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Research Paper

The expression of P63 protein in some keratinocyte original tissues and cells

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Abstract

Objective: To examine the expression patterns of p63 in tissues of particular keratinocyte original hyperproliferate diseases and variety cell types for determining if P63 is the marker of proliferative potential keratinocytes. **Methods:** P63 protein was detected and analyzed by immunoreactivity method and Western blot in biopsy specimens of keratinocyte original disorders including squamous cell carcinomas SCC, basal cell carcinomas BCC, Bowen's disease and other tissues or cells, such as psoriasis vulgaris, normal skin tissues, primary cultured keratinocytes, immortal HaCaT cells, and epidermoid carcinoma cells A431. **Results:** P63 protein was expressed in the nuclei of basal and suprabasal layer of the epidermis, germinative cells of sebaceous glands in normal epidermal. P63 was strongly and diffusely detected in the majority of tumor cells in BCC and poorly-differentiated SCC. In Bowen's disease, p63 expresses are remarkable in all cell layers. In the psoriasis plaque epidermal, p63 expressed mainly in basal cells and part of spinous cells. P63 expressed more strongly in primary cultured keratinocytes than in A431 cells or HaCaT cells. **Conclusion:** P63 is a nuclei marker of undifferentiated keratinocytes with the proliferative potential and may disrupt the terminal differentiation. The overexpression of p63 reflects immaturity of the tumor cells. The immunohistochemical staining of p63 may be useful for investigating the origin and differentiation of tumor cells.

Key words: P63 protein; keratinocyte; carcinoma, skin; psoriasis

INTRODUCTION

Skin is composed of two layers: epidermis and dermis. Mature epidermis consists of stratified squamous epithelium of keratinocyte type. It is capable of regenerating throughout life, which is accomplished by the proliferation of epidermal stem cells(SC) in the lower layers of the normal squamous epithelium[1]. The division of an epidermal SC produces another stem cell and a transient amplifying cell(TAC). Like epidermal SC, TAC also undergoes cell division, but at a greater rate and a finite proliferate potential and progress to differentiation[2]. So in the normal basal cell layer, differentiated cells are presented in addition to SC and TAC. P63 gene is the homologue of p53, and it exhibits a high sequence and structural homology to p53. But several studies find that p63 do not function as a tumor suppressor but rather as an oncogene[3]. It has also been postulated that p63 plays a role in maintaining the undifferentiated stem cell phenotype[4,5]. Here, we carried out an immunohistochemical study and Western blot methods on p63 protein expression. This was carried out in the skin biopsy specimen of normal human skin, psoriasis, some human cutaneous tumors (including basal cell carcinomas-BCC, in situ and invasion squamous cell carcinomas-SCC, cutaneous melanoma, primary cultured normal keratinocytes, immortal HaCaT cells and A431 cells) to investigate whether or not p63 can serve as a valuable immunohistochemical marker for proliferative potential

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keratinocytes.

MATERIALS AND METHODS

Antibodies and reagents

Mouse monoclonal antibody to p63 was purchased from Santa Cruz Biotechnology(clone 4A4,Santa Cruz, CA, USA). Peroxidase conjugated goat anti-mouse IgG was obtained from pierce(HB 987318). Fluorescein isothiocyanate(FITC)-conjugated immunoglobulin G (IgG) Ab(pierce,HI1029034, USA).Tissue culture reagents such as Serum-Free Keratinocyte medium for Culture of Human keratinocytes(Keratinocyte-FM, 17005); Bovine Pituitary Extract(BPE,13028); Recombinant Epidermal Growth Factor(rEGF,10450); and Dulbeco's Modified Eagle's Medium(DMEM) were all purchased from Gibco(invitrogen corporation), Newborn Calf Serum(FCS) from HyClone(SH30118. 02, USA), culture ware from Costar(Cambridge, MA). β-actin, Santa Cruz Biotechnology(Santa cruz, SC-47778). Other chemicals and reagents were of analytical grade and obtained from local suppliers.

Patients and samples

Fifty paraffin-embedded histologic sections were retrieved from the archives of the department of dermatology in the second affiliated hospital of medical school of Xian Jiao Tong University. Seven cases of frozen sections were collected from patients and one skin sample obtained from embryonic of 150 days. The best representative section was selected and 5 μm sections of the respective formalin-fixed paraffin-embedded tissues blocks were used for immunstains.The samples were as follows: Bowen's disease(n = 10), BCC(n = 10), poorly differentiated SCC(n = 5), well-differentiated SCC(n = 10), malignant melanoma(n = 5), psoriasis vulgaris(n = 10) and six cases of normal skin. The diagnoses of the above diseases were based on the clinical and histological characteristics of each kind of diseases.

Cell culture

The human epidermoid carcinoma cells A431 and the keratinocyte cell line HaCaT were both American Type Culture Collection(ATCC) and maintained in DMEM supplemented with 10% FCS and 1% penicillin/streptomycin, and kept in an atmosphere of 5% CO_2 in a 37℃ humidified incubator, in accordance with ATCC guidelines. In all experiments, 75%-80% confluent asynchronously growing cells were used.

Isolation and primary culture of keratinocyte from discarded surgical specimens of foreskin (deriving from 3 young men about twelve years of age). The methods following Keratinocyte-FM experimental protocol (Toma et al[6]) with minor modifications. Pieces of fresh human foreskin were washed with PBS without Ca^{2+} or Mg^{2+}, and cut into 4 to 6 mm pieces, and the epidermal layer was detached from the dermis by incubation with 0.25% dispase solution(Invitrogen Corporation, Carlsbad, CA,USA) at 4℃ overnight. The epidermis was manually removed from each tissue piece and incubated in 0.25 trypsin/ethylenediaminetetraacetic acid (EDTA) solution for 10 min at 37℃, DNase I was added for 1 min, and 10% FCS was added to inhibit the enzymes. The tissue was triturated with a pipette and passed through a 200 mm nylon mesh. The cells were then centrifuged at 1 000 g for 5 min, and resuspended in keratinocyte-FM. Cells not attached in 10 min were discarded to separate the rapidly attaching stem cells from the slower adhering keratinocytes that were liable to differentiate. The adhered cells were continually cultured in SFM medium supplemented with rEGF 5 ng/ml, BPE 50 g/L at 37℃ in a humidified atmosphere containing 5% CO_2. Upon reaching 60%-75% confluences, the medium was removed and resuspended in keratinocyte-FM for subculture. Glass coverslips were used after passage 2 for p63 immunofluorescence staining.

Immunohistochemistry

In the immunohistochemical staining for p63, paraffin-embedded sections(5 μm) were dewaxed, dehydrated and then subjected to antigen retrieval treatment in 0.01 mol/L citrate buffer(pH = 6.0) in microwave oven for 10 min at 100℃ followed by slow cooling for 50 min. 3% hydrogen peroxide blocks endogenous peroxidase activity for both paraffin-embedded sections and frozen sections. Slides were then incubated overnight at 4℃ temperature with an anti-p63 monoclonal antibody 4A4(1:200 dilutions). Peroxidase-conjugated anti-mouse IgG was added for 40 min then developed with 3-3-diaminobenzidine(DAB, ZhongShan, Beijing, China) to produce a brown-coloured signal. Cells were counterstained with haematoxylin. Negative controls were run on each sample by incubating tissue sections without the primary antibody. The stromal cells, which didn's show p63 positively, were used as an internal control in each case. As p63 is a nuclear protein, only nuclear positivity was assessed.

The Immunofluorescence of primary cultured keratinocytes, HaCaT, and A431 cells

For the immunofluorescence staining, colonies were fixed for 10 min with 4% paraformaldehyde and permeabilized for 30 min with 0.2% Triton X-100. Cells were blocked by 10% bovine serum albumin(BSA) and incubated with primary antibody overnight at 4℃. Primary antibody used for primary cultured keratinocytes, HaCaT and A431 cells was mouse Ab

for p63 at a dilution of 1:200. After three washes with PBS, cells were incubated with secondary goat anti-mouse, FITC-conjugated IgG Ab at a dilution of 1: 1 000 for 1 h at room temperature. Preparations were examined by fluorescence microscopy.

Preparation of cell lysate and Western blotting analysis

The cells were washed with ice-cold PBS and resuspended in an ice-cold lysis buffer containing protease inhibitors (1% SDS, 10 mmol/L Tris, pH 7.4, leupeptin 100 μg/ml, phenylmethylsulphonyl fluoride 100 μg/ml, aprotinin 100 μg/ml) for 20 s on ice. The protein was estimated using the BCA protein assay reagent(Pierce Warriner). Samples containing equal protein were boiled in reducing buffer(0.5 mol/L Tris-HCL PH 6.8, 10% SDS, 10% glycerol, 0.4%bromopgenol blue, 10% β-mercaptoethanol). Proteins were electro transferred onto nitrocellulose membranes(Hybond-C, Amersham, UK) in transfer buffer(20 mmol/L glycine, 25 mmol/L Tris,0.6 mmol/L SDS, 10% methanol) for 2 h at 50 V. After transfer, to prevent nonspecific binding, the blots were blocked for 1 h at room temperature in 10% skimmed milk powder in PBS 0.1% Tween TBS, followed by overnight incubation with primary mouse Ab for p63 at a dilution of 1:500 at 4℃. After washing the blots three times in TBS for 20 min, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-mouse secondary antibody(dilution: 1:10 000). The blots was washed three times in TBS for 20 min and were developed with enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Pierce, Warriner, USA). Blots were also probed for β-actin, as an additional loading control. These experiments were repeated for a minimum of three times.

RESULTS

Normal skin

P63 was consistently expressed in all normal skin tissues in several distinct sites. P63 immunohistochemically characteristically stained was always nuclear and consistently localized to basal and spinous cells of the epidermis, peripheral cells of eccrine dermal ducts, germinative cells of sebaceous glands and myoepithelial cells of the terminal portion of eccrine glands and apocrine glands(***Fig 1A,1B***). In the hair follicles, p63 expression was limited to the nuclei of the outer root sheath cells and hair matrix cells(***Fig 1C***). Expression of p63 in the embryonic epithelium(***Fig 1A***) and foreskin specimens were up to two-thirds to three-quarters of the epithelial thickness. The other body sites samples were lower to one-third or one half of the epithelium. In addition, a few cells in the epidermis were p63 negative.

SCC

The expression of p63 protein in SCC was associated with histological types. Grade Ⅰ and Ⅱ SCCs showed variable p63 reactivities in a basal layer like distribution, mainly in peripherally located tumour cells. Terminally differentiated squamous cells and keratin pearls were either negative or showed only focal immunoreactivity (***Fig 1D***); whereas undifferentiated cells of grade Ⅲ SCC were readily apparent, with up to full-thickness p63 positivity(***Fig 1E***).

Bowen's disease

In Bowen's disease, the expression of p63 was usually in a broad continuous banding multiple cell layers in thickness except in the granular cell layer(***Fig 1F***).

BCC

P63 expressed in the nuclears of all BCCs, whereas the majority of cases were up to three-quarters of the epithelial thickness(***Fig 1G,1H***). It seems that there were no significant differences between histologically differented subtypes and undifferentiated ones in p63 immunreactivity.

Psoriasis

In some cases of psoriasis, the expression of p63 in lesional psoriatic epidermis was in all cell layers, similar to Bowen's disease, but the staining signal and the number of positive cells were weaker and less than Bowen's disease. There seems no difference of p63 expression in uninvolved psoriatic skin compared to involved psoriatic skin. P63 expression showed up to two-thirds in the basal and the suprabasal layers and the discontinuous staining was noted focally or extensively in two cases of old plaque lesions(***Fig 1I***).

Melanoma

There was no p63 staining in melanoma skin cancer cells.

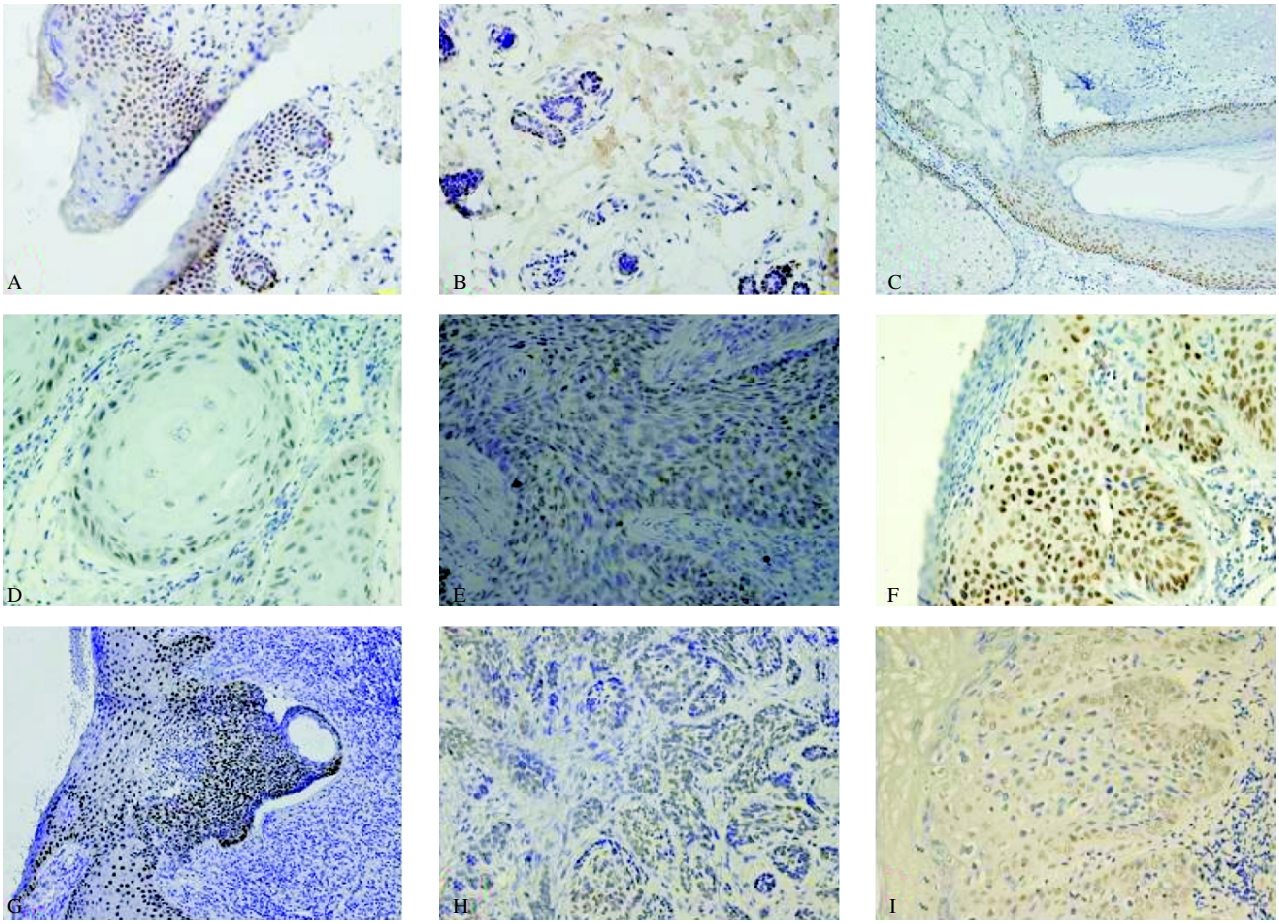
The expression of p63 in the tissues above was consistently observed in several replicate experiments(n = 3) and was confirmed by Western blot(***Fig 2***).

Primary cultured keratinocytes

P63 expresses strongly in the nuclear of the cultivation of foreskin keratinocytes of short-term(passage 3-5) colony formation(***Fig 3A***). The expression of p63 decreases after the cells were subcultured continuously to passage 6-8 and lost after passage 8(***Fig 3B***).

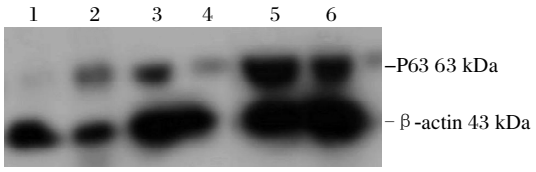
HaCaT

HaCaT cells almost did not express p63 protein when analyzed by immunofluorescence method(***Fig 3C***). But p63 protein showed positive by Western blot method (***Fig 4***).



In normal human epidermis, p63 is expressed in nuclei of (A)basal cells and spinous cells; (B)peripheral cells of the eccrine dermal duct; (C)germinative cells of the sebaceous glands,outer root sheath cells and hair matrix cells; In SCCs, p63 is expressed in (D)grade II showed peripherally or focally located immunoreactivity; (E)grade III showed diffused immunoreactivity. In Bowen's disease, p63 expresses in (F) all cell layer except in granular cells. (G)homogeneous diffuse p63 expression in BCCs in contrast to adjacent nontumorous skin; (H)strong diffuse nuclear p63 immunostaining in a solid type BCC. The expression of p63 protein in psoriasis; (I)shows the discontinuous staining. DAB staining. Magnification with × 20 in A,B,C,G,H and with × 40 in D,E,F.

Fig 1 Immunolocalization of p63 protein in some tissues of keratinocyte origin



1: Melanoma; 2: Foreskin epidermal; 3: SCC; 4:Psoriasis; 5: BCC; 6: Bowen's disease.

Fig 2 Western blot analyses p63 expression

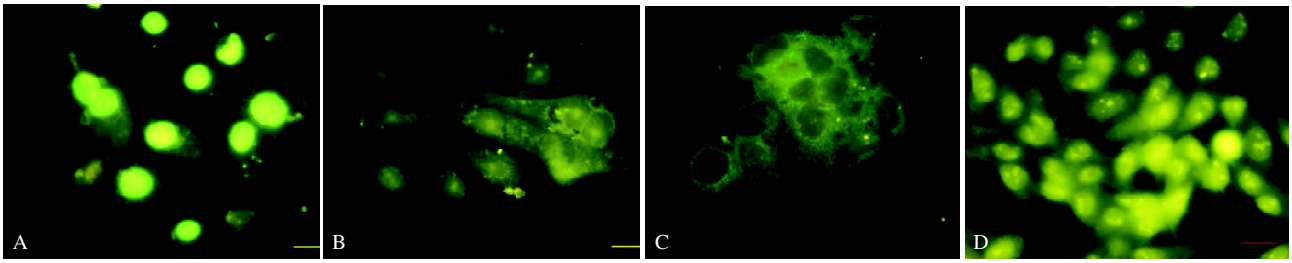
A431

P63 protein could be expressed in A431 cells nuclei, staining signals were weaker than short-term primary cultured keratinocytes(***Fig 3D***).

These results in the cells of primary cultured keratinocytes, HaCaT and A431 were repeated at least three times and confirmed by Western blot(***Fig 4***).

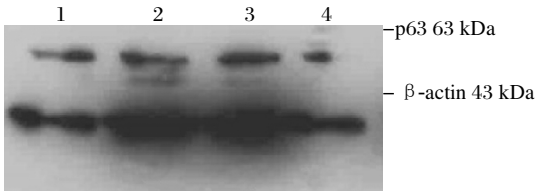
DISCUSSION

P63 is a transcription factor whose expression is thought to be specific for keratinocytes and related to epithelial cell types[4,7]. BCC, SCC, and Bowen disease are all tumors of keratinocyte origin. BCC is believed to be derived from undifferentiated cells of the basal layer of the epidermis and/or hair germ cells. It is locally destructive, but almost never metastasizes. In BCC, the expression of p63 is strongly and diffusely detected in the majority of tumour cells, regardless of histological type. Homogeneous p63 expression in BCC in this study is compatible with a basal/progenitor cell origin. In contrast to BCC, SCC shows variable expressions of p63 depending on the degree of anaplasia, with the majority of less well-differentiated SCC cells possessing p63. Thus, the over-expression of p63 reflects immaturity of the tumor cell lineage and may disrupt terminal differentiation. This concept is further supported by much greater abundance of p63 containing cells in poorly and moderately differentiated SCCs than in well-differentiated ones[8]. In order to study early molecular events leading to invasive SCC, we have examined



The expression of p63 protein (A)in primary cultured keratinocytes with strong p63 immunoreativity(3 passage); (B)in primary cultured keratinocytes with negative p63 immunoreativity(8 passage); (C)shows HaCaT cells with negative signals. (D)in A431 cells with weaker p63 immunoreativity(FITC-labelled fluorescence × 100).

Fig 3 p63 expresses and analyzes in cells of keratinocytes origin by immunofluorescence microscopy



1:primary cultured keratinocytes in passage 3; 2:HaCaT cells; 3:A431 cells; 4:primary cultured keratinocytes in passage 6.

Fig 4 Western blot analyses the P63 expression

Bowen's disease which is preinvasive lesions associated with skin SCC and the results showing that p63 expression occurred in all cell layers remarkably. This finding can be useful for differentiation of these lesions from others.

Melanocytes are derived from the neural crest. The small number of cells in the epidermis of normal skin tissues without positive reaction for p63 may contain melanocytes. All 5 cases of malignant melanoma show negative p63 expression, so we demonstrate that p63 positive cells are keratinocytes originally. Thus, it is of interest that the p63 negative cells do not originate from epidermal germinative cells. In contrast, skin appendages are derived from epidermal germinative cells in the embryonic stage. The observation that p63 expression is limited to myoepithelial cells both in the eccrine and the apocrine glands supports the hypothesis that myoepithelial cells of the sweat glands do not originate from mesenchymal cells but from primary epithelial germinative cells. Furthermore, the immunohistochemical staining of p63 may be useful for investigating the origin or differentiation of tumor cells[9].

Psoriasis is a chronic skin disease of yet unknown etiology, which is characterized by epidermal hyperproliferation and regenerative maturation. It is confirmed that the germinative cell population of SC and more differentiated TAC play a crucial role in the maintenance of the normal homeostasis in normal human epidermis and the balance is disturbed in psoriatic epidermis[10]. In our experiment, p63 expression in psoriasis plaque epidermis is weaker than those in other diseases. As the conversion of a psoriatic plaque to SCC or BCC is extremely rare, p63 seems not to be associated with tumor predisposition.

The method of culturing primary keratinocytes from foreskins in this experiment is for epidermal SC. As specific markers of the epidermal stem cells have not yet been established, it has been postulated that p63 plays a role in maintaining the undifferentiated stem cell phenotype[4,7,11]. The expression of p63 shows stronger positive nuclear staining in the early-term(3-5 passages). It is known that the detachment of keratinocytes from the epidermal basement membrane induces irreversible cell cycle withdrawal and initiates their terminal differentiation program[12]. It is generally accepted that as soon as cells leave the SC compartment, they are committed to the terminal differentiation, and this process is considered to be continuous and irreversible. So p63 expression becomes weaker and weaker until losing the nuclei staining as subcultured to later- term(6-8 passages). The results emphasize that p63 plays an essential role in maintaining the proliferative capacity of keratinocyte precursors and the results coincided with the research of epidermal in vivo that p63 is expressed in the nuclei of keratinocytes with proliferative potential. The results support the concept that p63 may be essential for regulating the differentiation of cells in normal human skin, and is critical for maintaining the germinative cells necessary to sustain epithelial development and morphogenesis[4,13].

HaCaT cells, though immortalized in vitro, closely resemble normal human keratinocytes in their growth and differentiation potential[14]. In surface transplants, HaCaT cells form a rather normal epidermis with all major differentiation markers[15]. But the full differentiation is delayed and the proliferation activity remains at a higher level as compared to normal keratinocyte transplants[16]. In our experiment, p63 expression in HaCaT cells shows almost negative by immunofluorescence staining. Thus, the immortal keratinocytes in single layer culture may represent a stable differentiation stage in the keratinocyte transformation process,

and may have lost the characteristic of epithelial stratification, although they might contain a few undifferentiated stage cells. This may explain why p63 can express in HaCaT by Western blot method.

Another immortal cell being used in this experiment is the epidermoid carcinoma cell line A431[17]. P63 expression in A431 is diffused but does not show strong positive signals in the nuclei compared with primary keratinocytes. The result is verified by Western blot, indicating that A431 cells may maintain the proliferative potential as tumor cells.

The presence of p63, which is in the basal cells of the epidermis, in the cells of the outer root sheath of the hair follicle, and in the small colony-forming cells of stratified epidermal cultures, suggests that p63 protein may have a function in maintaining the proliferative potential of keratinocytes and have a preventative action of terminal differentiation as well as the tendency for epithelial stratification. P63 expression in tumour cells of SCC, BCC, Bowen's disease demonstrate that p63 may be a valuable immunohistochemical marker for undifferentiated and poorly differentiated tumors of epithelial cell origin. Taken together, these findings support the ideas that p63 is the marker of skin keratinocyte in the undifferentiated state of SC and TAC fractions which have an intrinsically high ability to regenerate epithelium. P63 has an additional role in maintaining keratinocytes in the proliferative compartment and in preventing them entry into terminal differentiation. Ultimately, p63 research may lead to novel therapeutic approaches for the keratinocyte original hyperproliferative disease types that are linked to aberrant p63 expression.

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