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ORIGINAL ARTICLE

Elevated ornithine decarboxylase activity promotes skin tumorigenesis by stimulating the recruitment of bulge stem cells but not via toxic polyamine catabolic metabolites

Candace S. Hayes · Karen DeFeo-Mattox · Patrick M. Woster · Susan K. Gilmour

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Abstract Elevated expression of ornithine decarboxylase (ODC), the regulatory enzyme in polyamine biosynthesis, targeted to the epidermis is sufficient to promote skin tumor development following a single subthreshold dose of dimethylbenz(a)anthracene (DMBA). Since skin tumor promotion involves recruitment of hair follicle bulge stem cells harboring genetic lesions, we assessed the effect of increased epidermal ODC on recruitment of bulge stem cells in ODC-ER transgenic mice in which ODC activity is induced *de novo* in adult skin with 4-hydroxytamoxifen (4OHT). Bromodeoxyuridine-pulse labeling and use of K15.CrePR1;R26R;ODC-ER triple transgenic mice demonstrated that induction of ODC activity is sufficient to recruit bulge stem cells in quiescent skin. Because increased ODC activity not only stimulates proliferation but also increases reactive oxygen species (ROS) generation via subsequent induction of polyamine catabolic oxidases, we used an inhibitor of polyamine catabolic oxidase activity, MDL72527, to investigate whether ROS generation by polyamine catabolic oxidases contributes to skin tumorigenesis in DMBA-initiated ODC-ER transgenic skin. Newborn ODC-ER transgenic mice and their normal littermates were initiated with a single topical dose of DMBA. To assess tumor development originating from dormant bulge stem cells that possess DMBA-initiated mutations, epidermal ODC activity was induced in ODC-ER mice with 4OHT 5 weeks after DMBA initiation followed by MDL72527 treatment. MDL72527 treatment resulted in a shorter tumor latency time, increased tumor burden, increased conversion to carcinomas, and lower tumor levels of p53. Thus, elevated epidermal ODC activity promotes tumorigenesis by stimulating the recruitment of bulge stem cells but not via ROS generation by polyamine catabolic oxidases.

Keywords Polyamines · Ornithine decarboxylase · Skin carcinogenesis · Stem cell · Acetylpolyamine oxidase

C. S. Hayes · K. DeFeo-Mattox · S. K. Gilmour (✉)
Lankenau Institute for Medical Research, 100 Lancaster Avenue, Wynnewood, PA 19096, USA
e-mail: gilmours@mlhs.org

P. M. Woster
Department of Drug Discovery and Biomedical Sciences, The Medical University of South Carolina, Charleston, SC 29425, USA

Introduction

Polyamines are amino acid-derived polycations that are essential for all cell growth, differentiation, and cell survival. Optimal levels of polyamines are required for DNA replication, gene transcription, protein biosynthesis, and ion channel function. There is considerable evidence that elevated levels of polyamines play an important role in the development of a wide variety of neoplasia. The polyamine content in normal cells is tightly regulated through *de novo* biosynthesis, recycling via catabolic back-conversion, and transport mechanisms. The enzymes ornithine decarboxylase (ODC) and S-adenosylmethionine decarboxylase (AdoMetDC) control the rate-limiting reactions for polyamine biosynthesis. ODC catalyzes the production of putrescine in the first rate-limiting step in polyamine biosynthesis. AdoMetDC produces decarboxylated AdoMet which serves as the aminopropyl donor in the sequential conversions of putrescine to spermidine and spermidine to spermine. Polyamine levels are also influenced by the polyamine catabolic enzymes, spermine oxidase (SMO) and spermidine/

spermine-N^1-acetyltransferase (SSAT). SMO directly converts spermine to spermidine (Wang et al. 2003), while SSAT produces acetylated substrates for N^1-acetylpolyamine oxidase (APAO)-mediated production of spermidine and putrescine (Pegg 2009). The induction of polyamine catabolic pathways leads to the production of H_2O_2 and cytotoxic aldehydes (Pledgie et al. 2005; Zahedi et al. 2007).

In order to characterize the critical polyamine-associated biochemical events which promote epithelial tumorigenesis, many studies have used the well-known initiation–promotion carcinogenesis model in mouse skin. This model consists of initiation with a single application of the carcinogen, 7,12-dimethylbenz[a]anthracene (DMBA) followed by multiple topical treatments with a tumor-promoting agent such as 12-O-tetradecanoylphorbol-13-acetate (TPA). Repeated TPA treatment clonally expands keratinocytes having a DMBA-initiated c-Ha-*ras* mutation and leads to the growth of benign papillomas. Some of these papillomas eventually convert to malignant carcinomas. A variety of transgenic mice with keratin promoter-driven overexpression of key polyamine regulatory proteins have been used in this mouse skin chemical carcinogenesis model. Although TPA induces two rate-limiting enzymes in polyamine biosynthesis, ODC (O'Brien 1976) and AdoMetDC (Scalabrino et al. 1980), overexpression of these enzymes targeted to the skin of transgenic mice results in different effects on tumor development (O'Brien et al. 1997; Shi et al. 2012). Elevated levels of epidermal ODC are sufficient to promote the development of skin tumors following a variety of initiating events including DMBA (O'Brien et al. 1997; Chen et al. 2000), UV irradiation (Ahmad et al. 2001), and oncogenes (Smith et al. 1998; Lan et al. 2005) without the use of tumor-promoting agents. In contrast, increased epidermal AdoMetDC expression is not a sufficient stimulus to drive tumor promotion following DMBA initiation alone (Shi et al. 2012). Indeed, increased epidermal AdoMetDC expression suppresses skin tumor promotion in DMBA-initiated/TPA-promoted mice (Shi et al. 2012). Moreover, increased epidermal spermine synthase activity has no effect on skin tumor development in transgenic mice subjected to a DMBA/TPA carcinogenesis protocol (Welsh et al. 2012). Surprisingly, K6/SSAT transgenic mice with increased epidermal expression of SSAT are more sensitive to skin tumor development when subjected to a DMBA initiation/TPA promotion protocol (Coleman et al. 2002). Increased putrescine production resulting from increased synthesis or increased degradation of the higher polyamines appears to be a common factor in both the K6/ODC and K6/SSAT transgenic mouse models in which skin tumorigenesis is enhanced (O'Brien et al. 1997; Peralta Soler et al. 1998; Wang et al. 2007; Coleman et al. 2002).

Besides the production of putrescine, another common factor in both K6/ODC and K6/SSAT transgenic mice is the generation of reactive oxygen species (ROS) and cytotoxic aldehydes via the induction of catabolic polyamine oxidases. Elevated ODC activity not only stimulates proliferation of normal keratinocytes but also induces apoptotic cell death via generation of reactive aldehydes and H_2O_2, due at least in part to the induction of SMO, followed by the subsequent activation of the ATM-DNA damage response pathway (Wei et al. 2008). These findings suggest that cells with high ODC activity will be selectively sensitized to die when exposed to further oxidative or metabolic stress. On the other hand, ROS and toxic aldehydes, produced by ODC-induced catabolic polyamine oxidase (SMO and/or APAO) activity, may promote tumor development. Free radicals and ROS are known to stimulate skin tumorigenesis, and, conversely, antioxidants block tumor initiation, promotion, and progression (Slaga 1995; Bickers and Athar 2006). To investigate, to what extent ROS production by polyamine catabolic oxidases may mediate ODC-promoted skin carcinogenesis, we studied the effects of the SMO/APAO inhibitor 1,4-bis-[N-(buta-2,3-dienyl)amino]butane (MDL72527) on tumor development in ODC-ER transgenic mice after initiation with DMBA.

Materials and methods

Animals

ODC-ER transgenic mice, in which an involucrin promoter directs the expression of the inducible *ODC* cDNA fused in frame to a 4-hydroxytamoxifen (4OHT)-responsive mutant estrogen receptor ligand binding domain to the suprabasal epidermis, have been previously described (Lan et al. 2005). ODC-ER transgenic mice and their normal littermates have been backcrossed into either the FVB or C57Bl/6 background for at least 10 generations. ODC activity was induced in ODC-ER transgenic mice by topical application of 4OHT (Sigma, St. Louis, Missouri) dissolved in ethanol (1.0 mg/100 μl) applied each day to a shaved area of the dorsal skin.

An inducible Cre-activated bi-transgenic mouse system was used to track hair follicle bulge stem cells and their progeny. K15-CrePR1 transgenic mice (Jackson Laboratories, Bar Harbor, ME) that express CrePR1 under the control of a keratin 15 (K15) promoter were generated to target expression of genes to the adult epidermal stem cells located in the hair follicle bulge cells (Morris et al. 2004). The activity of the 5 kb K15 promoter is tightly restricted to the stem cells found in the bulge area of hair follicles in adult skin (Liu et al. 2003). CrePR1 is a fusion protein consisting of Cre recombinase and a truncated progesterone receptor in which Cre recombinase activity is induced following treatment with the progesterone antagonist

RU486 (Sigma, St. Louis, MO) (Berton et al. 2000). K15-CrePR1 transgenic mice were crossed with Cre-responsive R26R transgenic mice (Jackson Laboratories, Bar Harbor, ME) that express *lacZ* under the control of a ubiquitous promoter after Cre-mediated removal of an inactivating sequence. The resulting K15-CrePR1; R26R bitransgenic mice were treated topically with RU486 (80 μg of RU486 in 0.2 ml acetone) to induce expression of *lacZ* in the bulge stem cells of the skin (Morris et al. 2004). Previous reports have demonstrated that topical treatment of K15-CrePR1; R26R mice with RU486 for 5 days resulted in permanent expression of *lacZ* in the epidermal stem cells in the bulge and in all subsequent progeny of the labeled bulge cells to generate all epithelial cell types in the epidermis, hair follicle, and sebaceous gland (Morris et al. 2004).

Protocols using animals for this study were approved by Institutional Animal Care and Use Committee of the Lankenau Institute for Medical Research in accordance with the current US Department of Agriculture, Department of Health and Human Service regulations and standards.

Tracking of bulge stem cells in ODC-ER transgenic mice

ODC-ER transgenic mice were bred with K15-CrePR1;R26R mice to generate K15-CrePR1;R26R;ODC-ER triple transgenic mice. RU486 (80 μg in 0.2 ml acetone) or acetone vehicle control was applied topically to the dorsal skin of K15-CrePR1;R26R;ODC-ER triple transgenic mice and K15-CrePR1;R26R littermate controls twice daily for 3 days beginning when the mice were 50 days old. At this age, the hair follicles are just beginning a prolonged second telogen phase of the hair cycle when the hair follicle epithelium is quiescent for up to 6 weeks. One week later, the mice were topically treated with 4OHT (1.0 mg/0.1 ml ethanol) to induce ODC enzyme activity for 3 weeks until sacrificed 4 weeks after the initial treatment with RU486. Skin sections from the treated areas were evaluated for β-galactosidase expression by incubation with X-Gal substrate (Roche Diagnostics, Indianapolis, IN) prior to paraffin embedding (Liu et al. 2003). Pictures were acquired using a Zeiss Axiophot microscope (Carl Zeiss Inc., Oberkochen, Germany), with a digital color camera and corresponding software (Axiocam, Zeiss). All images were processed for printing using Adobe Photoshop software.

Detection of label-retaining cells

To label slow-cycling cells, neonatal ODC-ER transgenic mice and their normal littermates were injected subcutaneously (s.c.) beginning at day 3 of age with bromodeoxyuridine (BrdU Sigma, St. Louis, MO; 50 μg/g body weight) twice daily (8 a.m. and 5 p.m.) for 3 days. Cells retaining the label after 8 weeks were identified as label-retaining cells. Mice were treated topically with 4OHT once a day, beginning at 7 weeks of age until sacrificed at 10 weeks of age. Label-retaining cells were detected in skin sections by immunohistochemical staining for BrdU-stained nuclei using a rat monoclonal anti-BrdU antibody (Zymed Laboratories, San Francisco, CA).

Skin tumor induction

4-day-old ODC-ER.FVB transgenic mice and their normal littermates were initiated with a single topical application of 250 nmol DMBA in 50 μl acetone. Five weeks later, mice were topically treated with 4OHT (1.0 mg/0.1 ml ethanol) to induce ODC enzyme activity, and daily 4OHT treatment continued until the mice were sacrificed at 12 weeks of age. Half the mice were given drinking water containing 0.05 % MDL72527 (synthesized in the laboratory of Dr. Patrick Woster, University of South Carolina) beginning at the time when 4OHT treatment was initiated. MDL72527 was administered only 5 days/week to prevent toxic effects from the build-up of spermine in the blood which has been reported in mice exposed for long periods to MDL72527 (Seiler et al. 2002; Wang et al. 2007). At sacrifice, tumor tissue and nontumor-bearing skin was frozen for future analyses, and representative sections were also processed for routine histological staining by hematoxylin and eosin. Tumor diagnosis was confirmed histologically by the standard criteria of cellular atypia, tissue organization, and invasion.

Immunoblot analyses

Tumors were excised, frozen in liquid nitrogen, pulverized, and stored at −80 °C. Ground tumor tissue was homogenized in RIPA-DTT buffer (50 mM Tris–HCl, pH 7.5; 1 % NP-40; 0.25 % sodium deoxycholate; 0.25 % SDS; 150 mM NaCl; 1 mM EGTA and 1 mM DTT) containing 2 μg/μl each of aprotinin, leupeptin, and pepstatin, 1 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM Pefabloc, by passing through a syringe needle and incubating 30 min on ice. Debris was removed by centrifugation at 13,000 rpm for 10 min. Protein content was determined using the Bio-Rad D/C protein assay kit (Bio-Rad Laboratories, Hercules, CA). Protein lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA), and briefly stained with Ponceau S (Sigma, St. Louis, MO) to verify efficient transfer. Immunoblots were incubated overnight at 4 °C in blocking solution (phosphate-buffered saline (PBS) with 5 % non-fat dry milk and 0.05 % Tween 20) followed by 2 h incubation with a rabbit polyclonal

antibody directed against p53 (Santa Cruz, Santa Cruz, CA) followed by secondary antibody conjugated to horseradish peroxidase. Antibody binding was detected by enhanced chemiluminescence (ECL Plus Western Blotting Detection System, Amersham/GE Healthcare, Piscataway, NJ). Filters were reprobed with a mouse monoclonal antibody against tubulin (Calbiochem, San Diego, CA) to verify equal loading of protein.

Polyamine analyses

For polyamine analyses, ground tissues were homogenized in 0.2 N perchloric acid and incubated overnight at 4 °C. Dansylated polyamines were separated on a reversed phase C18 HPLC column (Koza et al. 1991). Polyamine values were normalized for the amount of DNA in the tissue extracts.

Statistical analysis

All experiments were performed at least in triplicate, and data were compiled from 2 to 3 separate experiments. Analyses were done using SAS Version 9.2 using a two-tailed Student *t* test. Values of $p \leq 0.05$ were regarded as being statistically significant.

Results

Elevated epidermal ODC activity is sufficient to recruit bulge stem cells

Elevated levels of ODC and polyamines have been shown to promote the development of tumors in carcinogen-initiated K6/ODC skin in which ODC is constitutively expressed throughout the animal lifetime (O'Brien et al. 1997; Chen et al. 2000; Ahmad et al. 2001). To separately test the effect of elevated ODC activity on tumor development in carcinogen-initiated skin but not on tumor initiation, we used ODC-ER transgenic mice in which involucrin-targeted ODC activity can be induced in adult epidermis with topical 4-hydroxytamoxifen (4OHT) treatment. Previous studies have shown that ODC activity and cutaneous putrescine levels rapidly increase and are significantly elevated within 2 days of 4OHT treatment, continuing to rise with daily 4OHT treatments (Lan et al. 2005). Studies with ODC-overexpressing primary keratinocytes isolated from ODC transgenic mice have confirmed high levels of putrescine with small increases in spermidine levels and decreased levels of spermine compared to keratinocytes from normal littermate mice (Wei et al. 2008).

Because tumors can be elicited in DMBA-initiated skin even with long intervals between initiation and promotion, it is believed that carcinogen target cells are long lived, slowly cycling stem cells found in the hair follicle bulge region (Morris et al. 2000; Owens and Watt 2003). Bulge region keratinocyte stem cells express high levels of α6-integrin, a proliferation-associated cell surface marker for basal keratinocytes (Tani et al. 2000). They also express the cell surface glycoprotein CD34 (Trempus et al. 2003), which plays a major role in hair follicle bulge stem cell activation (Trempus et al. 2007). $\alpha6^+CD34^+$ cells remain dormant during normal epidermal cell cycling, but are recruited from the bulge region in response to wounding or tumor promoters such as TPA (Ito et al. 2005; Trempus et al. 2007). To determine whether *de novo* induction of ODC activity in ODC-ER transgenic skin is a sufficient stimulus to activate hair follicle $\alpha6^+CD34^+$ stem cells in a similar manner to that seen with TPA, we compared the number of $\alpha6^+CD34^+$ cells in the epidermis of 10-week-old ODC-ER transgenic mice following 4OHT induction and in that of their normal littermates. As shown in Fig. 1, there was a significant reduction in the percentage of $\alpha6^+CD34^+$ cells in ODC-ER transgenic compared to that in normal littermate epidermis (7.9 vs. 12 %, respectively) following 3 weeks of 4OHT-induction of ODC activity. Since epidermal stem cells can also be distinguished by their ability to incorporate and retain 5-bromo-2′-deoxyuridine (BrdU) over a long time, we labeled newborn mouse skin with twice daily injections of BrdU for 3 days, which labels all proliferating skin epithelial cells, including those of the bulge (Cotsarelis et al. 1990; Morris and Potten 1999; Taylor et al. 2000). After 10 weeks, the only labeled cells that remained in the skin of ODC-ER transgenic or normal littermates were located in the bulge region of the hair follicles and none in the upper follicle, sebaceous gland, or epidermis. However, following 3 weeks of 4OHT-induction of ODC activity, 10-week-old ODC-ER skin showed fewer BrdU-labeled nuclei (Fig. 1b), reflecting recruitment of some of the labeled retaining cells out of the bulge region and that some of the resting follicles had entered into the anagen (growing) phase of the hair cycle. This recruitment of bulge stem cells was dependent on induction of ODC activity since there was no decrease in cutaneous BrdU-labeled nuclei and $\alpha6^+CD34^+$ cells in 10-week-old ODC-ER transgenic mice not treated with 4OHT.

To trace the emigration of keratin 15-expressing bulge stem cells following induction of ODC activity in ODC-ER transgenic mice, we generated K15CrePR1;R26R reporter mice that also possess the ODC-ER transgene. K15-CrePR1;R26R bigenic mice and K15-CrePR1;R26R;ODC-ER triple transgenic mice were treated with RU486 at 7 weeks of age to induce permanent expression of *lacZ* in the epidermal stem cells in the bulge and in all subsequent progeny of the labeled bulge cells (Morris et al. 2004). One week later, 4OHT treatment was initiated to induce ODC activity, and blue-stained cells expressing beta-galactosidase activity were examined in mice 3 weeks later. Blue cells

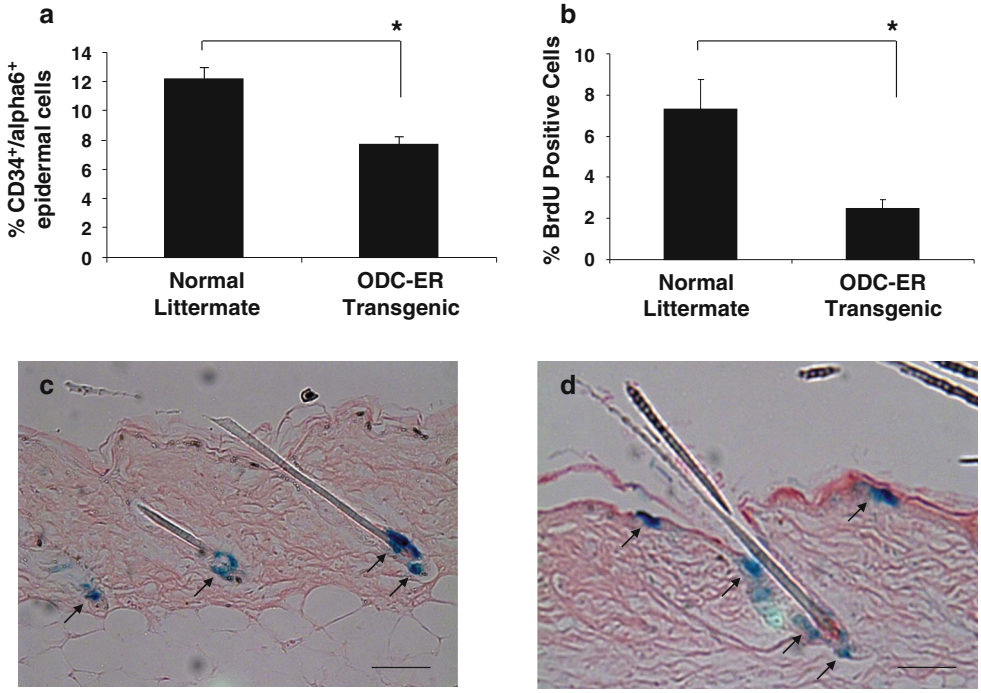

Fig. 1 Induction of ODC activity is sufficient to recruit hair follicle $\alpha6^{+}CD34^{+}$ stem cells. **a** Beginning at 7 weeks of age, ODC-ER transgenic mice and their normal littermates were topically treated every day for 3 weeks with 4OHT (1.0 mg/0.1 ml ethanol) to induce ODC enzyme activity. Cells were isolated from the skin following collagenase digestion, stained for α6-integrin and CD34, and analyzed by flow cytometry for the % $\alpha6^{+}CD34^{+}$ stained cells in the total viable cell population. Results are given as mean ± SD; $^{*}P < 0.01$. **b** Beginning at 3 days of age, ODC-ER transgenic mice and their normal littermates were subcutaneously injected twice a day for 3 days with BrdU. At 7 weeks of age, all mice were topically treated every day with 4OHT until sacrificed 3 weeks later. Skin sections were stained for cells that incorporated BrdU by immunohistochemical staining. BrdU-positive cells/1,000 cells were counted in 3–5 sections for each treatment group. Percentage of BrdU-positive cells is expressed as the mean ± SD; $^{*}P < 0.01$. **c**, **d** K15-CrePR1;R26R bigenic mice and K15-CrePR1;R26R;ODC-ER triple transgenic mice were treated with RU486 (80 μg of RU486 in 0.2 ml acetone) at 7 weeks of age to induce Cre recombinase activity and permanent expression of *lacZ* in K15-expressing epidermal stem cells in the bulge and in all subsequent progeny of the labeled bulge cells. One week later, daily 4OHT treatment was initiated to induce ODC activity, and 3 weeks later the mice were sacrificed. Skin sections from **c** K15-CrePR1; R26R bigenic mice and **d** K15-CrePR1; R26R; ODC-ER triple transgenic mice were evaluated for β-galactosidase (*lacZ*) expression (blue-stained cells indicated by *arrows*) by incubation with X-Gal substrate prior to paraffin embedding. *Scale bar* 100 μm

remained in the bulge region of telogen (resting) hair follicles in K15-CrePR1; R26R bigenic mice (Fig. 1c). However, blue cells were observed not only in the bulge region but also in the upper hair follicle and in the interfollicular epidermis in K15-CrePR1;R26R;ODC-ER triple transgenic mice after 3 weeks of elevated epidermal ODC activity (Fig. 1d). These data indicate that increased polyamine biosynthesis in the epidermis of ODC-ER transgenic mice stimulates the recruitment of bulge stem cells in a manner similar to wounding or treatment with the tumor promoter TPA (Ito et al. 2005; Trempus et al. 2007; Li et al. 2012).

MDL72527 treatment increases skin tumor growth and conversion to carcinomas in DMBA-initiated ODC-ER mice

To generate dormant stem cells harboring mutations that have the potential to develop into skin tumors later in life, newborn ODC-ER transgenic mice and normal littermates were DMBA-initiated at 4 days of age. To determine if *de novo* induction of suprabasal epidermal ODC activity is sufficient to promote skin tumor formation in mice with DMBA-initiated skin, ODC activity was induced with topical treatment with 4OHT beginning at 5 weeks of age (Fig. 2a). We reasoned that permanently altered bulge stem cells will still remain at 5 weeks after DMBA initiation since (1) a pulse-labeled basal epidermal cell requires 5 days to leave the basal layer in mouse skin and 8 days to reach the stratum corneum via terminal differentiation (Morris and Argyris 1983), and (2) carcinogen label-retaining cells were only found in a slowly cycling stem cell population around hair follicles 1 month following a single topical application of a carcinogen (Morris et al. 1986). Consequently, although all cells within the epidermis can sustain oncogenic mutations from exposure to a carcinogen, relatively few skin cancers develop because most cells that acquire mutations are lost through the normal process of terminal differentiation.

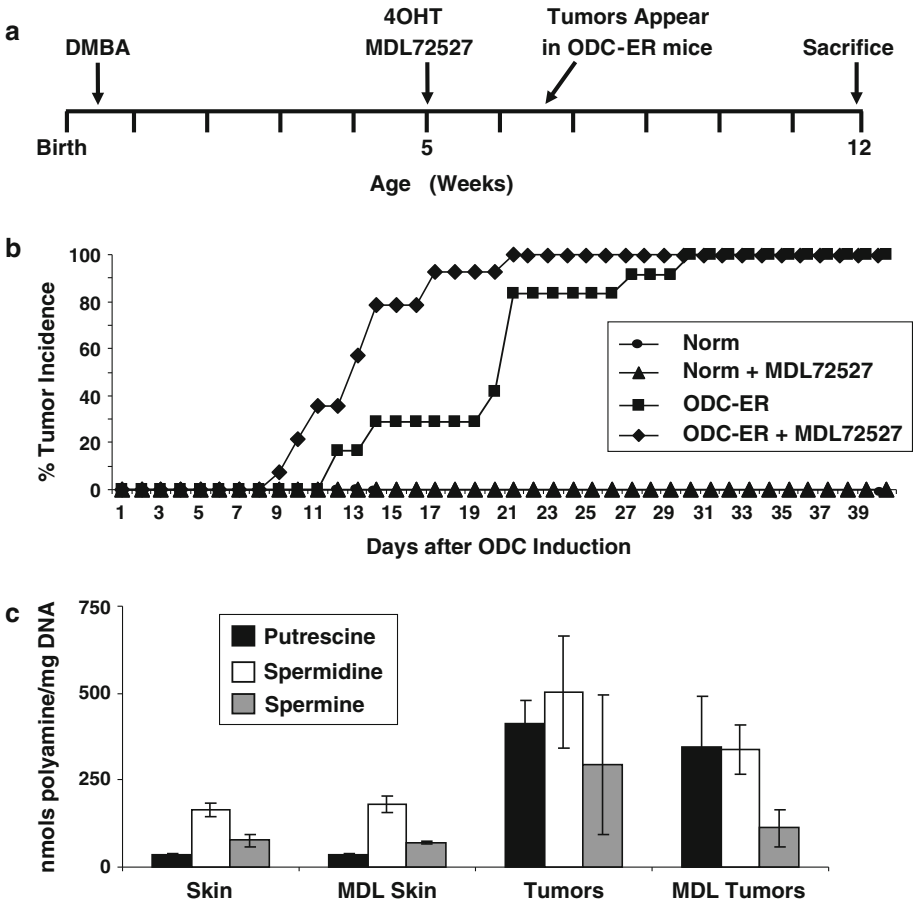

Fig. 2 MDL72527 treatment results in a shorter latency time in DMBA-initiated skin tumor formation in ODC-ER transgenic mice. At 4 days of age, ODC-ER transgenic mice and normal littermates were initiated with a single topical application of 250 nmol DMBA. Five weeks later, daily treatment with 4OHT (1.0 mg/0.1 ml ethanol) was initiated until the mice were sacrificed at 12 weeks of age. Half of the mice were administered 0.05 % MDL72527 in their drinking water beginning at 5 weeks of age. **a** Timeline of treatment and tumor induction in ODC-ER transgenic mice. **b** The number of mice with skin tumors/total number of mice in each group ($n = 8$ mice/group) $\times$ 100 % showing shorter tumor latency period in ODC-ER transgenic mice treated with MDL72527. **c** Tumor tissues and nontumor-bearing skins from ODC-ER transgenic mice treated or not treated with MDL72527 were extracted with 0.2 N perchloric acid, and dansylated polyamines were separated on a reversed phase C18 HPLC column. Polyamine values are expressed in nmol/mg DNA $\pm$ SEM

Previously we reported that elevated ODC activity in keratinocytes increases levels of the catabolic enzyme spermine oxidase with no detectable increase in SSAT, APAO, or AdoMetDC (Wei et al. 2008). This is consistent with our finding that cutaneous spermine levels are often slightly decreased and putrescine and spermidine levels increased following ODC induction in ODC-ER transgenic mice compared to polyamine levels in normal littermate skin (Lan et al. 2005). To test whether the generation of ROS and cytotoxic aldehydes via upregulation of polyamine catabolic oxidase activity plays a role in ODC-promotion of skin tumors in DMBA-initiated skin, half the mice were treated with MDL72527, an inhibitor of polyamine catabolic oxidases, beginning at 5 weeks when 4OHT treatment was initiated (Fig. 2a). Mice were administered drinking water containing 0.05 % MDL72527 only 5 days/week to prevent toxic effects from the build-up of spermine in the blood which has been reported in mice exposed for long periods to MDL72527 (Seiler et al. 2002; Wang et al. 2007). Without repeated treatment with a tumor-promoting agent such as TPA, no normal littermates (with or without MDL72527 supplemented water) developed skin tumors following initiation with this single, subthreshold dose of DMBA. Unlike 4OHT-treated normal littermates, ODC-ER transgenic mice developed skin tumors only following induction of ODC activity with 4OHT treatment (Fig. 2b). Treatment with the specific inhibitor of ODC activity, α-difluoromethylornithine (DFMO), prevented the development of skin tumors in 4OHT-induced ODC-ER transgenic mice (data not shown), indicating that DMBA-initiated tumor development in ODC-ER mice is dependent on elevated epidermal ODC

activity and polyamine biosynthesis. MDL72527 treatment resulted in a significantly shorter tumor latency time with tumors first appearing at 9 days after ODC induction in MDL72527-treated ODC-ER mice compared to 12 days after ODC induction in ODC-ER mice given tap water. At 19 days after ODC induction was begun, skin tumors had appeared in 90 % of MDL72527-treated ODC-ER transgenic mice compared to only 30 % of ODC-ER transgenic mice on tap water (Fig. 2b). After 30 days of ODC induction, all ODC-ER transgenic mice, both treated and not treated with MDL72527, had developed skin tumors, whereas no normal littermates had developed any skin tumors. As previously reported (Koza et al. 1991), both putrescine and spermidine levels were increased in tumor tissue compared to nontumor-bearing skin from the same ODC-ER mice (Fig. 2c). However, treatment with MDL72527 appeared to have no significant effect on tumor or skin polyamine levels (Fig. 2c).

Although MDL72527 treatment had no effect on the total number of tumors per mouse, MDL72527 treatment significantly increased the tumor burden (size of the tumors) (Fig. 3a). By 40 days of 4OHT treatment, MDL72527-treated ODC-ER transgenic mice had a significantly increased percentage of tumors converted to malignant carcinomas compared to ODC-ER mice given control tap water (Fig. 3b). Since levels of p53 are upregulated in tumor tissue compared to normal nontumorigenic tissue in response to DNA damage and stress signals, we looked for effects of MDL72527 treatment on tumor p53 levels. Whereas immunoblot analyses of nontumor-bearing skin revealed no detectable levels of short-lived, wild-type p53 protein (data not shown), p53 protein levels were elevated in the majority of tumors from mice not treated with MDL72527. In contrast, the increased incidence of carcinomas was accompanied by decreased tumor levels of p53 in MDL72527-treated mice (Fig. 3c). In all, MDL72527 treatment increased sensitivity of ODC-ER transgenic mice to DMBA-induced skin tumors with lower levels of tumor p53 and increased conversion to a malignant phenotype.

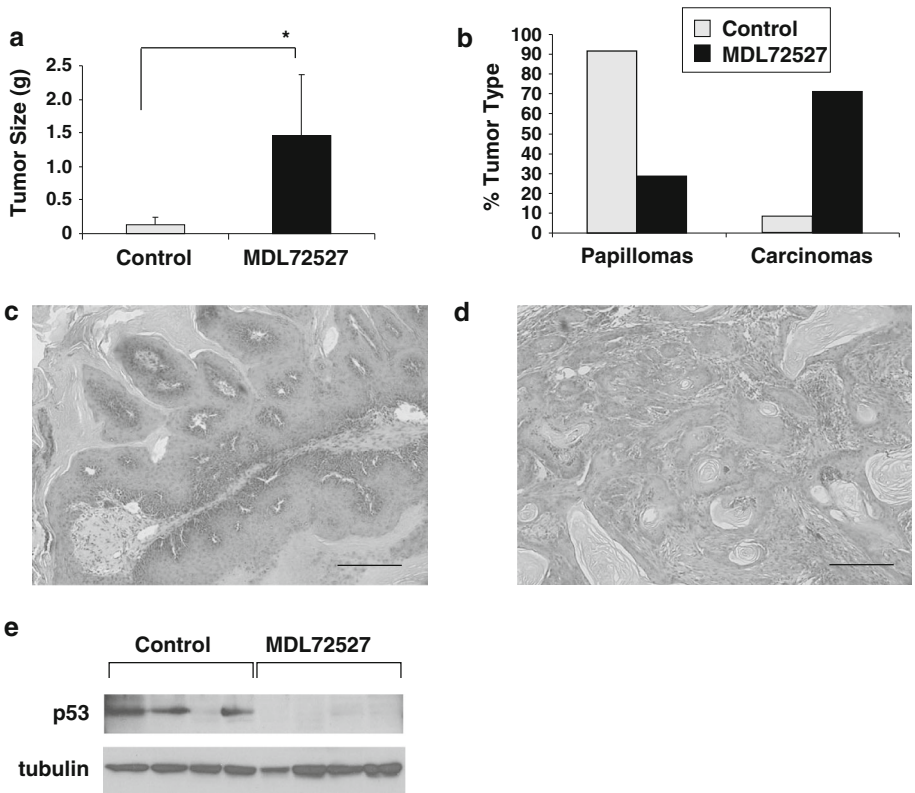

Fig. 3 Increased tumor burden and conversion to carcinomas in MDL72527-treated ODC-ER transgenic mice correlates with decreased tumor p53 levels. **a** Average tumor size in DMBA-initiated ODC-ER transgenic mice administered tap water (control) or water with 0.05 % MDL72527. Results are given as mean ± SD; $^{*}P < 0.01$. **b** Percentage of total tumors that were papillomas or carcinomas in DMBA-initiated ODC-ER transgenic mice administered tap water (control) or water with 0.05 % MDL72527. **c**, **d** Representative hematoxylin and eosin stained tumor sections from **c** an ODC-ER transgenic mouse not treated with MDL72527 and from **d** an ODC-ER transgenic mouse treated with MDL72527. *Scale bar* 200 μm. **e** RIPA lysates were prepared from tumors of mice administered tap water (control) or water with 0.05 % MDL72527. Immunoblot analysis of representative tumor protein lysates in which blots were probed with an antibody specific for p53 and reprobed for tubulin as a control for protein loading

Discussion

Elevated epidermal ODC activity is sufficient to promote skin tumorigenesis following a single topical subthreshold dose of a carcinogen such as DMBA (O'Brien et al. 1997; Chen et al. 2000). The ODC-ER transgenic mouse model offers a unique opportunity to study the effect of increased epidermal polyamine biosynthesis in carcinogen-initiated skin without the need for repeated treatments with a tumor-promoting agent such as TPA. TPA promotion recruits inflammatory cells and incites the production of ROS and a diverse array of pro-tumorigenic cytokines. Both TPA and wounding are also known to stimulate the recruitment of hair follicle bulge stem cells that harbor dormant mutations induced by initiation with a carcinogen (Ito et al. 2005; White et al. 2011; Li et al. 2012). In the absence of TPA treatment or wounding, *de novo* induction of ODC activity reduces the number of BrdU-labeled stem cells and the percentage of $\alpha6^{+}CD34^{+}$ stem cells in quiescent skin, reflecting increased recruitment of bulge stem cells with increased polyamine biosynthesis. This is the first evidence that elevated levels of polyamines alone can positively affect the recruitment of $\alpha6^{+}CD34^{+}$ stem cells which are then activated to give rise to a hierarchy of transit amplifying cells (Taylor et al. 2000). Since the stem cell population in the bulge region is thought to harbor mutations long after initiation with a carcinogen, the development of skin tumors in ODC-ER transgenic mice weeks after DMBA initiation but without TPA treatment is at least in part the result of the ODC-stimulated recruitment of these dormant stem cells harboring DMBA-induced mutations.

Whereas elevated ODC activity provides a strong proliferative stimulus, it can also paradoxically induce the expression of p53 (Gilmour et al. 1999; Wei et al. 2008), stimulate ROS generation via induction of polyamine catabolic oxidases, and activate the ATM-DNA damage response pathway, resulting in apoptotic cell death in keratinocytes (Wei et al. 2008). In contrast to DFMO-inhibition of ODC activity that completely blocks skin tumor formation in DMBA-initiated K6/ODC (Peralta Soler et al. 1998) and ODC-ER transgenic mice (Lan et al. 2005), we have shown that inhibition of polyamine catabolic oxidases with MDL72527 in DMBA-initiated ODC-ER mice leads to increased skin tumor growth and conversion to carcinomas. These findings indicate that PAO/SMO-production of ROS and cytotoxic aldehydes does not significantly contribute in a tumor-promoting role in ODC-ER transgenic mice.

The association of stimulated tumor progression and decreased tumor levels of p53 in MDL72527-treated ODC-ER mice suggests that the induction of polyamine catabolic oxidases and subsequent ROS generation in ODC-ER transgenic skin triggers a protective effect with the accompanying induction of p53 tumor suppressor activity. This is consistent with our previous report that both H_2O_2 and aldehyde/acrolein produced via spermine oxidase activity were responsible for the induction of the DNA damage response observed in keratinocytes with elevated ODC activity (Wei et al. 2008). Treatment of ODC-overexpressing keratinocytes with MDL72527 to inhibit polyamine catabolic enzymes (spermine oxidase and polyamine oxidase) and ROS generation also inhibited p53 induction and activation of the ATM-DNA damage response pathway, thus preventing their apoptotic death (Wei et al. 2008). Moreover, loss of p53 function protected ODC-overexpressing keratinocytes from early apoptotic cell death and significantly extended their survival (Wei et al. 2008). Our tumor results suggest that MDL72527 inhibition of polyamine catabolic oxidase-production of ROS and cytotoxic aldehydes prevents the induction of p53 and the ATM-DNA damage response pathway. This would presumably signal the death of keratinocytes harboring DMBA-induced mutations, thus protecting against tumor development. However, Casero et al. (Kwak et al. 2003) have shown that generation of reactive aldehydes and acrolein by polyamine catabolic enzymes induces phase 2 enzymes such as NAD(P)H:quinone oxidoreductase 1 (NQO1) via activation of the Nrf2-ARE pathway. The increased sensitivity of MDL72527-treated ODC-ER mice to DMBA-initiated skin tumorigenesis supports the belief that the induction of phase 2 enzymes provides a mechanism to protect cells from oxidative damage induced by polyamine metabolites (Kwak et al. 2003). Interestingly, mice deficient in NQO1 show increased sensitivity to carcinogen-induced skin tumorigenesis as well as lower induction of p53 and decreased apoptosis (Iskander et al. 2005). Indeed, immunoblot analyses of tumors from mice treated and not treated with MDL72527 reveal lower, but not statistically significant, levels of NQO1 protein in tumors from mice treated with MDL72527 (data not shown). It is possible that the levels of NQO1 are more dependent on the stage of tumor progression rather than on MDL72527 treatment. Moreover, the protective effect of phase 2 enzymes such as NQO1 may be most important at early stages of skin tumorigenesis and may serve to protect epidermal stem cells harboring mutations from apoptotic death.

Our tumor results suggest that ODC-upregulation of polyamine catabolic oxidation and production of mutagenic ROS triggers protective mechanisms, possibly involving p53-dependent cell cycle arrest and apoptosis in epidermal cells harboring mutations. MDL72527 inhibition of polyamine catabolic oxidases precludes the induction of p53 tumor suppressor activity in ODC-ER transgenic mice but does not inhibit other pro-tumorigenic effects of polyamines such as growth stimulation, angiogenic

activity, and effects on inflammatory infiltrates (Lan et al. 2005; Hayes et al. 2011). Indeed, the multiple modifying effects of polyamines on epigenetic pathways controlling chromatin remodeling (Hobbs et al. 2003, 2006) can impact cell proliferation, stem cell recruitment, angiogenesis, and inflammation, all of which play a key role in promoting tumorigenesis.

In contrast to the MDL72527-enhanced tumor progression in ODC-ER mice, MDL72527 partially prevented skin tumor development induced by a DMBA initiation and TPA promotion protocol in K6/SSAT transgenic mice in which the epidermal SSAT/APAO catabolic pathway is upregulated (Wang et al. 2007). This MDL72527 tumor inhibition supports the notion that the increased tumor susceptibility in K6/SSAT transgenic mice is dependent on production of ROS and toxic aldehydes. Indeed, it is likely that the oxidative damage induced by TPA promotion as well as by greatly increased production of polyamine catabolic metabolites in K6/SSAT transgenic mice overwhelms cellular protective mechanisms, thus permitting the survival of tumor cells and their progression. However, unlike ODC activity that is elevated in most tumors, SSAT/APAO catabolic pathways are not induced in all tumors, including DMBA-initiated/TPA-promoted tumors in non-transgenic mice. On the other hand, SSAT can be induced by hormones, toxins, drugs, and cellular stress, all of which can alter polyamine homeostasis and pathological outcomes (Wang and Casero 2006). Thus, inhibition of polyamine catabolic oxidases with MDL72527 can suppress or stimulate tumor progression depending on the tumor type and treatment. In addition, MDL72527 has cytotoxic effects that are independent of its inhibition of polyamine catabolic oxidases (Seiler et al. 2002). It has been suggested that MDL72527 can cause the release of lysosomal enzymes into the cytosol, thus producing oxidative stress and cell death (Agostinelli et al. 2010). Our data highlight the importance of understanding and targeting polyamine metabolism in different tumor types to better design chemotherapeutic strategies to treat cancer patients.

Our finding that elevated polyamine levels stimulate the recruitment of keratin 15-expressing bulge stem cells in quiescent skin is significant with regard to the stem cell origin of skin cancer. Previous studies have documented the contribution of keratin 15-expressing stem cells to the development of cutaneous papillomas as well as their recruitment following only wounding or treatment with tumor promoters (Ito et al. 2005; White et al. 2011; Li et al. 2012). The identification of polyamines as endogenous stimulators of stem cell recruitment is consistent with the increased susceptibility of ODC transgenic mice to skin tumorigenesis following subthreshold exposure to carcinogens and their subsequent, rapid development of keratoacanthomas and aggressive squamous cell carcinomas (O'Brien et al. 1997; Smith et al. 1998; Lan et al. 2005). Future experiments will exploit this important finding to characterize and target polyamine recruitment of stem cells that initiate invasive skin tumor development.

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Conflict of interest The authors declare that they have no conflict of interest.

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