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SP1-regulated *p27/Kip1* gene expression is involved in terbinafine-induced human A431 cancer cell differentiation: An *in vitro* and *in vivo* study

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ABSTRACT

In this study, the differentiation-promoting effects of terbinafine (Lamisil®, TB) were investigated in human epithelioid squamous carcinoma (A431) cells. The polyhydroxyethyl-methacrylate (poly-HEMA)- and type-I collagen-coated culture plate models were adapted to harvest the TB-induced differentiated cells by agitation of the suspension medium. We demonstrated that p27/Kip1, p21/Cip1 and the keratinocyte differentiation marker, human involucrin (hINV), were induced (>25 μM) in TB-induced differentiated A431 cells. Animal studies demonstrated that administration of TB (10 mg/kg body weight) inhibited A431-xenografted tumor growth through differentiation processes as evidenced by expression of pancytokeratin in tumor tissues. Immunocytochemical staining analysis showed that p27/Kip1, but not p21/Cip1, positive-stained cells were detected in the early-differentiated cells of TB-treated tumor tissues. SP1, which regulates p27/Kip1 expression, was induced by TB (>10 μM) in A431 cells. The TB-induced promoter activity and protein expression levels of p27/Kip1 were significantly attenuated by pretreatment with mithramycin A, a SP1 specific inhibitor. We also demonstrated that TB-induced differentiated A431 cells sorted from the poly-HEMA-coated culture plates were arrested in the G1 phase. TB-induced G1 arrest in the suspension-cultured cells was attenuated by mithramycin A pretreatment. Such results

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Abbreviations: BM, basement membrane; ChIP, chromatin immunoprecipitation analysis; CDK, cyclin-dependent kinase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ECM, extracellular matrix; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; FIL, filaggrin; hINV, human involucrin; LD, lower differentiated region; LOR, loricrin; NBT, nitro blue tetrazolium; NHEKs, Normal human epidermal keratinocytes; poly-HEMA, polyhydroxyethylmethacrylate; RT-PCR, reverse transcriptase-polymerase chain reaction; SC, stratum corneum; TB, terbinafine; TGase-1, tissue transglutaminase-1; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WD, well differentiated region.

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suggest that SP1 plays a critical role in the p27/Kip1 gene transcriptional activation that may be subsequently involved in the TB-induced A431 cancer cell differentiation process.
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1. Introduction

Terbinafine (Lamisil®, TB) is a newly synthesized oral antimycotic drug in the allylamines class: a fungicidal agent that inhibits ergosterol synthesis at the stage of squalene epoxidation [1]. The cream and oral tablet forms of TB have been approved for clinical use in the United States [2]. The oral formulation has been on the market in various countries for more than ten years and as of 1997, more than 7.5 million individuals had been treated with this drug [3]. It shows a good safety profile and relatively few drug interactions [4]. An *in vivo* study demonstrated that TB have potential as an anti-cancer agent by arresting human cancer cell growth at the G1 phase [5]. In this study, experiments using a human squamous tumorigenic cell line (A431) were performed to determine if this TB-induced anti-tumor effect was capable of inducing a full program of differentiation. In this context, it is important to note that human skin cancer may be retarded by either consumption or topical application of TB.

In human squamous skin cancer cells, drug-induced differentiation is a multi-step process in which irreversible growth arrest is an early event followed by the sequential expression of differentiation-associated genes, including keratin 1 and 3, human involucrin (hINV), loricrin (LOR), filaggrin (FIL), and transglutaminase-1 (TGase-1) [6]. Agents that inhibit cancer cell proliferation and enhance the conversion of malignant cells to differentiated cells are expected to terminate cancer development.

Consumption of dietary agents that reduce keratinocyte proliferation and enhance the conversion of premalignant cells to differentiated cells is expected to reduce cancer development. For example, recent studies have demonstrated that a bioactive polyphenol from green tea, (−)-epigallocatechin-3-gallate (EGCG), acts to increase hINV gene expression, suggesting that EGCG treatment enhances normal human keratinocyte differentiation but not apoptosis [7,8]. On the other hand, the cyclin-dependent kinase inhibitor (p21/Cip1) was induced by curcumin, which participates in the cell differentiation process in normal human keratinocytes [8]. In addition, it has been demonstrated that quercetin arrests primary human foreskin keratinocytes in G1 through p27/Kip1 induction [9]. The roles of increased p21/Cip1 or p27/Kip1 in skin tumor carcinogenesis have been investigated *in vivo* by utilizing the two-stage skin carcinogenesis model on p27/Kip1 and p21/Cip1 knockout mice. The results demonstrated that p27/Kip1 deficient mice displayed a more rapid clonal expansion of initiated cells during promotion. In contrast, p21/Cip1 deficient mice mainly displayed a higher grade of undifferentiated tumors [10]. The p21/Cip1 is considered to function as a specific inhibitor of tumor cell growth. Previous paper have demonstrated that p21/Cip1 inhibits tumor cell proliferation by participating in the activation of tumor cell differentiation as evidenced by a higher expression profile of p21/Cip1 during all-trans retinoic acid-induced differentiation in various types of human cancer cells [11]. Thus, the most important issue is to identify specific agents with dual mechanisms of action in human skin cancer cells that both decrease proliferation and increase differentiation.

In this study, the expression of cell cycle and differentiation regulatory proteins were determined during commitment of TB-induced squamous cancer cell differentiation. For example, increased levels of expression of p27/Kip1, p21/Cip1 and keratinocyte differentiation markers (such as TGase-1) were observed simultaneously, suggesting a close link between cell growth arrest and differentiation. Moreover, according to previous studies, a SP1 binding sequence appears in the *p27/Kip1* gene promoter [12]. In addition, differentiation-associated markers such as TGase-1 [13,14] and hINV [15] which are expressed in the suprabasal layers of the human epidermis, were also transcriptionally regulated by SP1. Interestingly, our results demonstrate that SP1 protein levels and its transcriptional activity were induced by TB treatment in A431 cells. The experimental findings reported below highlight the molecular mechanisms underlying TB-induced cell growth arrest and differentiation activity in human squamous carcinoma cells.

2. Materials and methods

2.1. *Terbinafine (TB)*

TB is manufactured by Patheon Whitby Inc. Whitby, Ontario, Canada L1N 5Z5. In this study, TB was purchased from Novartis Pharmaceuticals Corporation, East Hanover, New Jersey 07936. Chemically, terbinafine hydrochloride is (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynyl)-*N*-methyl-1-naphthalene-methanamine hydrochloride. Terbinafine hydrochloride is a white to off-white fine crystalline powder with 99.5% purity. It is freely soluble in DMSO, methanol and ethanol, and is slightly soluble in water.

2.2. *Determination of the cell growth curve*

Normal human epidermal keratinocytes (NHEKs) from neonatal foreskin were first cultured for maintenance in EpiLife® medium containing 0.06 mM $CaCl_2$ and EpiLife® Defined Growth Supplement (Cascade Biologics, Portland, OR) and used as normal cell control. The A431 human epitheloid squamous carcinoma cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum at 37 °C in an incubator containing 5% CO_2. Media with and without TB were changed daily until cell counting.

2.3. *Flow cytometry analysis*

Human NHEK and A431 cancer cells were synchronized as previously described [16]. After the cells had grown to 70–80% confluence, they were rendered quiescent by incubation for 24 h in medium containing 0.04% FCS and then challenged with 10% FCS. The population of nuclei in each phase of the cell cycle was determined using established CellFIT DNA analysis software (Becton Dickenson, San Jose, CA).

2.4. *Suspension detached and collagen-coated dish culture of A431 cells*

The suspension cultures were performed as described previously [17,18]. Briefly, A431 (2×10^6) cells were plated in 6-cm polyhydroxyethylmethacrylate (poly-HEMA)-coated dishes in supplemented culture medium without bovine hypothalamic extract. The A431 cells were harvested from the dishes by gently pipetting (suspension-cultured cells, presented as S) or with trypsin-EDTA (adherent-cultured cells, presented as A).

For collagen-coated dishes for adherent culture [17,18], A431 (2×10^6) cells were allowed to adhere to 6-cm type-I collagen-coated dishes for 18 h in culture medium without bovine hypothalamic extract. The A431 cells were then exposed to TB for an additional 24 h. Non-adherent (or detached, presented as D) cells were removed by gently washing the plates with PBS. The total number of adherent (or attached, presented as A) cells were harvested and counted from the dishes with trypsin-EDTA.

2.5. *Antibodies*

The following antibodies were obtained as indicated: p21/Cip1, p27/Kip1, GAPDH, SP1 and caspase 8 antibodies (Santa Cruz, Inc., CA); cyclin D1, D3, Rb-2, α-tubulin, and PCNA antibodies (Transduction Laboratories, Lexington, KY); Bad (Cell Signaling Technology, Beverly, MA, USA); Bax and Bcl-2 antibodies (Dako Corporation, Denmark); caspase 9 and caspase 3 antibodies (Stressgen Biotechnologies, Victoria, British Columbia, Canada); cyclin E polyclonal antibody (Transduction, San Diego, CA); hINV monoclonal antibody (SY5, Sigma Chemical Co., St. Louis, MO); filaggrin and TGase-1 (B.C1,) antibodies (Biomedical Technologies, Inc., Stoughton, MA, USA); human K1 and K10 antibodies (Chemikon Int., Hofheim) [19]; and loricrin antibody (PRB-145, Covance Inc.).

2.6. *Western blot analysis*

Western blot analysis was performed as previously described [5]. Immunodetection was performed by probing with proper dilutions of specific antibodies and the specific protein complexes were identified by using a Vistra ECF kit (Amersham Biosciences, Arlington Heights, IL) according to the manufacturer's instructions.

2.7. *Immunoprecipitation*

For immunoprecipitation, 300–1000 μg total cellular lysates were incubated with 3–5 μg of either p21/Cip1 (F-5; Santa Cruz Biotechnology) or p27/Kip1 (C-19) rabbit polyclonal antibodies or control rabbit immunoglobulin (Santa Cruz Biotechnology). The antigen/antibody complexes were precipitated using protein A-conjugated agarose beads, subjected to SDS-PAGE and detected with antibodies against CDK4 (DCS-156; BD Biosciences) or CDK2 (D-12, Santa Cruz Biotechnology) respectively. Results are representative of at least 3 independent experiments.

2.8. *Kinase activity assays*

CDK2 or CDK4 kinase assays were carried out as described previously with minor modifications [20].

2.9. *Chromatin immunoprecipitation analysis (ChIP)*

Chromatin immunoprecipitation assays of cultured cells were performed as described previously [21]. The SP1 antibody (sc-420, Santa Cruz Biotechnology Inc.) was used for the immunoprecipitation reactions. Primers specific for detection of the SP1 binding regions from −462 to −774 of the p27/Kip1 gene were adapted with the sense primer, CCGAACCT-CAGGCCCCGCCC, and anti-sense primer, GACGAAGAA-GAAAATGATTG.

2.10. *Plasmid construction*

The SP1 binding site of the p27/Kip1 gene promoter region (−549 and −523) [12] was synthesized such that the sequence repeated five times and was then was linked to a basic luciferase reporter gene. The 5× SP1 (GGGGCGGGGC) and 5× SP1 mutant (GGTTCGGTTC) DNA binding sequences were cloned into the pGL3 vector (Promega, Madison, WI, USA) with KpnI and XhoI restriction sites. The pGL3 vector contains the sequences encoding the Firefly luciferase gene which when fused with SP1 or its mutant promoter, were presented as PGL3(SP1)5 and PGL3(mut SP1)5, respectively. The pRL-TK vector encoded the Renilla luciferase gene, which was used as an internal control to normalize Firefly luciferase expression.

2.11. *Cell culture and dual luciferase reporter assay*

The A431 cells were co-transfected with 2 μg of pGL3 and 500 ng of pRL-TK vector using lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA) in a 6-well plate (5×10^5 well^{-1}) and then treated with TB 24 h later. The SP1 specific inhibitor, mithramycin A (400 nM) [12] (purchased from Sigma Chemical Co.), was added 1 h before TB treatment. After 24 h of drug treatment, the cells were lysed in passive lysis buffer (Promega). Cell lysates were then added to the luciferase substrate (Dual-Glo luciferase reporter system, Promega) at which point the Firefly and Renilla luciferase activities were measured with a 96-well luminometer (Hidex Chameleon, Finland).

2.12. *Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis*

The cDNA was amplified from 1 μg of total RNA using a SuperScript one-step RT-PCR with platinum Taq system (Life Technologies, Inc.). PCR was conducted for 32 cycles in a

thermal controller. Primers used for amplification were as follows: K1-specific primer, K1-f (5′-ACACCAGGTATCAGATGTGGG-3′) and K1-r (5′-TGAAGCCCCCTCCACTTCGGTA-3′). K10-specific primer, K10-f (5′-TTTGGTGGTGGATTCGGAGGAGAC-3′) and K10-r (5′-CAATTTGCATCTCTAGGTCGGC-3′). LOR-specific primer, LOR-f (5′-CGAAGGAGTTGGAGGTGTTTTCC-3′) and LOR-r (5′-TTTATTGACTGAGGCACTGGGG-3′). FIL-specific primer, FIL-f (5′-GATCTGGACACTCAGGGTCTCATC-3′) and FIL-r (5′-GCTCTGTCTTCTTGATGGGACCTG-3′). TGase-1-specific primer, TGase-1-f (5′-TGAATAGTGACAAGGTGTACTGGCA-3′) and TGase-1-r (5′-GTGGCCTGAGACATTGAGCAGCAT-3′). The GAPDH-specific PCR products from the same RNA samples were amplified and served as internal controls. Primers GAPDH-f (5′-ACCACAGTCCATGCCATCAC-3′) and GAPDH-r (5′-TCCACCACCCTGTTGCTGTA-3′) were used for amplification of GAPDH.

2.13. Animal experiments

The A431 cells (5×10^6) in 0.1 ml DMEM were transplanted subcutaneously between the scapulae of each nude mouse (10 mice in each group) according to our previous reports [5]. After transplantation, the tumor size was measured using calipers, and the tumor volume was estimated by the formula: tumor volume (mm^3) = $W^2 \times L \times 1/2$, where L is the length and W is the width of the tumor. Once the tumor reached a volume of 200 mm^3, animals received intraperitoneal injections of DMSO (25 μl) or TB (10 mg/kg) three times per week for 6 weeks. Half of the mice in each group ($n = 5$) were sacrificed at 3 weeks after TB or DMSO treatment. At the end of the experiment, all of these tumor tissues were dissected from the mice for immunohistochemical analysis.

2.14. Immunocytochemical staining analysis

As previously described [5], paraffin-embedded blocks were serially sectioned at 5–7 μm thickness and incubated overnight at 4 °C with the pan-cytokeratin, p21/Cip1, and p27/Kip1 antibodies, respectively. After a second incubation with a biotinylated anti-mouse antibody, slides were incubated with peroxidase-conjugated streptavidin (DAKO LSAB+ kit; Dako Corp., Carpinteria, CA). Reaction products were visualized by immersing slides in diaminobenzidine tetrachloride and were finally counterstained with hematoxylin.

2.15. Statistics

All of the data are expressed as the mean value ± S.E. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

3. Results

3.1. Anti-proliferation effect of TB on human skin cancer (A431) cells

In this study, we first examined the anti-proliferation effects of TB on human epithelioid squamous carcinoma (A431) cells. Normal human epidermal keratinocytes were used here because they secrete keratin and could be compared to A431 cancer cells to examine cell growth inhibitory effects in response to TB. The cells were cultured in the presence or absence of TB (30–120 μM) for 4 days, and the cell numbers were counted daily. Our results showed that TB significantly inhibits cell growth in human A431 cells (Fig. 1A, left panel). In contrast, the cell growth inhibition effect was less profound in human NHEKs (Fig. 1A, right panel).

3.2. TB-induced G1 cell cycle arrest effect in human A431 skin cancer cells

To more convincingly demonstrate the actions of TB on a specific phase of the cell cycle, A431 cancer cells were synchronized by switching them to media with 0.04% FCS for 24 h to render them quiescent [5]. After 24 h, 10% FCS and 0.05% DMSO (control) or 30 μM TB in 0.05% DMSO were added to the culture media and, at various time points thereafter, the cells were harvested for flow cytometry analysis. Fig. 1B shows representative FACS analyses of DMSO- (left panel) and TB-treated (right panel) A431 cells at various timepoints after release from quiescence. The results show that TB induced a significant accumulation (>80%) of A431 cells in the G1 phase of the cell cycle (Fig. 1B, right panel). Based on the FACS analysis results observed for A431 cells, the timepoints 0, 15, and 18 h after release from quiescence could symbolize G0/G1, S, and G2/M (Fig. 1B, left panel). Synchronized cells exposed to 10% FCS for more than 24 h were then trended to reach confluence (Fig. 1B, left panel). Fifteen hours (presented as the S phase) was selected as a specific timepoint for evaluation of the dose-dependent effect on TB-induced G1 arrest. Serum synchronized A431 cells were then challenged with complete medium containing 10% FCS with or without TB (1–100 μM) for 15 h, and cells were sorted for flow cytometric analysis. Our results show that TB (0–50 μM) dose-dependently blocked transition of the cells from G0/1 to S (Fig. 1C). We also found that the sub-G1 population of A431 cells was induced by 15 h of TB (>50 μM) treatment (Fig. 1C, $^{*}P < 0.05$).

3.3. Effects of TB on A431 cell cycle regulatory proteins

The A431 cells were treated with TB (10–100 μM) using the same conditions as in Fig. 1C described above. After drug treatment, the TB- and DMSO-treated cells were harvested for protein extraction, and Western blotting analysis was performed (Fig. 1D). As shown in Fig. 1D, p21/Cip1 and p27/Kip1 protein levels were increased, whereas cyclins A1, B, and D3 were significantly down-regulated in TB-treated A431 cells. The levels of other G1 phase regulatory proteins, including cyclins D1, D3, E, cdk2, cdk4, PCNA, and Rb-2 proteins, were not significantly changed in the TB-treated A431 cells (part of the results are presented in the Supplemental Fig. 1D).

Time-dependent experiments were performed and showed that significant inductions of p21/Cip1 and p27/Kip1 proteins were only observed in the TB-treated A431 cells (Fig. 1E, lower panel), suggesting that p21/Cip1 and p27/Kip1 proteins might play some important role in the TB-mediated cell-specific insult. Treatment of A431 cells with TB (25 μM) for 24 h caused an increased formation of the p21/Cip1-CDK4 and p27/Kip1-

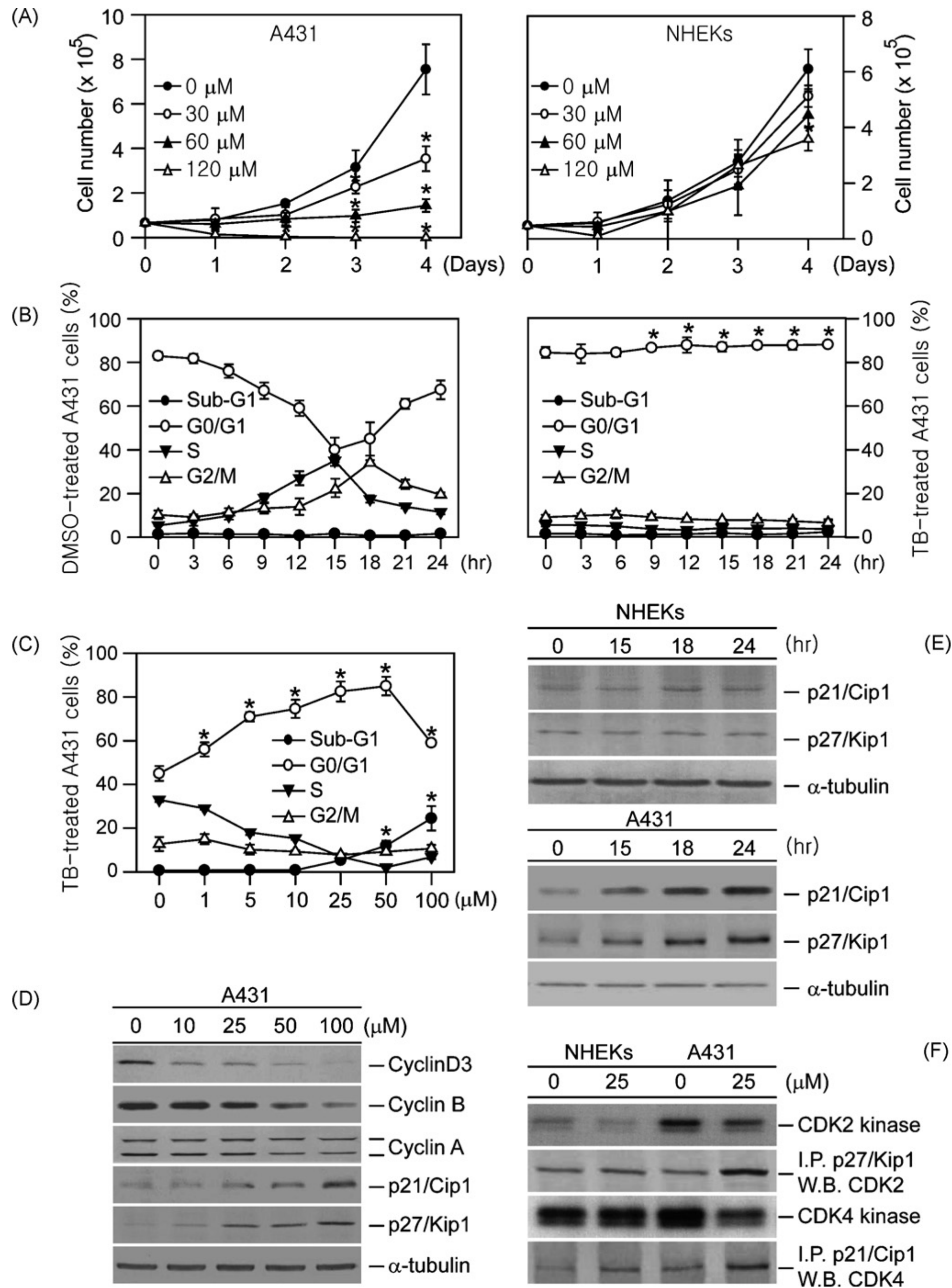

Fig. 1 – TB-induced cell growth inhibition effects in human skin cancer and normal cells. (A) Human A431 (left) and primary cultured NHEK (right) cells were time-dependently treated with TB. The cells were counted daily according to Section 2. (B) Serum-starved human A431 cells were time-dependently treated with 10% FCS in the presence (right) or absence (left) of TB (30 μM). Flow cytometry analysis was then performed as described in Section 2. (C) Serum-starved human A431 cells were treated with TB (0–100 μM) in the presence of 10% FCS for 15 h and then harvested for flow cytometry analysis ($^{*}P < 0.05$). (D) Serum-starved A431 cells were treated with TB (0–100 μM) for 15 h. Protein extracts of the cells were harvested for immunoblotting analysis and the G1 phase cell cycle regulatory protein levels were then determined. (E) Serum-starved NHEKs and A431 cells were time-dependently treated with TB (25 μM). Immunoblot analysis was performed to detect the TB-induced p21/Cip1 and p27/Kip1 protein levels. (F) The p27/Kip1- and p21/Cip1-associated CDK2 and CDK4 kinases were detected by I.P./W.B. analysis. In addition, CDK2 and CDK4 kinase activities from lysates of TB- or DMSO-treated cells were measured as described in Section 2.

CDK2 complexes (Fig. 1F). As we know that p21/Cip1 and p27/Kip1 are potent inhibitors of CDKs, our results also demonstrate that the total cellular CDK2 and CDK4 kinase activities in the TB-treated A431 cell lysates were significantly inhibited (Fig. 1F).

3.4. *In vivo* anti-tumor effect of TB on A431-xenografted tumors

Given the observations of the TB-induced G1 cell cycle arrest in A431 cells *in vitro*, we next determined whether administration

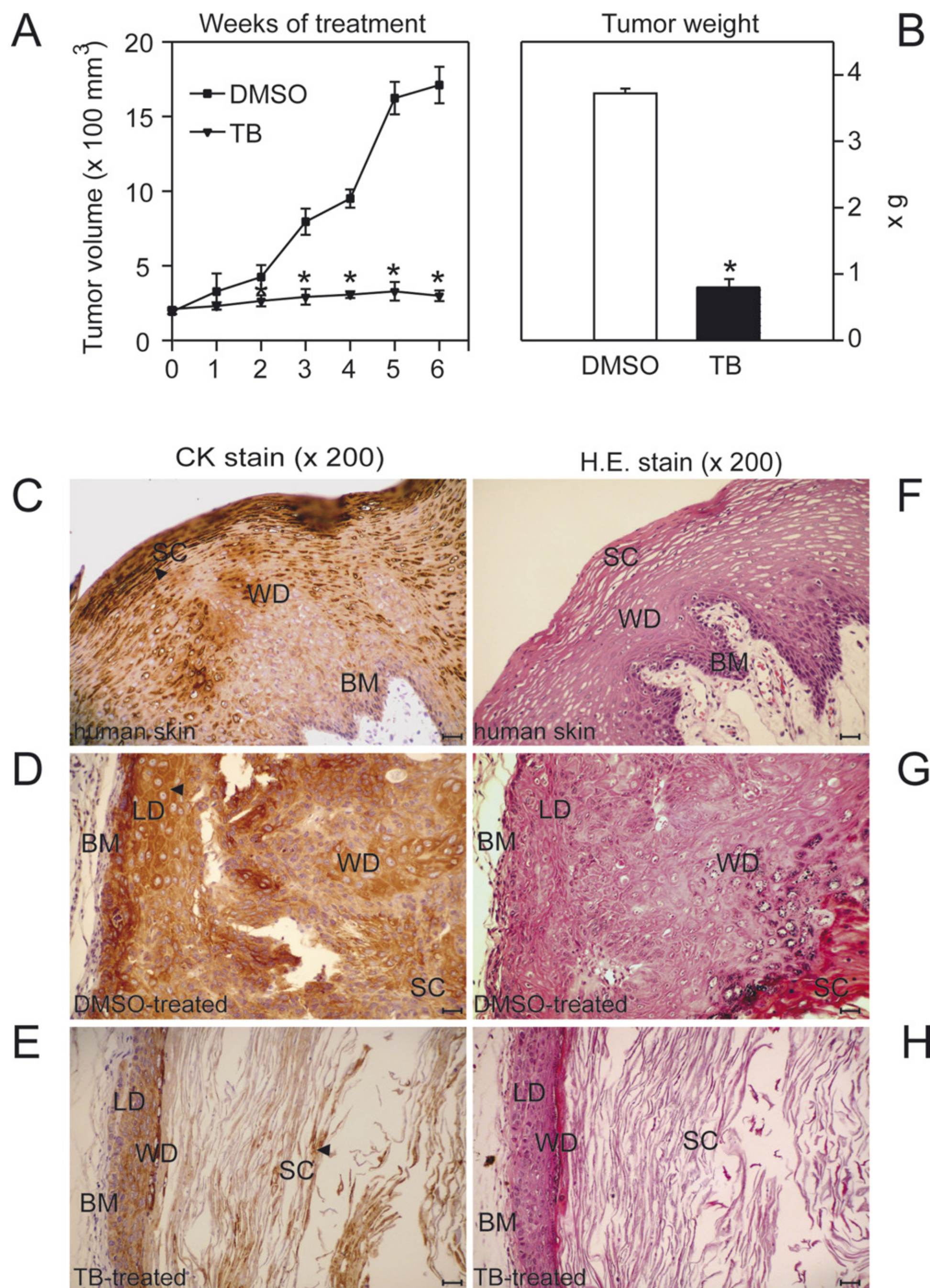

Fig. 2 – TB reduces the growth rate of A431-xenografted tumors in nude mice. (A and B) The tumor volume and weight of DMSO-treated (solid square) versus TB-treated (solid reverse triangle) nude mice were measured at the end of experiment. Five samples were analyzed in each group and values are represented as mean ± S.E. $^{*}P < 0.05$. (C–H) For immunohistochemical staining, normal human skin (C and F) and A431-xenografted tumor tissues (D–H) dissected from DMSO- or TB-treated mice were stained with a cytokeratin-specific antibody (C–E) and H.E. (F–H) for morphological observations. The arrowheads indicate the representative keratin immunoreactive (brown) cells (200×). Bar, 25 μm. TB, terbinafine; SC, stratum corneum; WD, well differentiated region; LD, lower differentiated region; BM, basement membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

of TB could decrease the tumor size of an A431-xenografted tumor in an *in vivo* setting (Fig. 2). By gross morphological observation, an apparent reduction in tumor volume between mice given vehicle versus TB was detected (data not shown). Importantly, the tumor volume/weight of the mice treated with TB was significantly reduced as compared with those treated with vehicle alone (Fig. 2A and B, $^{*}P < 0.01$).

3.5. *TB induces A431 tumor cell differentiation in vivo*

Recent studies have demonstrated that agents such as green tea polyphenol epigallocatechin-3-gallate (EGCG) possess anti-carcinogenic properties and were found to induce terminal differentiation in human A431 cancer cells [22] and in human normal keratinocytes [23]. To determine whether TB could induce the A431-xenografted tumor cells to differentiate, normal human skin and A431 tumor tissues were stained with a specific antibody against human pancytokeratin, a marker for differentiation of epithelioid squamous skin cancer cells (CK stain, Fig. 2C–E). Alternatively, another serial section of the tissues was also stained with H.E. for comparison with the CK stain (Fig. 2F–H). As shown in Fig. 2C, keratin immunoreactive cells (arrowhead) were detected in the stratum corneum (SC) of normal human skin. In the DMSO-treated A431 tumor tissue, keratin expression was evenly detected (Fig. 2D, arrowhead). In contrast, in the TB-treated A431 tumor tissues, the majority of the tumor cells were converted into a SC-like tissue (Fig. 2H, indicated as SC), which was very similar to the SC seen in normal human skin (Fig. 2F, SC). The keratin expression pattern in the SC-like tissue seen in the TB-treated A431 tumor was similar to the normal human skin in which deeply stained keratin was detected in the SC and in cells close to well differentiated (WD) regions (Fig. 2E, arrowhead).

3.6. *P27/Kip1 but not p21/Cip1 was induced by TB in the earlier differentiation stage of A431-tumor tissues*

Irreversible cell growth arrest is an early event of cell differentiation in normal human epidermal keratinocytes and is assumed to be influenced by the stringent control the expression of differentiation-specific genes. As seen in Fig. 1, the CDK inhibitors (p21/Cip1 and p27/Kip1) were induced by TB treatment in the A431 cells that arrested in G1. To investigate whether p21/Cip1 and p27/Kip1 were involved in earlier stages of TB-induced tumor cell differentiation, A431-xenografted tumor-bearing mice were treated with DMSO or TB and then sacrificed at week 3 before the full-scheme (6 weeks) therapy was completed. The short-term TB-treated A431 tumor tissues were dissected for immunohistochemical staining analysis and stained with specific antibodies against p21/Cip1 and p27/Kip1, respectively. In normal human skin tissue, p21/Cip1-stained cells were rare but located mainly around the basement membrane (BM), where the cells are less differentiated (LD) (Fig. 3B, green arrow). In contrast, the p27/Kip1-stained cells were located in regions close to the SC, where the cells are well differentiated (Fig. 3C, green arrow). Previous reports postulated that p27/Kip1 expression might be required for normal human squamous epidermis cell differentiation [24,25]. In the short-term (3 weeks) DMSO-treated A431 tumor tissues, p21/Cip1-stained cells were detected mainly in the LD region (Fig. 3E, green arrow). The expression patterns of the p21/Cip1-stained cells located in the A431 tumor tissues of both groups were quite similar (Figs. 3E and H). In contrast, p27/Kip1-stained cells in the short-term TB-treated A431 tumors were detected in both the LD and WD regions (Fig. 3I, red and green arrows). Interestingly, our results revealed that the expression patterns of p27/Kip1 in the A431 tumors tissues of both groups were completely different (Fig. 3F and I).

To calculate the percentage of tumor cells expressing p21/Cip and p27/Kip1 in different staging categories (WD and LD), cells in multiple fields of the microscopic observations of tumor sections were counted according to a previous paper [5]. Our results demonstrated that there are no significant differences in the expression patterns of p21/Cip1 in both groups (Fig. 3J). In contrast, p27/Kip1-stained cells detected in the LD region of the TB-treated tumor tissues were significantly increased when compared to the DMSO-treated group (Fig. 3K, solid bar, $^{*}P < 0.01$). Such results implied that, in short-term TB-treated A431 tumors, p27/Kip1 protein expression in the earlier differentiation area (LD) mediates a cell growth cycle arrest that might be involved in an early event of the differentiation process.

3.7. *In vitro differentiation marker expression in TB-treated A431 cells*

In this study, our results postulated that G1 cell growth cycle arrest might be an early event in A431 cell differentiation that participates in TB-induced tumor growth inhibition. However, its effects on the promotion of human A431 cell differentiation have not been adequately investigated. To evaluate the effects of TB-induced A431 cell differentiation, a suspension culture model was performed in which the interaction of differentiated keratinocytes with the extracellular matrix (ECM) is decreased and then detached in the suspension part. A431 cells were cultured in poly-HEMA-coated culture plates in the presence or absence of TB (25–100 μM) for 24 h. The TB-induced differentiated cells were detached and then harvested by agitation in the suspension medium. Our results reveal that the keratinocyte differentiation marker (hINV) and the CDK kinase inhibitor (p27/Kip1) were induced in TB-treated A431 cells in a dose-dependent manner (Fig. 4A, indicated as S25–S100). The adherent (indicated as A0) and suspension (indicated as S0) populations of A431 cells cultured in poly-HEMA-coated plates treated with DMSO were adapted as a negative control. The increased expression of hINV was detected in suspension-cultured (S0) A431 cells (Fig. 4A). However, a degraded form of hINV as well as increased p27/Kip1 protein expression was only detected in TB (>25 μM)-treated suspension-cultured A431 cells (Fig. 4A, S25–S100).

To confirm such observations, another cell culture model was used for investigation of TB-triggered A431 cell differentiation. The A431 cells were cultured in type-I collagen-coated plates in the presence or absence of TB [26]. At 24 h after TB (25–100 μM) treatment, the undifferentiated (attached, presented as A) versus the differentiated (detached, presented as D) A431 cells were harvested separately and immunoblot analysis was conducted. Our results show that the expression of p27/Kip1 was dose

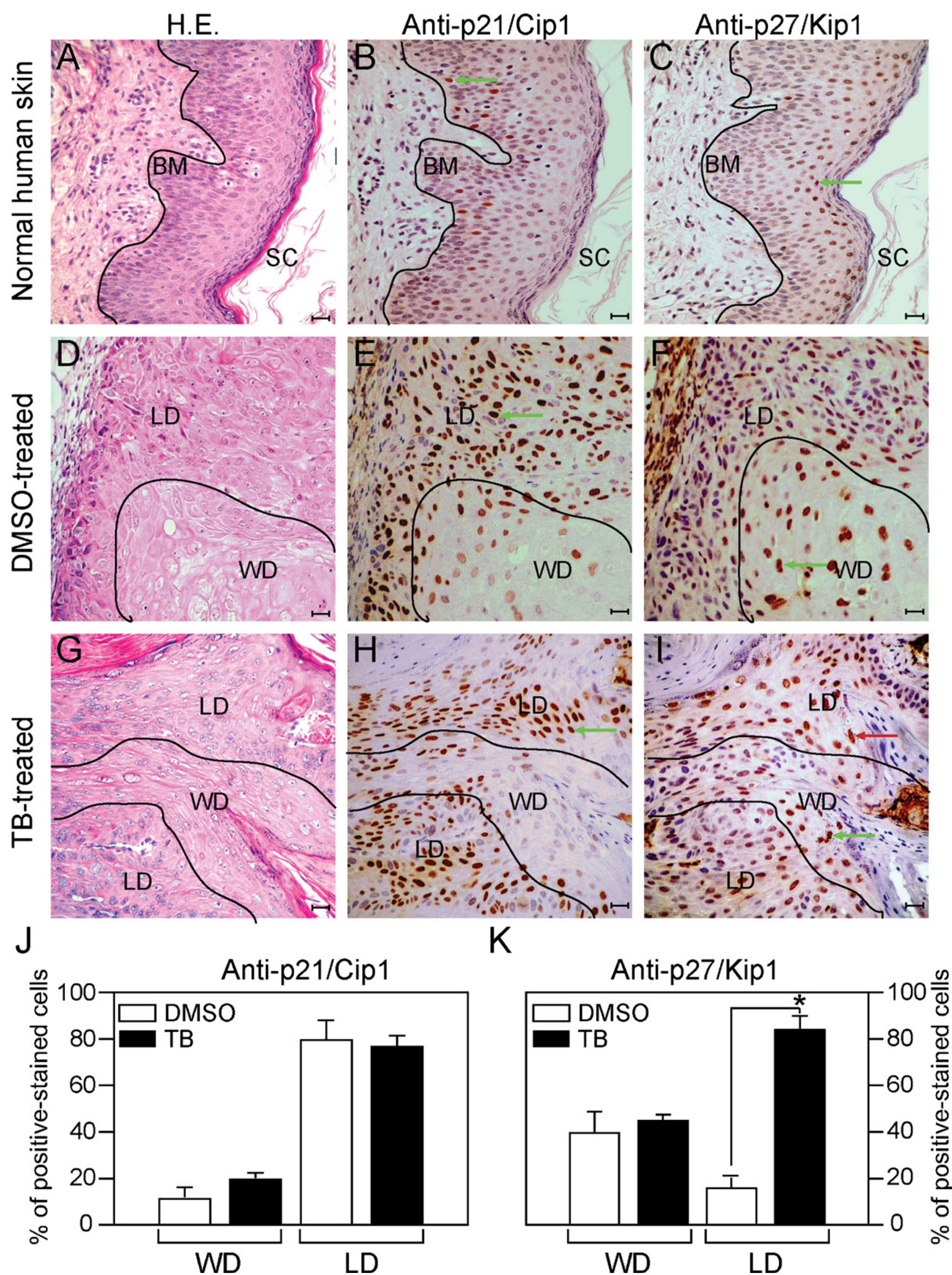

Fig. 3 – Immunohistochemical detection of p21/Cip1, and p27/Kip1 proteins normal human skin and A431-xenografted tumor tissues. (A–I) TB- and vehicle-treated mice were sacrificed at week 3. Serial sectioned A431-xenografted tissues were stained with H.E. or antibodies specific to p21/Cip1 and p27/Kip1. The immunoreactive (brown) cells are indicated with green or red arrows (200×). Bar, 20 μm. (J and K) The percentage of cells expressing p21/Cip1 (J) and p27/Kip1 (K) were calculated as described in Section 2. TB, terbinafine; SC, stratum corneum; WD, well differentiated region; LD, lower differentiated region; BM, basement membrane. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

dependently induced by TB in both attached and detached A431 cells. In the DMSO-treated cells, p27/Kip1 was also detected at a basal level in the detached cells, which means that p27/Kip1 is expressed in spontaneously differentiating A431 cells (Fig. 4B, lane 1, presented as D0). However, TB-induced p21/Cip1 expression was only detected in the undifferentiated (attached) A431 cells (Fig. 4B). Such results exclude the possibility of p21/Cip1 involvement in TB-mediated A431 cell differentiation.

As described above, degraded forms of hINV and p27/Kip1 were detected in the TB-treated A431 cells (Fig. 4A and B, arrowhead). Since cell growth inhibition, differentiation, and/or activation of cellular apoptotic responses are three major mechanisms that have an effect on anti-tumor agent-mediated tumor growth inhibition, changes in apoptosis regulatory proteins in TB-treated A431 cells were determined by immunoblot analysis. Our results show that the expression level of Bax protein was increased, whereas Bcl-2 protein

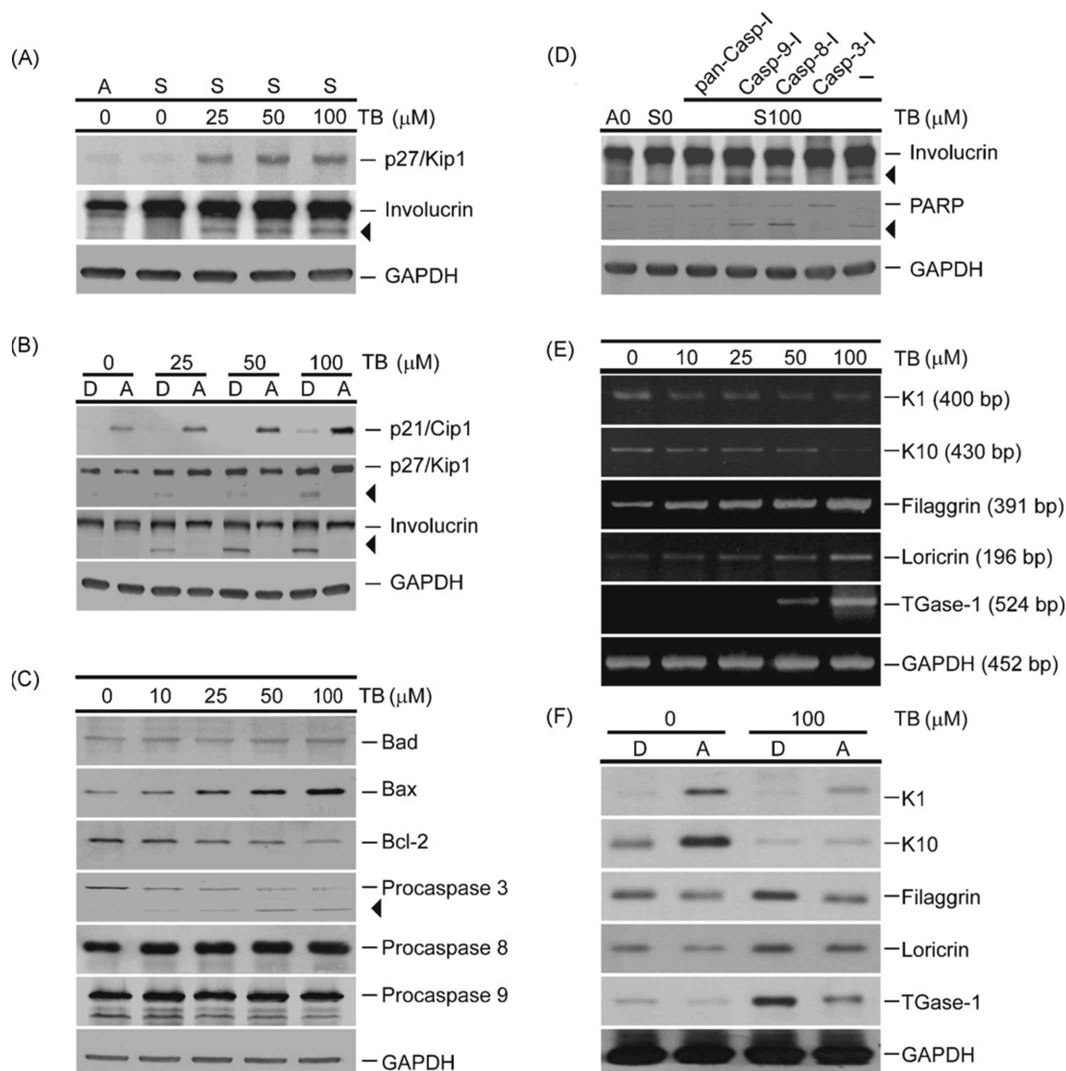

Fig. 4 – TB-induced changes in differentiation and apoptosis regulatory proteins in A431 cells. (A) Human A431 cells were cultured in poly-HEMA-coated culture plates. The suspension-cultured (presented as S0–S100) and adherent-cultured (presented as A0) A431 cells treated with TB were harvested separately by gently agitation. The expression of p27/Kip1 and hINV protein levels in A431 cells were then determined by immunoblotting analysis. (B) Human A431 cells were cultured in type-1 collagen-coated plates [26]. Detached (presented as D) versus attached (presented as A) A431 cells were harvested separately after 24 h TB treatment. Protein extracts were isolated for immunoblotting analysis. (C) Human A431 cells were treated with TB for 24 h and the changes in apoptosis regulatory proteins were determined by immunoblotting analysis. (D) Human A431 cells were cultured in poly-HEMA-coated culture plates pretreated with Z-VAD-FMK (pan-caspase inhibitor) or caspases 3-, 8- and 9-specific inhibitors for 24 h and then treated with TB for an additional 24 h (presented as S100). The mock-treated A431cells were sorted and divided into suspension (S0) and adherent (A0) cultured cells for immunoblot analysis. (E) Human A431 cells were treated with TB for 24 h. RT-PCR analysis was performed for detection of the expression levels of several structural epidermal markers. (F) Human A431 cells were cultured in type-1 collagen-coated plate in the presence or absence of TB for 24 h. After drug treatment, the detached (presented as D) and attached (presented as A) cells were harvested for immunoblotting analysis.

expression was decreased in A431 cells treated with TB (10–100 μM) for 24 h (Fig. 4C). We also found that procaspase 3 was activated, which may be the major mechanism invoked in TB-treated A431 cells leading to apoptosis (Fig. 4C). The levels of other regulatory proteins, such as Bad and procaspases 8 and 9, did not change in the TB-treated A431 cells. We suggest that TB-induced procaspase 3 activation is involved in hINV degradation, as seen in Fig. 4A and B.

To test this hypothesis, the suspension-cultured A431 cells were pretreated with Z-VAD-FMK (pan-caspase inhibitor), or caspases 3-, 8- and 9-specific inhibitors for 24 h, and then TB (100 μM) was added for an additional 24 h. As shown in Fig. 4D, caspase 3- and pan-caspase-specific inhibitors inhibited the degradation of both hINV and PARP, a downstream target of caspase 3 (Fig. 4D, lanes 3 and 6). In contrast, pretreatment with caspases 8 and 9 inhibitors could not reduce TB-induced hINV degradation in the suspension-cultured A431 cells (Fig. 4D, lanes 4 and 5).

We further examined the effects of TB treatment (10–100 μM, for 24 h) on the expression of keratinocyte differentiation markers found in adult human skin, i.e., keratins (K1, K10), filaggrin, loricrin, and transglutaminase-1 (TGase-1) (Fig. 4E). TGase-1 is the primary enzyme responsible for catalysis of inter-protein covalent isopeptide bond formation that assembles the cornified envelop [27–29]. LOR is a major component of the epidermal cornified cell envelope and is only detected in terminally differentiated keratinocytes. In addition, FIL, a basic histidine-rich protein synthesized by cells of keratinizing epithelia, was also determined. As shown in Fig. 4E, the mRNA levels of late stage epidermal cell differentiation markers, including TGase-1, LOR, and FIL, were dose dependently increased in TB-treated A431 cells. In contrast, the early stage keratinocyte differentiation markers, including K1 and K10 [30], were significantly inhibited in TB-treated A431 cells.

To further confirm that these markers were induced by TB and to confirm TB's association with the keratinocyte differentiation process, A431 cells were cultured in type-I collagen-coated plates in the presence or absence of TB (100 μM) for 24 h. The detached (D) and attached (A) cells were then harvested for immunoblotting analysis (Fig. 4F). Our results demonstrate that the proteins expression of later stage differentiation markers, including TGase-1, LOR, and FIL, was significantly induced by TB in detached A431 cells (Fig. 4F, lane 3). In contrast, the proteins expression of early-differentiation markers, including K1 and K10, was slightly inhibited by TB treatment in detached A431 cells (Fig. 4F, lane 3).

3.8. *The TB-induced p27/Kip1 expression in differentiated A431 cells was transcriptionally up-regulated by SP1*

As indicated in the previous report, p27/Kip1 and hINV can act as differentiation markers in keratinocytes [31]. Many studies have shown that SP1 is one of the transcriptional factors involved in the expression of p27/Kip1 [12,32], TGase-1 [13,14], and hINV [15]. We next determined whether the SP1 DNA binding sites that appeared in these genes' promoters were regulated by SP1 in TB-treated A431 cells. To explore this hypothesis, cells were transfected with pGL3(SP1)5, a luciferase reporter plasmid with a SP1 DNA binding site from the p27/Kip1 gene promoter (−549 and −523) that is repeated 5 times [12]. Our results showed that TB dose-dependently increased SP1-linked luciferase activity (Fig. 5A, left panel, lanes 4–6), and this effect could be significantly abrogated with mithramycin A, a specific SP1 DNA binding inhibitor (Fig. 5A, right panel, lane 4 vs. 6). The mutated SP1 DNA binding sequences were cloned to create PGL3(mut SP1)5 and transfected into A431 cells as a negative control for TB treatment (Fig. 5A, lanes 7–9).

We further explored the effect of TB on SP1 protein levels in A431 cells. As shown in Fig. 5B, the SP1 protein level was significantly elevated in A431 cells treated with TB (1–100 μM) for 24 h. To determine the effect of TB on SP1/DNA binding activity, nuclear extracts were prepared from DMSO- and TB-treated A431 cells in the presence or absence of mithramycin A. Chromatin immunoprecipitation was performed using the SP1-specific antibody to precipitate the SP1-associated DNA complex sequence. Increased binding of SP1 to the p27/Kip1 promoter (−462 and −774) was detected by PCR analysis in TB-treated A431 cells (Fig. 5C, lanes 1–3). The SP1 binding activity was significantly inhibited by mithramycin A pretreatment (Fig. 5C, lanes 5–6). Furthermore, our results demonstrate that TB-induced p27/Kip1 protein expression was almost completely reversed by mithramycin A pretreatment (Fig. 5D, lane 4). These data suggest that the TB-induced SP1 expression in A431 cells plays a critical role in p27/Kip1 gene transcriptional activation.

3.9. *TB-induced G1 arrest in differentiated A431 cells was attenuated by mithramycin A pretreatment*

As described above, p27/Kip1 inhibition of CDK2 kinase activity was linked to TB-induced cell cycle arrest in A431 cells (Fig. 1). However, the role of p27/Kip1-mediated G1 arrest involvement in the TB-induced A431 cell differentiation process has not been adequately investigated. A431 cells were cultured in poly-HEMA-coated culture plates pretreated with mithramycin A (400 nM) for 1 h, and then TB (10–100 μM) was added for an additional 24 h. The TB-induced differentiated A431 cells were agitated in the suspension medium and then harvested for flow cytometry analysis (Fig. 5E, indicated as S0–S100). Our results reveal that the TB-treated cells (S10 and S100) were arrested in the G1 phase of the cell cycle (Fig. 5E, bars 2–3). Interestingly, the TB-induced G1 arrest effects in the suspension-cultured A431 cells were nearly completely attenuated by pretreatment with mithramycin A (Fig. 5E, bars 5–6). In the same conditions, apoptotic cells were found in the suspension-cultured A431 cells when treated with a higher concentration of TB (>100 μM) (Fig. 5F, bar 3). However, the TB-induced apoptosis effects observed in A431 cells could not be reversed by mithramycin A pretreatment (Fig. 5F, bar 6). Our results indicate that SP1-induced p27/Kip1-mediated G1 arrest effects were directly linked to the differentiation process in TB-treated A431 cells.

4. Discussion

In this study, our *in vitro* studies demonstrated that TB induced a G1 cell growth cycle inhibition and differentiation in cultured human epithelioid squamous carcinoma (A431) cells.

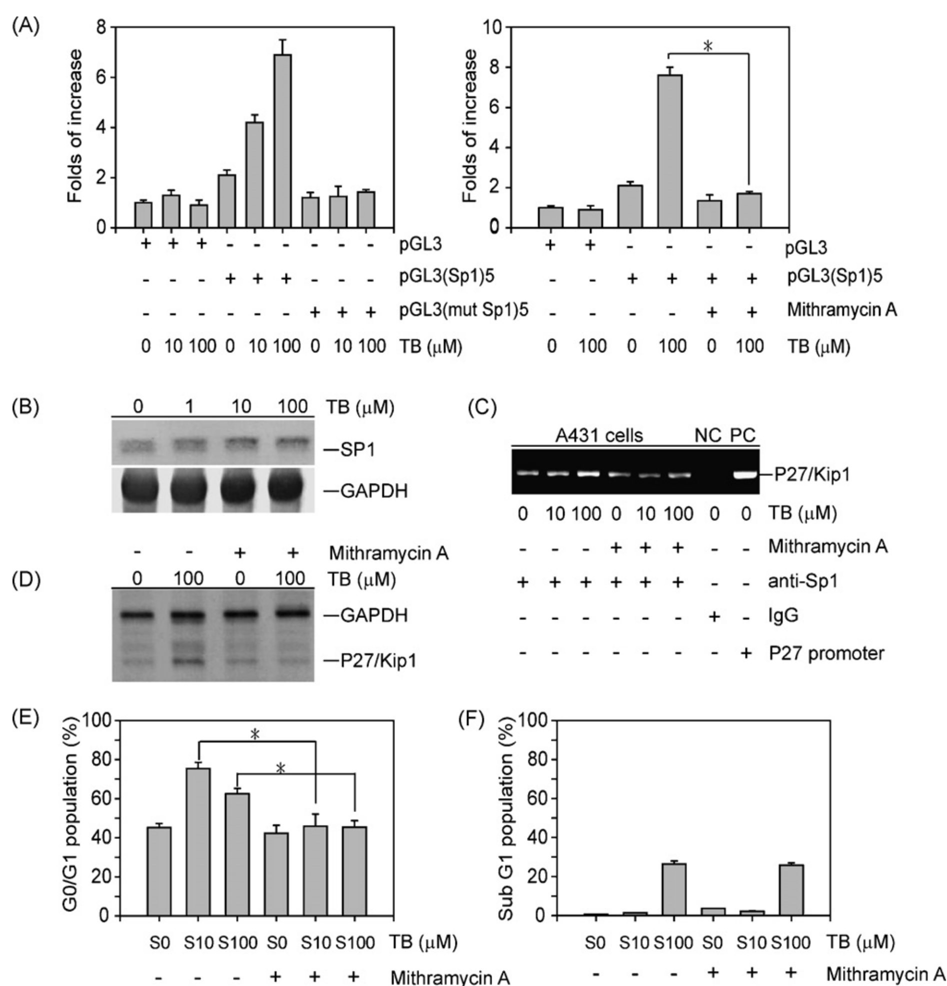

Fig. 5 – TB-induced p27/Kip1 gene expression in human A431 cells was transcriptionally regulated by SP1. (A) Human A431 cells were transiently transfected with either PGL3(SP1)5, PGL3(mut Sp1)5, or empty (PGL3) plasmids for 24 h and then treated with TB for an additional 24 h (left). In the right panel, human A431 cells were transiently transfected with either PGL3(SP1)5 or empty (PGL3) plasmid vector for 24 h. After transfection, the SP1 inhibitor (mithramycin A, 400 nM) was added to the cells for 1 h, and the cells were then treated with TB for an additional 24 h. Luciferase activity was assayed and normalized to pRL-TK expression according to Section 2. The results shown indicate triplicate experiments and are presented as mean ± S.E. *P < 0.05. (B) Sp1 protein expression level was dose-dependently induced by TB in A431 cells. (C) The A431 cells were pretreated with mithramycin A for 1 h and then treated with TB for an additional 24 h. The cellular nuclear extracts were sorted for Chip assay. Shown are representative data from two independent experiments with similar results. (D) TB-induced p27/Kip1 expression in A431 cells was attenuated by mithramycin A pretreatment. (E and F) Mithramycin A attenuated TB-induced G1 cell cycle arrest in poly-HEMA-coated suspension-cultured A431 cells.

Increased expression of p27/Kip1 and the presence of keratin in the A431-xenografted tumor from the TB-treated mice suggest that cancer cell growth inhibition and differentiation contribute to the anti-tumor effects of TB (summarized figure was presented as Fig. 6). Our results using the immunohistochemical stain method revealed that p27/Kip1-positive-stained cells were detected in the region close to the SC of normal human skin tissue (Fig. 3C, green arrow). Such results were similar to previous studies indicating that p27/Kip1 induction might be associated with differentiation processes in normal human squamous epidermis cells [24,25]. Among these changes shown above, we suggest that TB-induced p27/Kip1 expression in cells located in the early-differentiated (LD) region of A431 tumors seems to have a major contribution in triggering cell growth arrest (Fig. 6A, step 1). The TB-induced p27/Kip1 expressing cells found in the LD region were then converted to WD cells by differentiation (Fig. 6, step 2). If the animals were exposed to TB consecutively (>6 weeks), the WD cells were then differentiated into the SC, causing significant regression of the tumor size (Fig. 6, step 3).

In this study, our results provided direct evidences that SP1-mediated p27/Kip1 expression is involved in the TB-induced G1 arrest. Such effects may then accomplish the full differentiation program in A431 cells. The *in vitro* results can be used for the interpretation of the *in vivo* observations, which indicated that p27/Kip1 but not p21/Cip1 was expressed and played some important roles in the early stage (LD) differentiation process of the A431 tumors (Fig. 3K). The role of p27/

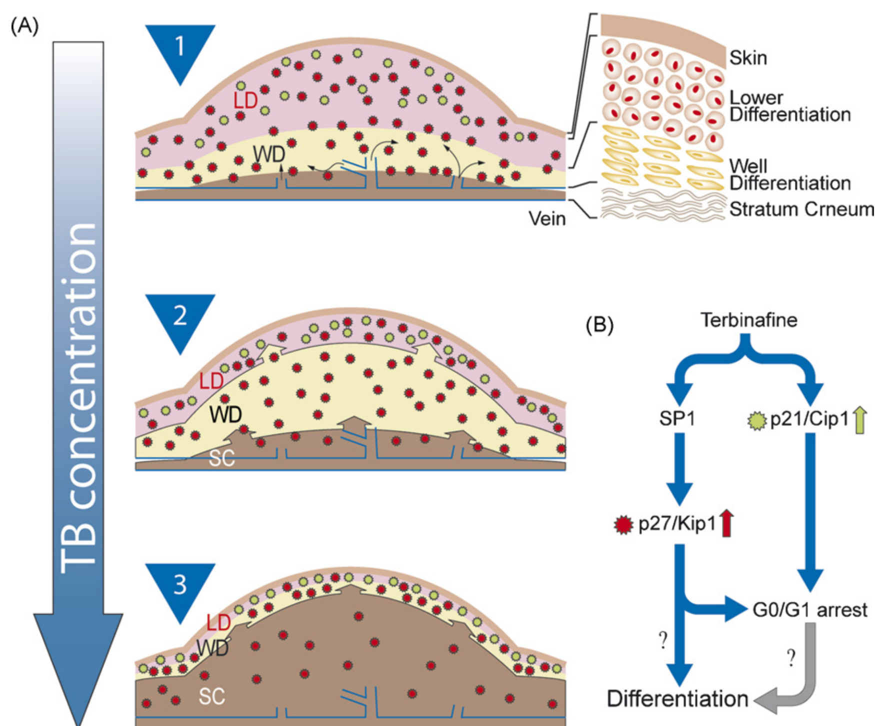

Fig. 6 – The suspected molecular mechanisms of the TB-induced differentiation processes in A431-xenografted tumor cells. (A) Human A431 cells were injected subcutaneously between the scapulae of each nude mouse. During the initial stage (3rd week) of TB therapy, p27/Kip1 (presented as a red spot) and the p21/Cip1 (presented as a green spot) positively stained A431 tumor cells were detected in the LD region, which triggered cells to undergo a G1 cell cycle arrest (pink area shown in step 1). In the next stage, most of the p27/Kip1-expressing LD cells became WD cells. In contrast, p21/Cip1 positively stained cells were not detected in the WD region (yellow area shown in step 2). In the final stage (TB-treated for more than 6 weeks), most of the WD cells were converted to SC (brown area shown in step 3). In the SC area, high concentrations of keratin, which attracted keratinophilic TB to accumulate in this area, were produced. (B) The postulated molecular mechanisms of TB-induced A431 cell differentiation. LD, lower differentiation; WD, well differentiation; SC, stratum corneum. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Kip1 during keratinocyte differentiation remains uncertain, although some investigations have demonstrated that p27/Kip1 is a differentiation-associated marker for keratinocytes in *in vitro* [24,33] and *in vivo* models [31]. However, the more undisputed role of p27/Kip1 is in the induction of cell growth arrest. As shown in Fig. 4B, the degraded form (~p23 kDa) of p27/Kip1 protein was detected in TB-induced differentiated cells harvested from type-I collagen-coated plates. A similar phenomenon was observed in the previous paper demonstrating that a caspase-3-like protease is involved in the degradation of p27/Kip1, which is involved in G1 cell growth arrest in human myeloma cells [34]. The biological functions of the degraded (p23 kDa) p27/Kip1 induced by TB in A431 cancer cells need to be further investigated. It was found that p27/Kip1 was significantly up-regulated by the SP1 transcription factor through binding sequences −549/−523 located in the p27/Kip1 gene promoter [12]. The SP1 binding sequence also appeared in the promoter regions of other differentiation markers, such as TGase-1 [13,14] and hINV [15]. Such results suggest that the TB-induced up-regulation of these differentiation-associated markers could also be transcriptionally regulated through activated SP1. The roles of SP1 in the activation of TGase-1 and hINV needed to be further investigated.

Terbinafine (Lamisil®, TB) is a newly synthesized oral antimycotic drug in the allylamine class of drug. It shows a good safety profile and has relatively few drug interactions [4]. Pharmacokinetic studies demonstrated that approximately 70% of TB is absorbed after an oral (250 mg) administration and reaches maximum plasma concentrations of 0.5–1.5 μg/ml within 2 h [35–38]. Another human study showed that the plasma level of TB was 1.7 ± 0.77 μg/ml (5.83 μM) after daily oral doses of TB (250 mg) for 4 weeks [37]. Our previous studies demonstrated that a lower concentration of TB (5 μM) irreversibly arrested cultured COLO 205 cells at the G1 phase of the cell cycle. In the present study, we further demonstrated that p27/Kip1 and hINV were significantly induced in differentiated A431 cells by 25 μM TB treatment (Fig. 4A, lane 3). Such results imply that continued administration of a lower dose of TB could reach therapeutic concentrations in the plasma. Cellular viability analysis also showed the cancer cell specificity of TB action (Fig. 1A, right).

TB is highly keratinophilic and preferentially accumulates in keratin-rich tissues such as the dermis, epidermis, and nails after long-term administration [39,40]. Our *in vivo* study demonstrated that A431-xenografted tumors differentiated into keratin-rich tissues (Fig. 2E, SC). The keratin-rich tissues

were suitable for massive accumulation of TB, causing additional A431 cancer cell differentiation (symbolized figure was presented as Fig. 6A, steps 1–3). The dose of TB (10 mg/kg body weight) used in the present *in vivo* study was not toxic to the vital organs (data not shown). Although animal studies of TB-induced anti-tumoral action are still ongoing, the findings from the present *in vitro* and *in vivo* studies strongly suggest the potential applications of TB in the treatment of human epithelioid squamous carcinoma. The universality of TB in the inhibition of cancer cell proliferation and induction of differentiation would make it a very attractive agent for cancer chemotherapy.

In this study, we found that the apoptosis regulatory genes including caspase 3, Bad, Bax, and Bcl-2 were changed in response to TB (Fig. 4C and D). However, caspases 8 and 9 were not activated during TB treatment. The p53-mediated apoptosis signaling pathway may not be essential in p53-mutated A431 cells [41]. A review of the literature revealed that loss of attachment to the basement membrane triggers keratinocyte differentiation, while in other epithelial cells, detachment from the extracellular matrix leads to rapid programmed cell death or anoikis [42,43]. Another study further demonstrated that fibronectin, a large ECM glycoprotein found in the plasma and other body fluids, regulates many cellular processes, including migration, proliferation, differentiation, and survival in human squamous cell carcinoma (SCC) cells. When fibronectins are altered by inflammation, wound healing, or metastatic processes, cell adhesion to the ECM can be disrupted, triggering a specific form of detachment-induced apoptosis called anoikis [44]. Additional studies should be performed to investigate whether TB could induce anoikis and unveil possible mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at 10.1016/j.bcp.2008.02.005.

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