

Regulatory Effect of Cannabidiol (CBD) on Decreased β -Catenin Expression in Alopecia Models by Testosterone and PMA Treatment in Dermal Papilla Cells

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Objectives: The hair follicle is composed of more than 20 kinds of cells, and mesoderm derived dermal papilla cells and keratinocytes cooperatively contribute hair growth via Wnt/ β -catenin signaling pathway. We are to investigate β -catenin expression and regulatory mechanism by CBD in alopecia hair tissues and dermal papilla cells.

Methods: We performed structural and anatomical analyses on alopecia patients derived hair tissues using microscopes. Pharmacological effect of CBD was evaluated by β -catenin expression using RT-PCR and immunostaining experiment.

Results: Morphological deformation and loss of cell numbers in hair shaft were observed in alopecia hair tissues. IHC experiment showed that loss of β -catenin expression was shown in inner shaft of the alopecia hair tissues, indicating that β -catenin expression is a key regulatory function during alopecia progression. Consistently, β -catenin expression was decreased in testosterone or PMA treated dermal papilla cells, suggesting that those treatments are referred as a model on molecular mechanism of alopecia using dermal papilla cells. RT-PCR and immunostaining experiments showed that β -catenin expression was decreased in RNA level, as well as decreased β -catenin protein might be resulted from ubiquitination. However, CBD treatment has no changes in gene expression including β -catenin, but the decreased β -catenin expression by testosterone or PMA was restored by CBD pretreatment, suggesting that potential regulatory effect on alopecia induction of testosterone and PMA.

Conclusion: CBD might have a modulating function on alopecia caused by hormonal or excess of signaling pathway, and be a promising application for on alopecia treatment.

Keywords: alopecia, dermal papilla, wnt/ β -catenin, testosterone, phorbol 12-myristate 13-acetate (PMA), cannabidiol (CBD)

INTRODUCTION

Mammalian hair growth is begun from hair follicles in the dermis, which are composed of more than 20 kinds of cells [1]. It is known that hair growth is regulated through proliferation

and differentiation of keratinocytes by the Wnt/ β -catenin pathway from dermal papilla cells that is in the stemness niche of the hair follicle [2]. Cyclic hair growth is progressed by anagen, catagen and telogen by which are multicellular signaling in hair follicle [3]. At the anagen stage, β -catenin is translocated into

the nucleus and stimulates the expression and releases of FGF growth factors such as FGF7 and FGF10 [4, 5]. FGF growth factors activate the FGF receptor 2 (FGFr2) of keratinocytes in order to progress hair growth [6-8]. Ubiquitination of β -catenin has been suggested as pivotal regulatory mechanism to hair growth cycle [9, 10]. Alopecia is a degenerated disease of hair growth that could be affected by environmental or genetic factors [11]. Especially, cyclic hair growth is defected in the steps from telogen phase to anagen reentry in alopecia [12-14].

Testosterone is the primary steroidal hormone of males in human population, and promote secondary sexual characteristics such as muscle, or bone formation [15]. Hormonal action of testosterone is known to regulate the Wnt/ β -catenin pathway in dermal papilla cells [16]. Testosterone is converted to dihydrotestosterone (DHT) by 5α -reductase (5α -R) in cytoplasmic region of dermal papilla cells [17, 18]. DHT shows higher receptor binding capacity than testosterone, and increases the expression of GSK-3 β , DKK-1, IL-6, and TGF- β 1, IL-6 and TGF- β 1 [2]. DHT inhibits the entering into catagen stage of keratinocytes during cyclic hair growth, indicating that defect of testosterone may be the cause of alopecia [19, 20]. On the other hand, phorbol 12-myristate 13-acetate (PMA) also induces early catagen by regulating the PKC isoforms (α , ϵ , λ , ι , γ) of dermal papilla [21].

Cannabidiol (CBD) is a phytocannabinoid extracted from Hemp (*Cannabis sativa*), and is used as a medicine for epilepsy treatment [22, 23]. Whereas Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the main psychotropic phytocannabinoid of Hemp species (*Cannabis marijuana*), is a partial agonist CB₁ and CB₂ receptor, CBD act as an antagonist of CB₁/CB₂ agonists [24]. In contrast to Δ^9 -THC, The possibility as therapeutic agents has been increased due to CBD lacks detectable psychoactivity [25]. CBD inhibits apoptosis by inducing Erk and AKT/mTOR pathways in SH-SY5Y cell lines, suggesting that CBD has a potential therapeutic effect for parkinson's disease [26]. Moreover, CBD treatment regulates proliferation via activation of CB₁ receptor of keratinocytes in hair follicles [27].

In this study, we are to investigate a possible regulatory mechanism of CBD in testosterone or PMA induced alopecia model. We performed microscopic analyses using alopecia patients derived hair. In addition, pharmacological effect of CBD was evaluated by β -catenin expression using RT-PCR and immunostaining experiment.

MATERIALS AND METHODS

1. Cell cultures, and reagents

Human dermal papilla cells were kindly obtained from Dr. YK Seo, Dongguk university, Ilsan, Korea. Cells were cultured in DMEM medium containing 10% fetal bovine serum and maintained in a humidified incubator at 37°C/5% CO₂. Testosterone and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Aldrich, and cannabidiol (CBD) from Cayman Inc, USA. The drugs were dissolved in appropriate solvents according to the manufacturer's protocol. Cell counting was performed using trypan blue staining (Gibco, USA).

2. Hair tissues analyses

Normal and alopecia hair tissues were collected from volunteers, and used for H&E staining and Immunohistochemistry. This study was performed after approval from the Institutional Review Board (IRB) of Kangwon National University (KWNUN IRB-2020-06-010-001). Hair tissues were fixed with 4% formaldehyde, and then paraffin blocks were prepared. The sliced tissue sections were prepared using a microtome. The tissues were stained with hematoxylin and eosin, and observed with optical microscope (CX-200TM, COXEM, Korea). For immunohistochemistry experiment, the tissues were undergone for antigen retrieval using sodium citrate buffer (10 mM sodium citrate, 0.05% tween 20, pH 6.0) to heat for 15 minutes. The tissues were incubated with β -catenin antibody (1:100; Bdsience, USA) and then followed by secondary peroxidase antibody. The tissues were exposed for 1 minute using DAB substrate kit (Vector, USA). The hairs were also subjected to scanning electron microscopic (SEM) analysis (CX-200TM, COXEM, Korea).

3. RNA extraction and RT-PCR

Human dermal papilla cells (4×10^5) were seeded in 100mm culture dish, and was treated with the drugs. Total RNA was isolated using the TRIZOL kit (Duchefa, Netherlands). Complementary DNA (cDNA) was synthesized with Oligo-dT primer and Reverse transcriptase (RTase). cDNA was mixed with specific primer sets in 0.2 mM dNTP, Kntaq polymerase, buffer containing 1.5 mM MgCl₂ (Enzynomics, Korea). PCR reactions were carried out in a Perkin Elmer Thermal Cycler 9600 (Applied Biosystems, MA, USA). PCR products were resolved

in 2% agarose gels.

4. Immunostaining

Dermal papilla cells were cultured on the coverslips and fixed with 100% methanol solution. After washing with PBS, the cells were permeabilized with 0.2% Triton X-100 solution for 10 min. Blocking was performed using 3% bovine serum albumin in PBS for 1 hr, and followed by incubation with β -catenin antibodies (1:300) at room temperature for 2 hr. Cells were then washed with PBS, and followed by incubation with secondary antibodies Alexa 488 goat anti-mouse IgG (1:500; Abcam, USA). Cells were stained with DAPI for counter staining. The coverslips were mounted on the slide glass with anti-fade reagent. Fluorescent images were examined with confocal microscopes (Ts2, Nikon, Japan) of Kangwon center for system imaging (KCSI).

RESULTS AND DISCUSSION

Growth and degeneration of hair is cyclically regulated via various molecular signaling pathway [28]. Alopecia hair loss could be progressed by various reasons such as aging or hormonal changes on cells in hair follicle tissues [11, 12]. The Wnt/ β -catenin pathway of dermal papilla cells regulates growth of hair, as well as is known as a key signaling pathway for proliferation and differentiation of keratinocytes in hair follicle [9, 10].

In this study, we investigated structural analyses and β -catenin expression between normal and alopecia hair tissues, as well as how CBD regulates β -catenin expression which is induced by testosterone or PMA in human dermal papilla cells. One of the characteristics of alopecia is changes in length or diameter of hair. We compared morphological characteristics between normal and alopecia hair tissues using SEM. The bulb of the alopecia hair tissues was severely deformed, and the surface of the hair shaft was irregular, suggesting that alopecia hair was severely damaged compared to normal hair (Fig. 1A). We also compared cell composition between normal and alopecia hair tissues using H&E staining (Fig. 1B). Cell density was less compact in alopecia hair tissues than normal hair tissues. Cells without nuclei were even observed in alopecia hair tissues, implying that the hair tissues and cell density were impaired in alopecia. Therefore, this phenomenon could be explained as induction of cell death mechanism such as apoptotic pathway by a decrease in differentiation and proliferation of keratinocytes during alopecia progression [29]. Immunohistochemistry experiment was performed to determine whether there was a difference in β -catenin expression in the shaft of normal and alopecia hair tissues. Interestingly, β -catenin was highly expressed in inner hair shaft region of normal hair tissues, but no expression in alopecia hair shaft (Fig. 1C). β -catenin expression is closely associated with proliferation and differentiation of dermal papilla cells but there have been few studies on functional roles of β -catenin expression of keratinocytes in the hair shaft region [30]. Our current data suggest that β -catenin is

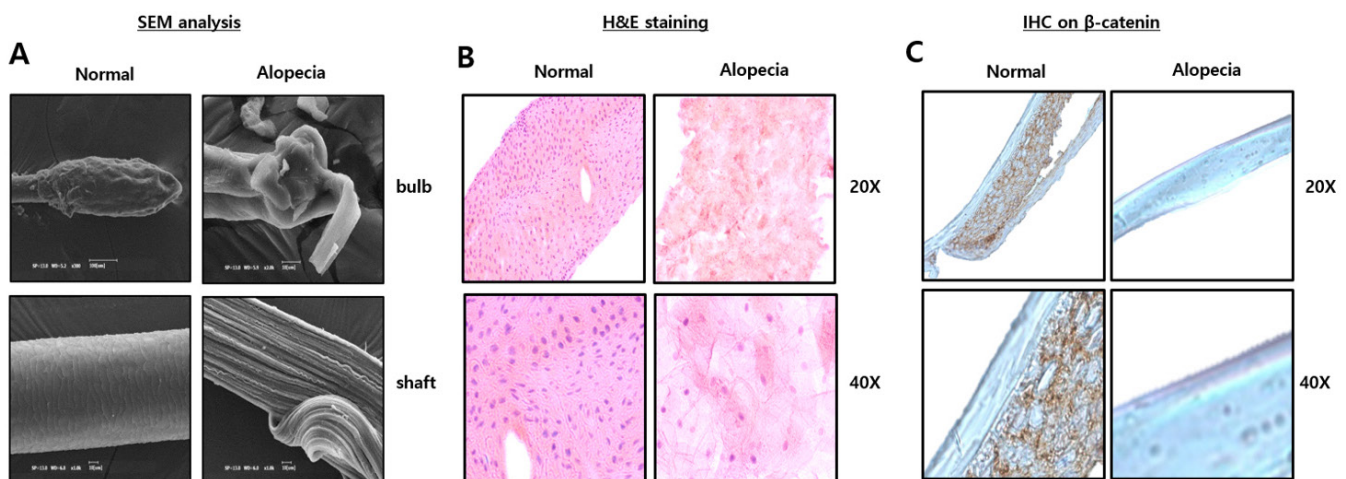


Figure 1. Alopecia hair is deformed and does not express β -catenin. (A) Normal and alopecia hairs were sliced into bulb and shaft, and then observed through an scanning electron microscope (SEM). (B) Normal and alopecia hairs was used for H&E staining. (C) The expression of β -catenin was measured through immunohistochemistry.

highly expressed to maintain proliferation of keratinocyte, but the loss of β -catenin expression might be a reason for alopecia progression.

Testosterone has been known as causative androgenic hormones of male alopecia in human population [15, 17]. High levels testosterone is convert into DHT, which can reduce growth and size of cells in hair follicles [18]. Testosterone was treated on culturing dermal papilla cells to examine molecular changes of gene expression. There was no significant growth inhibitory effect by testosterone treated dermal papilla cells (Fig. 2A). However, RT-PCR analysis showed that expression of *wnt5a* and β -catenin were decreased by testosterone treatment. We also performed immunostaining experiment on β -catenin expression in testosterone treated dermal papilla cells. Expression of β -catenin was decreased in this experiment, suggesting that β -catenin is regulated by testosterone. On the other hand, PMA, a PKC activator, has been also known as regulating

β -catenin expression, and PMA was treated in dermal papilla cells. Using RT-PCR and immunostaining experiment, we obtained similar data that β -catenin expression was regulated by PMA treatment (Fig. 2B). These data suggest that β -catenin has an essential function for the growth and maintenance of dermal papilla cells in hair follicles. Moreover, appropriate β -catenin expression could be required for the differentiation into keratinocytes of hair tissues [3]. In the absence of β -catenin, dermal papilla cells are fail to adopt the fate of hair keratinocytes and instead, differentiate into epidermal keratinocytes [31]. We also performed immunostaining experiment using proteasome inhibitor MG132 to examine on the decreased β -catenin level. Decreased β -catenin expression by testosterone was increased in MG132 treatment (Fig. 3). Therefore, β -catenin level is tightly regulated by ubiquitination pathway in dermal papilla cells.

There are some reports that CBD in dissolved in hemp oil can promote the growth of the hair, but this pharmacological

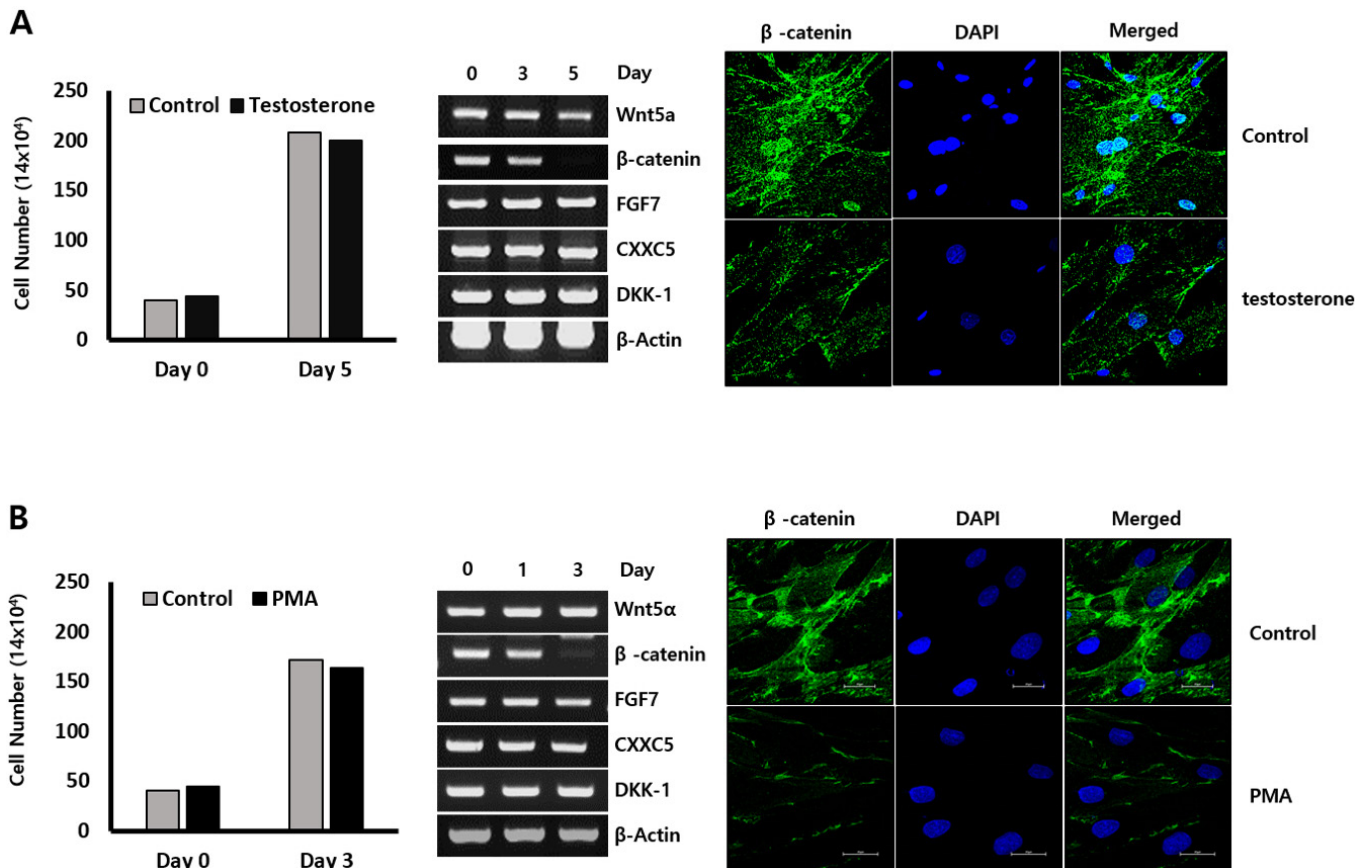


Figure 2. β -catenin is decreased by testosterone and PMA treatment in dermal papilla cells. (A) Dermal papilla cells were treated with testosterone for 5 days, and then analyzed on cell growth and gene expression. It was confirmed that β -catenin expression was decreased in testosterone treated dermal papilla cells using RT-PCR and immunostaining experiment. (B) PMA also treated for 3 days in dermal papilla cells, and followed by the same experiments as shown in testosterone treated dermal papilla cells.

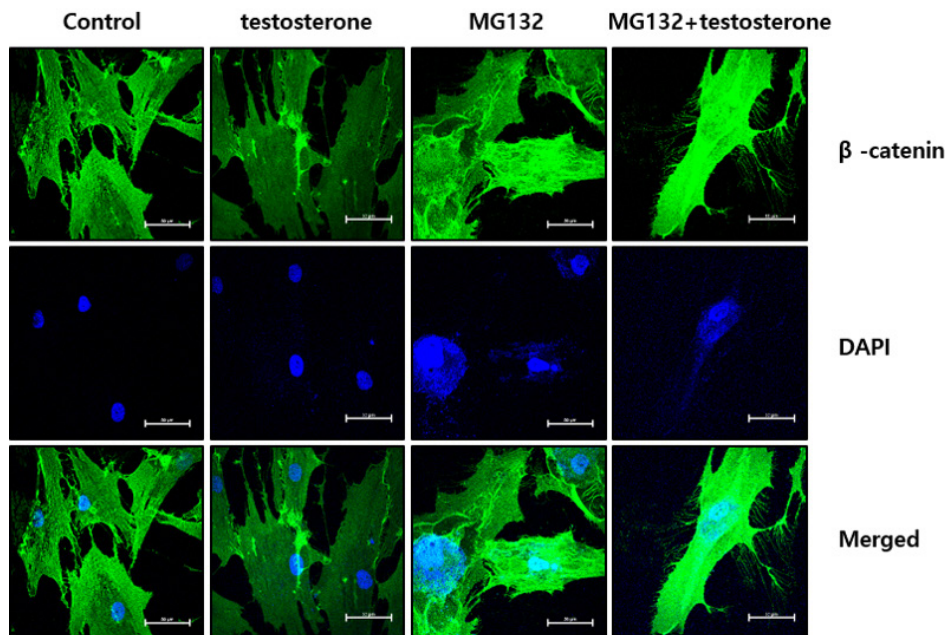


Figure 3. Ubiquitination pathway involves in the β -catenin down-regulation. Dermal papilla cells were pre-treated with MG132 for 6 hr, and then treated with testosterone. MG132 pretreatment restore the decreased β -catenin expression by testosterone.

effect might be resulted from the vitamins in oil components [32]. CBD oil contains high concentration of vitamin E, which can stimulate hair growth and help improvement of alopecia [33]. Although preclinical evidence suggests topical application of CBD may be efficacious for some skin disorders, and inflammatory diseases, but elucidation of underlying molecular mechanisms have not yet to be fully identified [34]. We treated CBD to investigate molecular efficacy on dermal papilla cells. CBD treatment showed growth inhibitory effect of dermal papilla cells. Unexpectedly, CBD showed little changes on RNA level including β -catenin (Fig. 4A). No changes of β -catenin expression were also observed in immunostaining experiment. We are to examine whether CBD may have a regulatory effect in the case of combinatory treatment with testosterone or PMA. CBD was pretreated for 6 hours in dermal papilla cells and followed by treatment with testosterone or PMA. Immunostaining experiment showed that decreased β -catenin expression by testosterone or PMA was restored by CBD pretreatment (Fig. 4B, C). This phenomenon implies that CBD has a modulating effect on alopecia progression induced by testosterone or PMA. CBD can inhibit cell proliferation and induce apoptosis via TRPV receptor in dermal papilla cells [35]. TRPV activation secretes the proteins such as HGF, IGF1, and SCF, and promotes cell death as CBD dose-dependent manner [34]. Our current data suggest that novel efficacy of CBD is considered to have a modulating mechanism on decreased expression of β -catenin by testosterone or PMA. Numerous cell culture and animal studies showed

antitumor effects of cannabinoids in various cancer types [36, 37]. CBD may enhance uptake or increase the potency of certain drugs used to treat cancer suggesting that CBD might have promising efficacy for treatment for many types of diseases including alopecia and cancers [38].

CONCLUSION

In this paper, not only we showed loss of β -catenin expression in hair tissues derived alopecia patients, but also CBD pretreatment could restore the decreased β -catenin expression by testosterone or PMA. Once focusing on β -catenin expression, CBD might be a modulating function for alopecia caused by hormonal or excess of signaling pathway. Therefore, CBD might have a modulating function on alopecia caused by hormonal or excess of signaling pathway, and be a promising application for on alopecia treatment.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

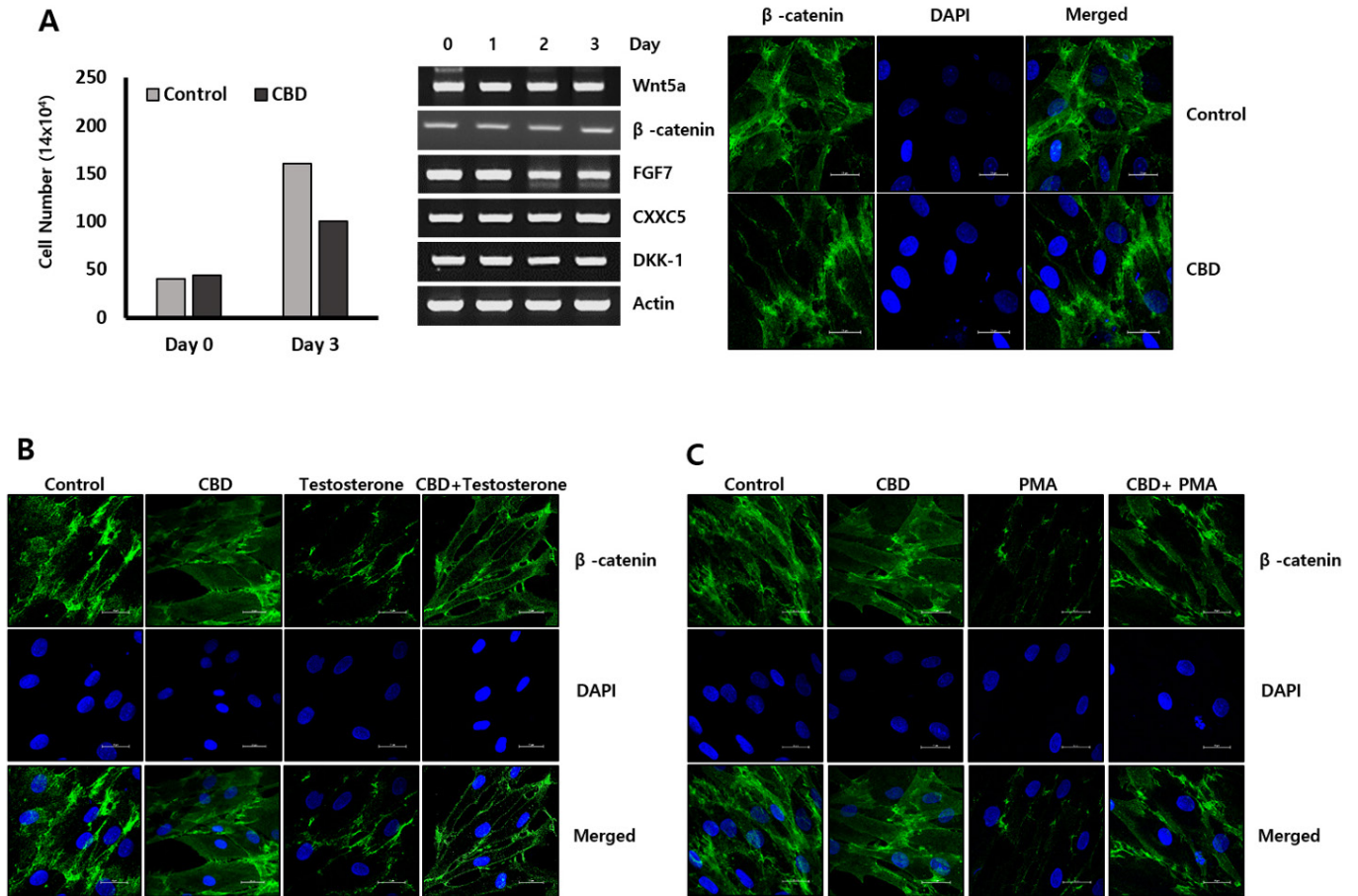


Figure 4. CBD treatment has no effect on gene expression, but restores the decreased β -catenin expression by testosterone and PMA. (A) Dermal papilla cells were treated with 20 μ M CBD. Cell growth was slightly inhibited at 3 day incubation. RT-PCR and immunostaining experiment shows no distinct changes on β -catenin expression. (B) CBD was pretreated for 6 hr, and then treated with testosterone in dermal papilla cells. Immunostaining experiment was performed with β -catenin antibody. (C) PMA was also treated for 3 days after CBD pretreatment in dermal papilla cells.

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