

Roles of Testosterone in the Growth of Keratinocytes Through Bald Frontal Dermal Papilla Cells

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A coculture of dermal papilla cells (DPCs) from the bald frontal scalp of stump-tailed macaques with keratinocytes derived from human neonatal foreskin revealed that testosterone inhibited keratinocyte proliferation, and that the antiandrogen RU58841 abolished this response. This testosterone-induced keratinocyte growth inhibition was not observed when either type of cells was cultured alone. We also examined conditioned media from the coculture system and demonstrated the identical testosterone-induced growth inhibition on keratinocytes, and this inhibitory effect was conditioned media concentration-dependent. These results suggested that the testosterone-mediated suppression on keratinocyte proliferation might proceed through some diffusible growth mediators in conditioned media. Differential display reverse transcriptase polymerase chain reaction allowed us to isolate several genes from frontal DPCs that can be either suppressed or induced by testosterone. Supervillin, a membrane-associated, F-actin-binding protein, was identified as one of the testosterone downregulated genes in frontal DPCs. Further characterization of these testosterone-target genes may reveal the mechanism by which testosterone inhibits the growth of follicular cells in androgenetic alopecia.

Key Words: Dermal papilla cells; keratinocytes; androgen; differential display; supervillin.

Introduction

The concept of stromal involvement in the hormonally induced growth responses of normal epithelium was

demonstrated *in vivo*. The embryonic prostate epithelium derived from the urogenital sinus was stimulated to grow and participate in morphogenetic responses when it was closely associated with the inductive urogenital sinus mesenchyme (1). Ductal morphogenesis and proliferation of epithelial cells are clearly under androgenic control. Androgen action is mediated by the androgen receptor (AR), a member of the steroid/thyroid hormone receptor superfamily (2–4). The underlying mesenchymal cells of the urogenital sinus seem to be the actual targets and the mediators of androgen action since the presence of AR in the stroma was required for epithelial growth (5,6), and this androgen-elicited stimulation could be blocked by antiandrogens such as hydroxyflutamide (7). In addition to the prostate, there is increasing evidence that mesenchymal-epithelial interaction plays an essential role in the early development of other androgen target tissues, such as the mammary gland, the seminal vesicles, and hair follicles (8–12). In the hair growth system, some evidence also suggests that the action of testosterone (T) on the epithelial cells is via the dermal papilla cells. For instance, the localization of ³H-T and detection of AR were only in the dermal papilla cells (DPCs) of hair follicles (13,14). Our previous data suggested that the AR protein was not found in keratinocytes (15). Taken together, the effect of androgen has been suggested to be due to the paracrine interactions between the AR-positive mesenchyme and AR-negative epithelium.

Depending on the site, androgens have paradoxically different effects on human hair follicles. Androgens stimulate hair growth in the beard and pubic areas but can cause hair regression on the scalp in genetically disposed individuals (14). Tissue-specific gene expression may be ascribed to different combinations of coregulators (coactivators/corepressors) that bind to AR (16–19). The alopecia in the frontal scalps of postpubertal stump-tailed macaques manifests a progressive follicular regression similar to human androgenetic alopecia, and treatment with antiandrogen can prevent or reverse this epigenetic alopecia (20–24).

In our previous studies, we used the coculture system to demonstrate the inhibitory actions of T on the proliferation

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of outer root sheath cells when cocultured with DPCs derived from the bald frontal scalp of stump-tailed macaques. In the present study, we further demonstrated that the T-induced growth inhibition could also be exerted when keratinocytes, another type of epithelium, replaced the outer root sheath cells. Moreover, the conditioned media (CM) collected from the same coculture system also showed the inhibitory effect on the proliferation of keratinocytes. Cell-specific gene expression by frontal DPCs probably plays a critical role in the paracrine regulation of epithelial cell growth. Based on this theory, the key T-regulated bald frontal DPC genes have not been identified, yet the elucidation of such genes is fundamental for defining the role of androgens in growth control and the role of mesenchyme in hair follicles. Therefore, differential display reverse transcriptase polymerase chain reaction (RT-PCR) followed by cloning was used to isolate and identify eight T-regulated frontal DPC genes, which may be potential candidates for the T-dependent inhibition on epithelial cell proliferation.

Results

Effects of T on Proliferation of Keratinocytes and/or DPCs Cultured Alone or Cocultured

To determine androgen (T) and antiandrogen (RU58841) regulation on keratinocyte or DPC (both frontal and occipital) proliferation, growth assays were performed in chemically defined medium keratinocyte growth medium (KGM medium) supplemented with T and/or RU58841. RU58841 had been defined as a potential antiandrogen for the treatment of androgenetic alopecia in our previous studies (25). Figure 1 shows that neither T nor RU58841 exerted any effects on the proliferation of either type of cells cultured alone (frontal DPC, occipital DPC, and keratinocyte). In the absence of T, the total cell number of cocultured keratinocytes and DPCs increased compared with the sum of keratinocytes and DPCs cultured alone. Addition of T, however, significantly decreased the total number of cocultured keratinocytes and frontal DPCs, and this T-elicited growth inhibition can be antagonized by RU58841. By contrast, the total number of KC cocultured with occipital DPC was not affected by T (Fig. 1). These results suggested that some mitogenic factors were produced from coculture of DPCs and keratinocytes. However, the presence of T in the coculture of frontal DPCs and keratinocytes either suppressed the production of mitogenic factors or produced some negative growth mediators.

Effects of CM on the Proliferation of Keratinocytes

CM prepared from various culture conditions were examined to determine whether diffusible mediators were involved in the T-inhibited growth of keratinocytes. Figure 2 shows the effects of medium conditioned by frontal DPC or keratinocytes culture alone (CMI and CMII) or cocultured (CMIII mixed-well and CMIV transwell), with or without 1 nM T, on the proliferation of keratinocytes. Results showed that keratinocytes

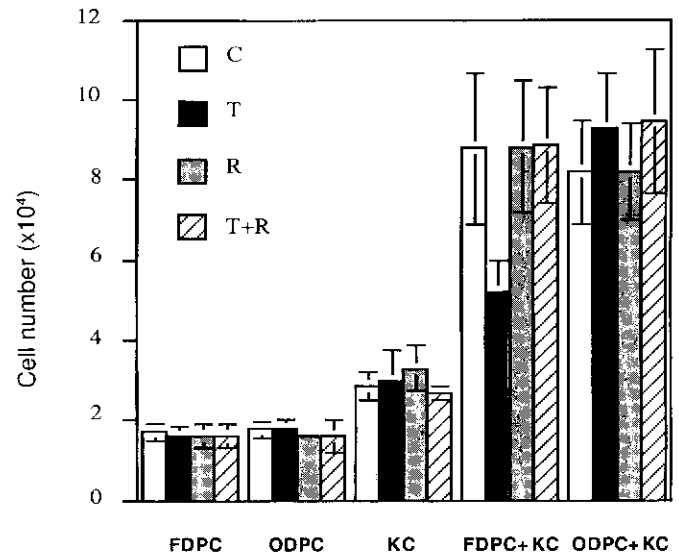


Fig. 1. Effects of T on proliferation of DPCs and keratinocyte cultures alone and cocultured. DPCs (FDPC, frontal; ODPC, occipital) and/or keratinocytes (KERATINOCYTES) with or without testosterone (T; 1 nM) and/or RU58841 (R; 1 nM) were cultured alone or cocultured. Cells were counted 7 d after the start of cultivation (C: control incubation, without T or RU58841). Each cell number ($\times 10^4$) value represents the mean \pm SD of three separate experiments performed in duplicate using 12 male and female animals.

proliferation was reduced when compared to control (C, KGM alone, without preinoculation with frontal DPCs or keratinocytes). This could be due to the consumption of growth factors in the CM prior to addition to the keratinocyte culture. Addition of 1 nM T in the CMIII or CMIV induced further suppression of keratinocyte proliferation. By contrast, addition of T in the CMI or CMII (frontal DPCs or keratinocytes alone) did not show any additional inhibitory effect on keratinocyte growth. Figure 3 demonstrates that CMIV inhibited keratinocyte proliferation in a CM concentration-dependent manner, when CMIV was serially diluted with normal KGM and then applied to keratinocytes. Compared to the control (100% KGM), the growth of keratinocytes was inversely proportional to the percentage of CMIV presented in the mixture. Keratinocyte proliferation was markedly reduced by 100% CMIV in the presence of T. The inhibitory effect from CMIV minus T and CMIV plus T exerted a 40% difference.

These results indicate that some T-regulated soluble factors were secreted into the CM and affected the cell growth. To determine whether the diffusible growth mediators in the CM were heat sensitive, CMIV was heat-treated and keratinocyte proliferation assay was performed. Figure 4 shows that some normal growth factors (from CMIV, without T) were heat sensitive because the growth effect was attenuated from 50 to 60°C, and completely abolished from 60 to 100°C. For the T-regulated negative growth mediators, they still showed the inhibitory effect at

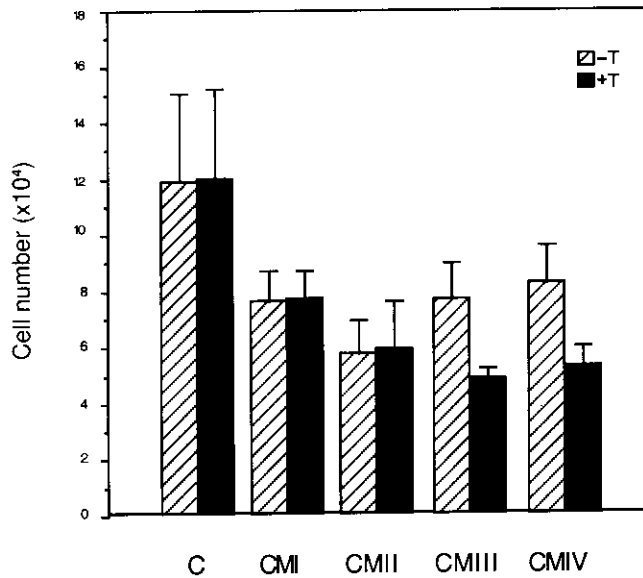


Fig. 2. Effects of CM from culture of frontal DPCs and/or keratinocytes, with or without T, on the proliferation of keratinocytes. CM produced by frontal DPCs alone (CMI), keratinocytes alone (CMII), or mixed co-cultured (CMIII), and transwell coculture (CMIV), with or without 1 nM T, were transferred to keratinocytes (4×10^4 cells/well) and incubated further for 3 d, as described in Materials and Methods. C, control media is KGM alone, without preinoculation with FDPC or keratinocytes. keratinocytes were counted by hemocytometer chamber 3 d after CM cultivation. Each cell number ($\times 10^4$) value is the mean \pm SD of three separate experiments performed in triplicate using 12 male and female animals.

50°C, and may or may not maintain the same activity from 60 to 100°C.

Differential Display RT-PCR Analysis

Differential display RT-PCR was performed in order to identify genes from frontal DPCs, which may play important roles in the T-elicited growth inhibition. Frontal DPCs were cocultured with keratinocytes in transwell plates for 3 d, with or without 1 nM T. Then the total RNA from frontal DPCs was harvested from multiple transwells. cDNA was synthesized and then used for replicate differential display RT-PCR analysis, as described in Materials and Methods. Repeated differential display RT-PCR from separate analyses revealed 12 potential candidate transcripts that were either repressed (Fig. 5A) or stimulated by T (Fig. 5B). The potential cDNA fragments (clones 1–12) were then isolated from the differential display RT-PCR gels individually, reamplified with appropriate primers, and subcloned into *pGEM-T* easy vector. The size of these clones ranged from 300 to 600 bp (Fig. 5C), and sequence analysis of each cloned species revealed that clone 1 showed 95% homology with human supervillin mRNA whereas clones 2–12 showed no perfect matches to any known genes in databases (data not shown) and may, therefore, represent novel genes.

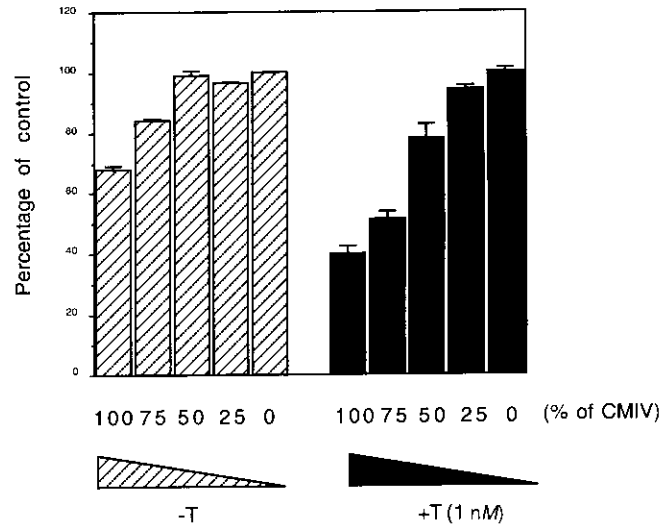


Fig. 3. Dose-dependent inhibition of media conditioned by transwell coculture of frontal DPCs and keratinocytes (CMIV), with or without T, on proliferation of keratinocytes. CMIV, with or without 1 nM T, were serially diluted with normal KGM before being transferred to keratinocytes. keratinocyte cells were counted 3 d after cultivation. Cell growth is expressed as the percentage of the cell number found when treated with only 100% normal KGM (control). Each value is the mean \pm SD of three separate experiments performed in triplicate using 12 male and female animals.

Reverse Northern Analysis of Differential Display RT-PCR-Identified Genes

To confirm further the T dependence of the clones, microarray reverse Northern dot-blot analysis of all candidate clones was performed. Total RNA from frontal DPCs after coculture with keratinocytes, with or without 1 nM T, were isolated and labeled as probes, respectively. Twelve differential display cDNA clones were equally dot-blotted onto duplicated membranes, as described in Materials and Methods. As shown in Fig. 6, reverse Northern analysis demonstrated that T repressed 4 of the 12 clones (Fig. 6A, clones 1–4) and induced another 4 of the 12 clones (Fig. 6B, clones 5–8). These results corresponded to the T regulation of each clone that appeared on the gel. However, the last 4 of the 12 clones (clones 9–12) failed to show the T influence (data not shown). Clone 1, supervillin, appeared to be the most potent one, with its expression in frontal DPCs suppressed by T up to 2.5-fold. Together, these data suggest that at least one known gene and seven different novel genes could be either induced or repressed by T in frontal DPCs. Whether these T-target genes can play any vital roles in the mediation of T suppression on cell growth remains to be determined.

Discussion

We have described herein the effects of T on the suppression of keratinocyte growth when cocultured with DPCs derived from bald frontal scalp of stump-tailed

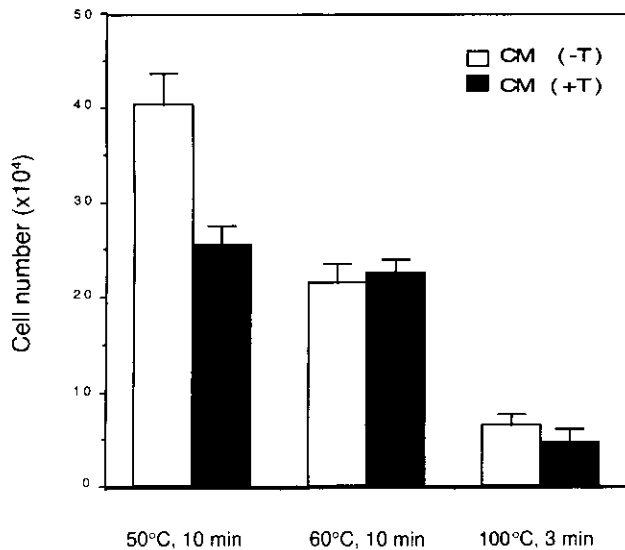


Fig. 4. Effects of heat-treated CMIV on proliferation of keratinocytes. CMIV, with or without 1 nM T, were treated by heating at (1) 50°C, 10 min; (2) 60°C, 10 min; or (3) 100°C, 3 min; and then applied to keratinocytes. Keratinocyte cells were counted 3 d after cultivation. Each cell number ($\times 10^4$) value is the mean \pm SD of three separate experiments performed in triplicate using 12 male and female animals.

macaques and identified eight T-regulated transcripts in frontal DPCs through the use of differential display RT-PCR strategy. Keratinocytes are follicular epithelial cells that differentiate to form various layers of the hair shaft and its inner and outer root sheaths. At the follicular bulb, the mesenchyme-derived papilla is surrounded by bulbar matrix cells in anagen follicle. The cyclic follicular growth provides a good model for the stromal-epithelial interaction under the control of androgen in androgen-sensitive follicles.

Our previous data suggested that bald frontal DPCs play an important role in the inhibitory actions of T on proliferation of the outer root sheath cell (26). In the present study, we also demonstrated the T-dependent inhibition on keratinocyte growth in a cell-cell direct interaction manner (Fig. 1), or CM from direct interaction (CMIII mixed-well coculture; Fig. 2) or indirect interaction (CMIV transwell coculture; Figs. 2 and 3). However, these data indicated that DPCs cocultured without T could stimulate proliferation of keratinocytes. One explanation is that some mitogenic substances were secreted, and those factors were shown to be heat sensitive (Fig. 4).

Insulin-like growth factor-1 (IGF-1) and keratinocyte growth factor (KGF) were previously reported as the most important growth factors stimulating keratinocyte growth and were also shown to be heat sensitive (27–31). By using RT-PCR, we showed that the expression of IGF-1 and KGF in keratinocytes was significantly induced by T when keratinocytes were cocultured with frontal DPCs (data not shown). These data suggested that T-elicited growth inhibition was not due to suppression on IGF-1 and KGF expression.

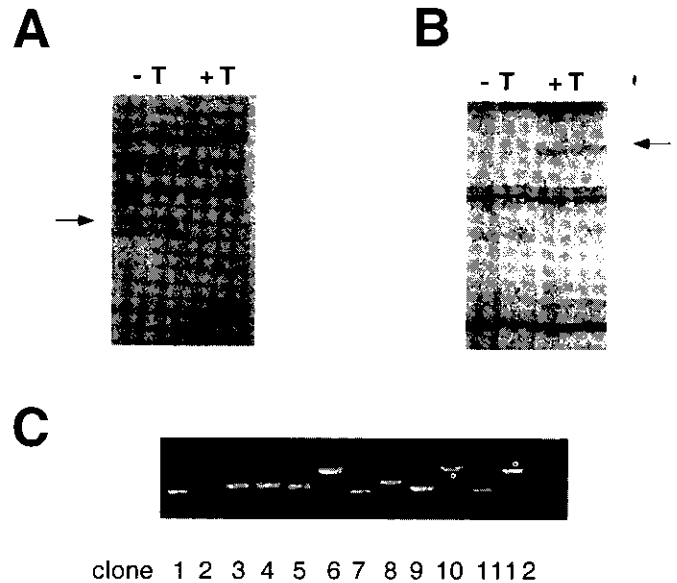


Fig. 5. Differential display RT-PCR analysis of T-regulated mRNA transcripts. Frontal DPCs were transwell cocultured with keratinocytes in serum-free KGM media, with or without 1 nM T, for 3 d. Total RNA was isolated from frontal DPCs and reverse transcribed using H-T₁₁A, H-T₁₁C, and H-T₁₁G as primers. Aliquots of RT reaction mixture were amplified by PCR using primers as described in Materials and Methods. Duplicated reactions for each RNA condition were loaded in adjacent lanes. Shown is a typical differential display gel pattern. (A) Some bands were observed suppressed by T; (B) some bands were observed induced by T. Twelve T-target bands were isolated from the differential display gel, reamplified with respective primers, and cloned into the *pGEM-T* easy vector for subsequent sequencing and reverse Northern analysis. Arrows show examples of selected bands differentially displayed in the lanes with or without T. (C) Colony-PCR products from the cloned cDNA. The size of the colony-PCR products was compared with 1-kb DNA marker (Gibco-BRL, Gaithersburg, MD).

In 1992, Tanaka et al. (32) defined a novel FGF-like growth factor called androgen-induced growth factor, which is produced and secreted by cancer cells in response to androgenic stimuli and is essential for the androgen-dependent growth of SC-3 cells. Chung et al. (33) also isolated and characterized one novel soluble growth factor from the human bone marrow stromal cell line, termed bone-prostate growth factor-1, which can stimulate prostate cancer epithelial growth in a paracrine manner.

Therefore, we are interested in knowing whether there are other novel androgen-regulation growth mediators in frontal DPCs that may contribute to the T-elicited growth inhibition. Differential display RT-PCR was performed by using RNA from bald frontal DPCs as templates for comparisons with and without T. Eight differentially displayed T up- or downregulated genes were obtained (Figs. 5 and 6). After sequencing these clones, we found that the one with the most significant downregulation by T is a human supervillin homolog. Supervillin is one of the membrane skeleton proteins, which stabilize and shape the plasma membrane

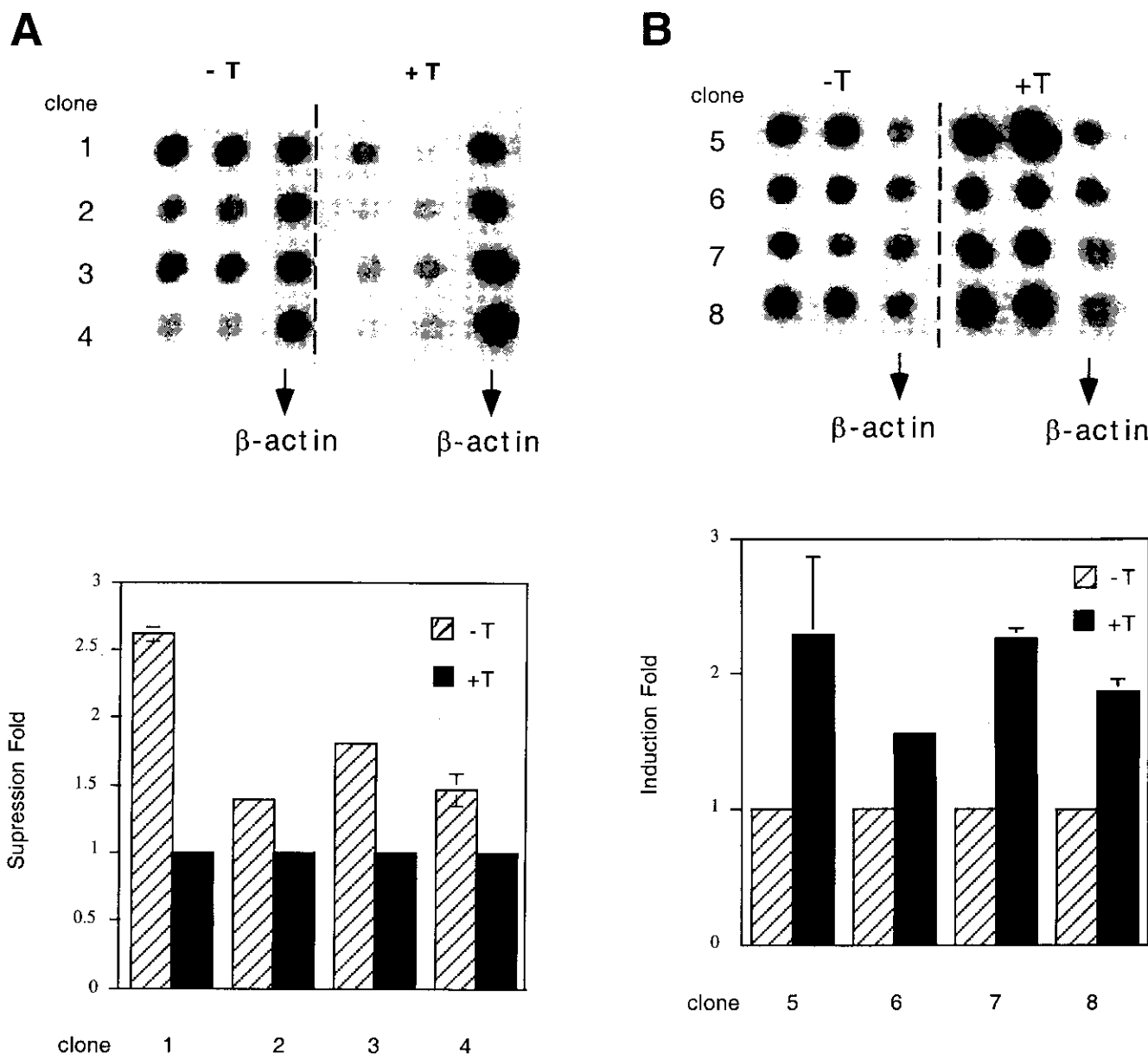


Fig. 6. Reverse Northern dot-blot of the cloned cDNA products from differential display RT-PCR. The candidate clones we isolated from the differential display gel were PCR reamplified, subcloned into pGEM-T vectors, and equally double-spotted onto duplicated membranes. The clone for β -actin was also spotted onto each membrane as internal controls (the right-hand four dots on each membrane) as described in Materials and Methods. Total RNA from frontal DPCs after coculture with keratinocytes, minus or plus 1 nM T, were isolated and labeled as probes, respectively. After hybridization with (-T) RNA probe or (+T) RNA probe, the radioactive intensity of each clone on two membranes was measured by PhosphorImager and quantified by Image Quant Software (Molecular Dynamics, Sunnyvale, CA). Radioactive intensity obtained for each individual clone was normalized with β -actin intensity. The intensity exerted from each clone represents the original RNA intensity in each RNA probe. The suppression fold for clones 1-4 (A) or induction fold for clones 5-8 (B) by T were obtained after quantification. Data are presented as the mean \pm SD of a duplicate determination.

and organize the cell surface into functional domains. Moreover, they control the formation or stabilization of dynamic cell surface extensions and/or are involved in transient interactions with the substratum and with other cells (34). Depending on the cell type, supervillin may play a role in the nucleus, as well as in the actin-based membrane skeleton. The gene map of supervillin coincides with one or more prostate tumor suppressor genes, which are located

on chromosome 10, and its expression levels are high in some carcinoma cell lines such as HeLa S3 (35). Thus, supervillin was believed to play multiple roles during cell growth, adhesion, and mobility (36). We believe this is the first report that androgen can suppress the expression of supervillin; whether androgen regulates cell growth directly via supervillin is an interesting subject for future study. All seven of the other androgen-regulated genes did not match

any known sequence, and their potential roles in T-mediated hair cell growth/baldness remains to be further characterized. In summary, our data suggest that T may either induce or suppress some growth mediators that may play important roles in hair growth and baldness.

Materials and Methods

Animal Subjects

Twelve adult male and female stump-tailed macaques (*Macaca arctoides*), ranging in age from 5 to 15 yr, were used to obtain the DPCs from frontal bald scalp and occipital nonbald scalp. Each animal was housed in our animal care facility (University of Wisconsin Research Animal Resource Center, accredited by the American Association for Accreditation of Laboratory Animal Care). The animal care protocol number is A-34-8800-G00267.

Isolation and Primary Cell Culture of DPCs and Keratinocytes

Under anesthesia with ketamine hydrochloride (20 mg/kg, intramuscularly), biopsies of the scalp skin were obtained from the bald frontal and hairy occipital scalp of the macaques. DPCs were manually isolated from the scalp skin, then transferred and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL). Following trypsin treatment, the cells were subcultured for 4 wk after the beginning of primary culture. The fourth passage of DPCs was used for studies. Normal keratinocytes were isolated from newborn human foreskin and cultured in KGM supplemented with bovine pituitary extract (Clonetics, San Diego, CA). The fourth and fifth passages of keratinocytes were used for studies.

CM Assay and Proliferation Assay

DPCs were inoculated at a density of 1×10^5 cells/well into type I collagen-coated six-well multiplates (Corning, New York) and cultured with DMEM supplemented with 10% charcoal-treated FBS. After 24 h of incubation, the medium was discarded and keratinocytes (2×10^5 cells/well) were seeded with KGM without bovine pituitary extract into the same well or in the upper compartment of transwell plates. DPCs and keratinocytes were cultured alone or cocultured and treated with or without 1 nM T (Sigma, St. Louis, MO) or 1 nM RU58841 (Roussel UCLAF, Romainville, France) for 3 d. Then all CM were collected and transferred onto 24-h-old keratinocytes, which had been inoculated at 4×10^4 cells/well in a 24-well cluster (Corning). The keratinocytes were then incubated for an additional 3 d and counted by a hemocytometer. For the proliferation assay, cells were prepared as above except they were refed on d 3 with fresh media, and then the total cell number was counted 7 d later.

Isolation of RNA

The guanidium isothiocyanate/cesium chloride method was used to isolate total RNA from frontal DPCs or keratinocytes grown in the transwell coculture system (37,38). After ultracentrifugation for 16 h, the RNA pellet was treated with phenol/chloroform extraction and precipitated with ethanol. The RNA was dissolved in diethylpyrocarbonate-treated water, and the amount was determined by the optical density at 260 nm.

Differential Display RT-PCR

Differential display RT-PCR was conducted following procedures previously described (39,40). All reagents were purchased from Gene Hunter (Brookline, MA). AmpliTaq DNA polymerase was obtained from Perkin-Elmer (Branchburg, NJ). Two microliters of cDNA template (from previous cDNA synthesis, use H-T₁₁A, H-T₁₁C, and H-T₁₁G as primers) (Gene Hunter), 2 µL of degenerate anchor primer (use the corresponding primer, H-T₁₁A, H-T₁₁C, and H-T₁₁G, that was used during cDNA synthesis), 2 µL of 5'-arbitrary primer (H-AP primers, Gene Hunter), 2 µL of 10X PCR buffer, 1.6 µL of 25 µM dNTP mix, 1 µL of [α -³⁵S]dATP, 0.2 µL of AmpliTaq, and 9.2 µL of H₂O were mixed in a 0.2-mL thin-wall PCR tube. PCR was performed using the following program for 40 cycles—94°C, 30 sec; 40°C, 2 min; 72°C, 30 sec—, then incubated at 72°C for 10 min (GeneAmp PCR system 2400, Perkin-Elmer). Then 3.5 µL of PCR product was mixed with 2 µL of sequencing loading dye, heated to 80°C for 2 min, and loaded 2.5 µL of duplicate reactions for each RNA condition in adjacent lanes onto an HR-1000 6% sequencing gel (GenomexLR Sequencer, Foster City, CA). Next the gel was run at 60 W for 3.5 h, dried, and exposed to X-ray film according to the operating manual. The amplified transcripts, which were scored as differentially expressed in androgen-regulated lanes, were precisely excised from the gel, soaked in dH₂O, and subjected to PCR reamplification by using the appropriate primer pairs under identical PCR conditions containing 250 µM dNTP. All reamplified transcripts were detectable on a 1% agarose gel after a single round of amplification.

Subcloning and Reverse Northern Dot-Blot

An aliquot of the PCR reaction was directly ligated into the pGEM-T easy vector (Promega, Madison, WI) following the manufacturer's specifications. 10 µL of the ligated mix were transformed with *Escherichia coli* competent cells. After overnight incubation at 37°C, colonies were randomly selected and placed into lysis buffer, boiled for 5 min, spun for 5 min, and the lysates saved. We performed the PCR for 30 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) for 50-µL reactions, with 5 µL of 10X PCR buffer, 1 µL of 10 mM dNTP, 1 µL of T7 primer (10 µM), 1 µL of SP6 primer (10 µM), 1 µL of the colony lysates, 40.5 µL of dH₂O, and 0.5 µL of Taq DNA polymerase in each tube.

Following a 5-min chain extension at 72°C, the size of the inserts was checked with 5 µL of the PCR products on a 1% agarose gel.

To prepare for blotting, 30 µL of each colony-PCR product were mixed with 5 µL of 2*N* sodium hydroxide, the mixture was boiled for 5 min to denature the DNA, and then neutralized with 5 µL of 3*M* sodium acetate (pH 5.0). After bringing the total volume to 210 µL with dH₂O, 50 µL of each sample were double dot-blotted onto duplicated nylon membranes using the Bio-Dot microfiltration system (Bio-Rad, Hercules, CA). β-actin was spotted as an internal control and the vector alone was spotted as a negative control. The membranes were then ultraviolet crosslinked and rinsed in 6X saline sodium citrate (SSC) before prehybridization.

[³²P]-labeled RNA probe was prepared by reverse transcription in a 20 µL- reaction by using 10 µg of RNA (from frontal DPCs cocultured with keratinocytes plus or minus T, respectively), 0.5 µL of dNTP-C (10 mM), 5 µL of α[³²P]dCTP, and 2 µL of RNase inhibitor (10 U/µL). After 4 h of prehybridization of the dot-blot membranes, the RNA probes were boiled for 5 min and then equal counts (10⁷ cpm) was added to the respective dot-blot membrane and hybridized overnight at 42°C. The blots were washed twice at room temperature, for 15 min each, with 1X SSC and 0.1% sodium dodecyl sulfate and exposed to an X-ray film overnight at -70°C. The radioactive intensity of each clone after hybridizing with RNA probe was measured by PhosphorImager, quantified by ImageQuant Software (Molecular), and normalized by radioactive intensity of β-actin.

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