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Keratin-6 driven ODC expression to hair follicle keratinocytes enhances stemness and tumorigenesis by negatively regulating Notch

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ABSTRACT

Over-expression of ornithine decarboxylase (ODC) is known to be involved in the epidermal carcinogenesis. However, the mechanism by which it enhances skin carcinogenesis remains undefined. Recently, role of stem cells localized in various epidermal compartments has been shown in the pathogenesis of skin cancer. To direct ODC expression in distinct epidermal compartments, we have developed keratin 6 (K6)-ODC/SKH-1 and keratin 14 (K14)-ODC/SKH-1 mice and employed them to investigate the role of ODC directed to these epidermal compartments on UVB-induced carcinogenesis. K6-driven ODC over-expression directed to outer root sheath (ORS) of hair follicle was more effective in augmenting tumorigenesis as compared to mice where K14-driven ODC expression was directed to inter-follicular epidermal keratinocytes. Chronically UVB-irradiated K6-ODC/SKH-1 developed 15 ± 2.5 tumors/mouse whereas K14-ODC/SKH-1 developed only 6.8 ± 1.5 tumors/mouse. K6-ODC/SKH-1 showed augmented UVB-induced proliferation and much higher pro-inflammatory responses than K14-ODC/SKH-1 mice. Tumors induced in K6-ODC/SKH-1 were rapidly growing, invasive and ulcerative squamous cell carcinoma (SCC) showing decreased expression of epidermal polarity marker E-cadherin and enhanced mesenchymal marker, fibronectin. Interestingly, the number of CD34/CK15/p63 positive stem-like cells was significantly higher in chronically UVB-irradiated K6-ODC/SKH-1 as compared to K14-ODC/SKH-1 mice. Reduced Notch1 expression was correlated with the expansion of stem cell compartment in these animals. However, other signaling pathways such as DNA damage response or mTOR signaling pathways were not significantly different in tumors induced in these two murine models suggesting the specificity of Notch pathway in this regard. These data provide a novel role of ODC in augmenting tumorigenesis via negatively regulated Notch-mediated expansion of stem cell compartment.

1. Introduction

Polyamines are small organic cations that are essential for normal cell growth, cell survival and skin homeostasis in eukaryotes. Dysregulated polyamine metabolism may be involved in the pathogenesis of skin cancer [1,2]. Ornithine decarboxylase (ODC) is the rate-limiting enzyme which catalyzes the most committed step in the polyamine biosynthetic pathway [3,4]. Under normal conditions ODC enzyme activity is tightly regulated. However, its expression and activity are induced in response to various external stimuli including tumor promoting agents [4,5]. ODC is over-expressed in human non-melanoma skin cancers (NMSCs), including squamous cell carcinomas (SCCs) and basal cell carcinomas (BCCs), which are the most common human neoplasm [6]. Solar ultraviolet B (UVB), the major etiologic factor for NMSCs in humans induces ODC in the skin. Over-expression of ODC has been demonstrated as an early event in the neoplastic transformation both in murine and human skin keratinocytes [4]. In this regard, employing various animal models of skin carcinogenesis and other epithelial tumors a direct link between increased ODC activity and promotion of neoplastic growth has also been established [7,8].

Keratin 14 (K14) is an intermediate filament produced in squamous epithelia. K14 promoter has been extensively used to direct the expression of various transgenes to the proliferating mouse

Abbreviations: NMSC, non-melanoma skin cancer; SCC, squamous cell carcinoma; BCC, basal cell carcinoma; ODC, ornithine decarboxylase; UVB, ultraviolet B; K6, keratin 6; K14, keratin 14; EMT, epithelial–mesenchymal transition; CK15, cytokeratin 15; CK5, cytokeratin 5; mTOR, mammalian target of rapamycin.

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skin keratinocytes [9]. For example, K14-MEK mice overexpressing MEK protein manifested increased expression of ODC in spontaneously developed skin tumors [10]. Over-expression of ODC in the outer root sheath (ORS) of the hair follicle using both keratin 5 (K5) and keratin 6 (K6) promoters was shown to stimulate skin tumor promotion in transgenic animal models [11–13]. The subthreshold doses of carcinogens topically applied to these animals lead to squamous papillomas in the absence of further treatments with a tumor promoter suggesting that ODC over-expressing animals are pre-promoted [11]. We showed that K6-ODC over-expressing *Ptch*$^{+/-}$ mice manifest augmented development of both BCCs and SCCs upon chronic UVB irradiation [14]. These studies unambiguously demonstrate the ability of ODC to augment proliferation of initiated skin keratinocytes contributing to the pathogenesis of NMSCs.

The bulge stem cells are known to play major role in maintaining the skin homeostasis and tumorigenesis [15]. The origin of SCCs may occur from the slow proliferating stem cell populations located either in inter-follicular epidermis or in the bulge region of hair follicle [16]. Oncogenic mutations or mutational inactivation of tumor suppressor genes in the epidermal keratinocytes are known to drive the pathogenesis of skin cancers. A recent study using ODC-ER transgenic has shown that augmented epidermal ODC activity in the hair follicle bulge stem cells promotes skin chemical carcinogenesis [17].

In the epidermis, Notch and its ligands are abundantly expressed which play an essential role in postnatal hair follicle differentiation and homeostasis. In addition, activated Notch signaling pathway controls stem cell self-renewal [18]. Conditional deletion of Notch1 resulted in development of spontaneous BCC-like lesions in newborn mice [19]. Notch1 expression in non-melanoma skin cancer varies differentially depending on the anatomical site and the tumor histotype. Inhibition of Notch in primary human keratinocytes expressing activated *ras* gene leads to formation of SCCs [20]. Notch1 is down-regulated in UVB-induced invasive SCC, possibly as a consequence of mutational inactivation of p53 [21].

We have generated two novel murine models over-expressing ODC driven by K14 and K6 promoters in SKH-1 genetic background. These promoters respectively target gene expression to inter-follicular epidermis and ORS of hair follicles [7,17]. Chronic UVB-irradiation of these animals showed significant differences in the tumor phenotype, tumor numbers and tumor volume. These differences in K6-ODC/SKH-1 mice and K14-ODC/SKH-1 mice were correlated with the ability of ODC in various epidermal compartments to differentially expand stem cell populations. We also show that Notch which plays a key role in stem cell renewal was down-regulated more effectively in K6-ODC/SKH-1 than in K14-ODC/SKH-1 mice. These data indicate that ODC over-expression regulates stem cell compartment by negatively regulating Notch leading to significant alterations in UVB-induced tumorigenesis.

2. Materials and methods

2.1. Animals

K6-ODC/SKH-1 mice were generated by breeding male (6–7 weeks old) hemizygous ODC transgenic B6.Cg-Tg (K6-Odc) 55Tgo strain (Taconic, Germantown, NY, USA) with female SKH-1 (Jackson Laboratory, Bar Harbor, ME, USA). These mice were back-crossed to SKH-1 for nine generations. K14-ODC/SKH-1 mice were generated by microinjecting the ODC gene carrying a K14 promoter into SKH-1 zygotes with support obtained from the transgenic core facility at University of Alabama at Birmingham. These mice were then crossed with SKH-1 to develop a suitable size colony. Tail biopsies obtained at day 11 were used for genotyping using K6-ODC and K14-ODC primers (Supplementary Table S1). The animal experiments were conducted using protocols approved by the IACUC of the University of Alabama at Birmingham.

2.2. UVB irradiation protocol

Both K6-ODC/SKH-1 and K14-ODC/SKH-1 mice were divided into two groups consisting of non-UVB-irradiated age-matched control and UVB-irradiated experimental animals ($n = 10$). These animals were chronically exposed to UVB radiation (180 mJ/cm^2) three times/week for 29 weeks using UV irradiation Unit (Daavlin Co., Bryan, OH, USA). Tumors were counted and measured at every two week intervals. At the end of the experiment, tumors and skin were harvested and randomly selected for the immunohistochemical and western blot analysis.

2.3. Histology, immunohistochemical or immunofluorescence staining of tissue sections

Histology, immunohistochemical or immunofluorescence staining of skin and tumor sections were performed according to the standard protocol as described earlier [22].

2.4. Western blot analysis

Tissue lysates were prepared in ice-cold lysis buffer containing 50 mmol/L Tris pH, 1% Triton X 100, 0.25% NaF, 10 mmol/L β-glycerophosphate, 1 mmol/L EDTA, 5 mmol/L sodium pyrophosphate, 0.5 mmol/L Na_3VO_4, 10 mmol/L dithiothreitol, 1% phenylmethylsulfonylfluoride, and protease inhibitors cocktail. For Western blot analysis, proteins (60–80 μg) were resolved on 4%, 10% and 15% Tris–glycine gel based on their molecular weights and transferred onto a nitrocellulose membrane (Bio-Rad) as described previously [22]. For sequential antibody reprobing, blots were stripped using Restore western blot stripping buffer (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's instructions. List of antibodies is summarized in Supplementary Table S2.

2.5. Qualitative and quantitative polymerase chain reaction (PCR)

RNA was extracted using Trizol, and reverse transcribed using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, NY, USA). Quantitative PCR was performed using Taqman Fast Advanced Master Mix Product Insert (Applied Biosystems, NY, USA). Relative quantification of the steady-state target mRNA levels was done after normalization of total amount of cDNA to GAPDH (glyceraldehyde-3-phosphate dehydrogenase), an endogenous reference. The list of primers used in this study is described in Supplementary Table S1.

2.6. Statistical analysis

Tumor numbers and volumes are expressed as mean ± SE. Statistical analysis was performed using Microsoft Excel software 2007. The significance between two test groups was determined using the Student t test. A value of $p < 0.05$ was considered to be significant.

3. Results

To address the tissue context-dependent role of ODC over-expression, we developed K6-ODC/SKH-1 and K14-ODC/SKH-1 hairless mice in SKH-1 genetic background. SKH-1 is a highly susceptible genetic background for UVB-induced carcinogenesis. These animals carry homozygous mutations in hairless gene which