTor signaling is not sufficient to sustain epidermal hyperplasia and wound-induced tumors in ODC transgenic skin. However, treatment with an anti-inflammatory agent dramatically reduced the epidermal hyperplasia in wounded ODCER skin without significantly reducing the high proliferation rate in the basal epidermal layer. Furthermore, inhibition of ODC activity with DFMO only normalized the wound healing response when given from the time of abrasion but not when initiated a few days after abrasion. DFMO suppressed not only the epidermal proliferation rate but also the early infiltration of neutrophils in newly abraded skin. Thus, although elevated polyamine levels stimulate epidermal proliferation both in non-wounded (11) and wounded skin, recruitment of inflammatory cells is necessary to sustain epidermal hyperplasia and wound-induced tumors.

The persistent infiltration of inflammatory cells in abraded ODC transgenic skin is also accompanied with a significant increase in wound MCP-1 production. MCP-1 levels strongly influence the infiltration of macrophages into wounds as demonstrated by the strong influx of macrophages following topical application of recombinant MCP-1 to wounds (43). Interestingly, mice that are deficient in MCP-1 exhibit defective wound repair but with no change in the number of wound macrophages, implicating a role for MCP-1 in macrophage activation and in promoting epithelial and vascular growth (44). It is possible that elevated levels of MCP-1 alters the effector state of macrophages and other inflammatory cells, thus contributing to the dramatic hyperplastic wound response and wound-induced tumor formation. For instance, macrophage polarization from an M1 proinflammatory phenotype to an M2 pro-growth phenotype (45) may be induced in a microenvironment with elevated epidermal levels of polyamines. Besides the beneficial role of inflammatory cells in killing invading bacteria in the wound bed, a persistant inflammatory microenvironment found in a chronic wound can also be a risk factor for malignant transformation (36, 46). Our data suggest that elevated polyamine biosynthesis contributes to 'a wound that never heals' phenotype that Dvorak (16) has likened to a tumor.

Chronic wounds are characterized by a persistent inflammatory response at the wound site, accompanied by unbalanced proteolytic activity and a highly pro-oxidant microenvironment (36). Infiltrating leukocytes, including neutrophils and macrophages, are a rich source of various reactive oxygen species that are released into the wound. In addition, the polyamine catabolic enzyme spermine oxidase, that generates both reactive oxygen species and cytotoxic aldehydes, is induced in ODC overexpressing keratinocytes (47). Since we found that the polyamine catabolic enzyme spermine oxidase is upregulated in the hyperplastic epidermis of abraded ODCER transgenic mice, it is possible that increased polyamine metabolism may contribute to the generation of a more pro-oxidant wound environment and the resulting prolonged hyperplasia and tumor growth in wounded ODCER mice. However, we have found that treatment with neither the reactive oxygen species scavenger, *N*-acetyl cysteine (48) nor with MDL-72527, an inhibitor of spermine oxidase activity (49, 50), prevented the wound-induced tumor formation in ODCER transgenic mice (data not shown). Our data suggest that increased epidermal polyamine biosynthesis exacerbates the wound healing process via multiple mechanisms that stimulate both epithelial proliferation and recruitment of inflammatory cells.

This study shows that wounding cooperates with increased epidermal ODC activity in non-dividing suprabasal epidermal cells to initiate tumor formation. Arwert *et al.* (51) also recently reported benign skin tumor growth following wounding in transgenic mice in which expression of activated mitogen-activated protein kinase kinase 1 is directed to suprabasal, non-dividing epidermal cells. The wound-initiated tumor formation in their transgenic mouse model was dependent on inflammatory cells including γδ T cells and macrophages (51). A similar wound healing phenotype is found in ODCER transgenic skin which is characterized by epidermal hyperproliferation with prolonged activation of translation-associated proteins, a prolonged infiltration of inflammatory cells, activation of fibroblasts and increased vascularization, all of which provide a growth factor and cytokine-enriched stroma that can both support and promote

tumorigenesis. We propose that the ODCER transgenic mouse model of wound healing provides a novel animal model to study the role of chronic inflammation in malignant transformation. In addition to stimulating proliferation of newly regenerated epidermal cells, this study highlights the far-reaching epigenetic effects that elevated epidermal polyamines impose on the dermal microenvironment to activate underlying stromal cells, enhance recruitment of inflammatory cells and skew a normal wound healing response into pathology involving tumor growth. Because these activating, modulatory effects of polyamines act as an accelerating switch in tissue remodeling events such as wound healing, inhibition of polyamine metabolic pathways is an important consideration in designing not only chemopreventive therapy but also adjunct chemotherapy in the treatment of skin cancer.

Supplementary material

Supplementary data and Figures S1 and S2 can be found at http://carcin.oxfordjournals.org/

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