A DOCTORIAL DISSERTATION

The hair growth promoting effect and action mechanism of 4-O-methylhonokiol from *Magnolia officinalis*

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후박에서 분리한 4-O-methylhonokiol의 육모 효능 및 작용기전

지도교수 강 희 경

김 상 철

이 논문을 의학 박사학위 논문으로 제출함

2010년 2월

김상철의 의학 박사학위 논문을 인준함

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제주대학교 대학원

2010년 2월

The hair growth promoting effect and action mechanism of 4-O-methylhonokiol from *Magnolia officinalis*

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A thesis submitted in partial fulfillment of the requirement for degree of doctor of philosophy in Medicine

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Dec. 15. 2009

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ABSTRACT

Hair loss, regardless of its type, is a common and distressing phenomenon. Recently, there has been increasing number of people suffering from hair loss or thinning. It is very important to develop new therapeutic materials. To prevent hair loss and to enhance hair growth, therefore, we screened the extracts of plants that have traditionally been used in oriental medicine and discovered that *Magnolia officinalis* (*M. officinalis*) potentially promoted the hair growth among them. *M. officinalis* has been used as a traditional remedy in China and Japan for treatment of gastrointestinal disorders, anxiety and allergic diseases including bronchial asthma. There have been many pharmacological reports of the activities of extracts or constituents from the bark of *M. officinalis* such as anti-inflammatory, antiallergic, antibacterial, and neurite spouting activities.

In the present study, we investigated the promotion effect of *M. officinalis* as well as 4-O-methylhonokiol, a neolignan compound from *M. officinalis*, on the growth of hair. When rat vibrissa follicles were treated with a EtOH extract of *M. officinalis*, the hair-fiber lengths of the vibrissa follicles increased significantly. In particular, 4-O-methylhonokiol, a principal of the extract, also increased the hair-fiber lengths of vibrissa follicles. In addition, after daily topical application of 4-O-methylhonokiol onto the back of C57BL/6 mice, anagen progression of the hair shaft was induced. Moreover, 4-O-methylhonokiol increased both the

expression of proliferating cell nuclear antigen (PCNA) in the bulb region and proliferation of immortalized vibrissa dermal papilla cells. In order to determine the mechanism by which 4-O-methylhonokiol promotes hair growth, we examined the expression levels of transforming growth factor-β1 (TGF-β1) and TGF-β2 which have been known to play an important role in anagen to catagen transition via the induction of keratinocyte apoptosis. When the vibrissa follicles in the anagen phase were treated with 4-O-methylhonokiol for 7 days, the expressions of TGF-β1 in cells of outer root sheath (ORS) and epithelial strand, i.e., in the epithelial residue of the regressing hair bulb during catagen were found to be lower than those of the control follicles that were expected to be in the anagen-catagen transition phase. Also, 4-O-methylhonokiol decreased the expression of TGF-β2 in the bulb matrix region of the 7-day cultured follicles. These results suggest that 4-O-Methylhonokiol has the potential to promote hair growth via down regulation of TGF-β1 and TGF-β2, as well as the proliferation of dermal papilla.

To investigate how the 4-O-methylhonokiol could inhibit the TGF-β1-induced keratinocyte growth arrest, human keratinocyte HaCaT cell was used. When HaCaT cells were pretreated with 4-O-methylhonokiol, the TGF-β1-induced p21 expression was decreased. Moreover, 4-O-methylhonokiol inhibited nuclear translocation of smad2/3 and smad4 and Sp1activation by TGF-β1. We observed that ERK activation by TGF-β1 was significantly attenuated by

treatment with 4-O-methylhonokiol. On the other hand, TGF-β has been reported to increase reactive oxygen species (ROS) intracellular content in different cell types as well as the effects of TGF-β on the cell growth arrest and apoptosis have been kwon to be mediated by oxidative stress. 4-O-Methylhonokiol inhibited TGF-β1-induced ROS production and suppressed mRNA expression of NOX4. These results indicate that 4-O-methylhonokiol could inhibit TGF-β1-induced cell growth arrest through down regulation of smad2/3, smad4, and NOX4 in Human keratinocyte HaCaT cell.

Key word: *Magnolia officinalis,* hair growth, 4-O-methylhonokiol, dermal papilla cells, TGF-β1, TGF-β2, PCNA, p21, Sp1, Smad2/3, Smad4, NOX4

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Ⅰ. INTRODUCTION

Hair loss is a common and distressing phenomenon. Recently, the number of people who suffered from hair loss or hair thing is increasing. Thus, it is very important to develop new therapeutic materials to stop hair loss and to enhance hair growth.

1. Structure of hair follicle

Hair is a primary characteristic of mammals, and exerts a wide range of functions including thermoregulation, physical protection, sensory activity, and social interactions. Hair shaft is produced through a complex differentiation process from the hair follicle (Schneider MR *et al*., 2009). Hair follicle can be divided into many parts according to their developmental origin, location and morphology. These include the outer root sheath (ORS), inner root sheath (IRS), matrix, and dermal papilla (DP). Most of these parts are composed of ectoderm-derived epithelial cells except for the DP, which is originated from the mesenchyme. The ORS is outmost structure of hair follicle, which surrounds whole follicle tissue. It is driven by the invagination of epidermis during embryogenesis (Hardy MH, 1992). The IRS locates inner side of ORS and surrounds hair shaft. The IRS is derived from the matrix keratinocytes and can be classified as Henry's layer, Huxley's layer, and innermost

layer according to their location and morphology. It is thought that IRS functions as a mold hair shaft production, and then determines shape of hair such as curl or straight (Powell BC *et al*., 1991). The matrix is mainly composed of keratinocytes and other ectoderm-derived cells, melanocytes. The matrix keratinocytes proliferate, move upwardly and differentiate into various types of structural cells including hair shaft and the IRS (Paus R *et al*., 1999). In the lower end bulb region of the hair-producing follicle, a permanent population of mesenchyme-drived fibroblast cell called dermal papilla is located. Dermal papilla is known to have a regulatory role in the hair growth control (Oliver RF, 1996). All these hair follicle-constituting cells are interacting each other coordinately, and then making a hair shaft.

1.1 Hair shaft

Hair shaft is generally made of three layers, which is derived from the differentiated and keratinized matrix cells: medulla (the innermost part), cortex (middle part) and cuticle (outmost part of hair shaft). In some hairs, however, medulla is absent. The major structural proteins of hair fiber are cysteine-rich keratins (MacKinnon PJ *et al*., 1990). In the follicle bulb, there is a pool of cells called matrix that are committed to hair growth and have a sustained but finite proliferative capacity (Cotsarelis G *et al*., 1990). There is a rapid differentiation of the matrix into the cells of the cortex and cuticle. The cortex is the major histological component of hair shaft, accounting for up to 90% of the cellular mass of the

hair. The cuticle is composed of hair shaft and, in general, accounts for less than 10% of the volume of the hair shaft (Orwin DF, 1979).

1.2 Root sheath

The outer root sheath (ORS) is the outmost structure of hair follicle. The ORS cells are ectoderm-derived keratinocytes that are known to have specified roles in maintaining hair follicle structure and regulating hair growth cycle. Especially, ORS keratinocyte located in the bulge area are well known to have several properties consistent with their being the stem cell (Cotsarelis G *et al*., 1990). Stem cells are present in all self-renewing tissues, and are believed to be long lived and have great potential for cell division. These cells are ultimately responsible for homeostasis of continually renewing tissues (Lajtha LG, 1979). In addition, stem cells play a central role in wound healing, aging and carcinogenesis (Lavker RM *et al*., 1991). Thus, it is important to know where stem cells are resided and then to understand the self-renewing nature of hair follicle. Stem cells are normally slow-cycling and can be identified experimentally as the label-retaining cell (Cotsarelis G *et al*., 1990). Using this method, Cotsarelis *et al*. (1990) have found that all the follicular label-retaining cells are exclusively confined to the bulge area. Moreover, the existence of stem cells in bulge area was supported by the finding that the bulge keratinocytes had growth potential superior than other keratinocytes of the skin cultured *in vitro* (Yang JS *et al*., 1993).

1.3 The dermal papilla cell

The dermal papilla (DP) is a condensate of mesenchyme-derived cells and its origin is distinguishable from other cell types in hair follicle, since rest of all are derived from ectoderm. The DP plays an important regulatory role in determining the type of hair produced (Oliver RF *et al*., 1988). It may be due to the production of paracrine factors and extracellular matrix (ECM) proteins (Randall VA *et al*., 2001).The morphology of the DP can be altered through the hair growth cycle, being maximal in volume in growing phase (anagen) and least at resting phase (telogen). This is, in fact, a result of changes in the amount of ECM and cell number within the DP. One of the important roles of ECM on hair growth is supported by the finding that the volume of the DP is directly proportional to the diameter and length of the resulting hair. A sort of ECM molecules has been found to be expressed in the DP, and one of the special characteristics is the domination of basement membrane components such as fibronectin, laminin, type iv collagen and proteoglycans (Elliott K *et al*., 1999). The hair follicle inductive properties of the DP have been well recognized. For instance, Oliver (Jahoda CA *et al*., 1996) has developed microsurgical implantation techniques and manipulated individual components. Series of his elegant experiments revealed the inductive properties of the DP. When the microsugically-dissected DP was inserted into lower part of amputated hair follicle *in vivo*, new hair follicle was

emerged several weeks after transplantation. It appears that follicular papilla is inherently able to induce follicle formation (Pisansarakit P *et al*., 991). Oliver and others also showed that adult dermal papilla cells cultured *in vitro* could retain their inductive properties. When mouse vibrissae DP cells were implanted into inactivated follicles, new hair follicle tissue was regenerated (Jahoda CA *et al*., 1984) It has been also reported that sheep DP cells cultured *in vitro* could induce hair follicle formation in heterotypic skin equivalent (Watson SA *et al*., 1994). The molecules responsible for inductive properties of the DP remain largely unknown. Several growth factors including insulin-like growth factor, fibroblast growth factor, epidermal growth factor and transforming growth factor families are suggested to be produced by DP cells and to play important roles in hair growth regulation (Stenn KS *et al*., 1996).

1.4 The matrix

The matrix keratinocytes are dedicated to hair shaft formation through proliferation, differentiation and keratinization. Although the fate and morphological change of putative hair shaft are well described, the molecular characteristics of these cells are poorly understood. This is partly due to difficulties in cultivation of hair matrix cells, in spite of several reports describing successful culture of follicular matrix cell (Fusenig NE *et al*.,

1994). Interestingly, Jahoda and his colleague have proposed that there are germinative epidermal cells in the most deeply embedded bulbar portion of follicle, which shows phenotypically and behaviorally specialized characteristics compared to the upper matrix cells surrounding the DP. They also shows that recombinations of germinative epidermal cells with low passage dermal sheath cells, or high passage dermal papilla cells of vibrissa follicles both consistently stimulated the morphogenesis of very large vibrissa-type follicles and fibers (Reynolds AJ *et al*., 1996). It is thought that germinative epidermal cells are derived from the stem cells in bulge area, and then continually give semi-differentiated matrix cells that are committed to putative hair shaft. Recently, several regulatory factors governing in the matrix differentiation are found. For example, HOXC13, but not other HOX multigene family, strongly activated the promoters of the keratin genes in the matrix (Jave-Suarez LF *et al*., 2002). Lymphocyte enhancing factor-1 (Lef-1) has been also known to be involved in this process, since a DNA binding consensus sequence of Lef-1 was present in the promoter region of almost all hair keratin genes (DasGupta R *et al*., 1999). Other important signaling systems include the bone morphogenic protein (BMP). The differentiation of the hair shaft but not the inner root sheath is severely impaired in transgenic mice of which BMP signaling is inhibited by the expression of Noggin in the hair

matrix, suggesting that BMP may be a key regulator in the genetic program controlling hair shaft differentiation in postnatal hair follicles (Kulessa H *et al*., 2000.).

2. Morphogenesis of hair Follicle

The first hair follicles are formed from the ectoderm, an epithelial layer that will give rise to epidermis, and the underlying mesoderm, a mesenchymal layer that formsx the dermis. During the period of hair follicles formation, the precise distribution of hair follicles over the surface of the body is established and the future phenotype of each hair is determined (Paus Rand Cotsarelis G., 1999). It has been well recognized that the generation of hair follicle relies on a reciprocal exchange of signals between epithelial and mesenchymal cells. The first signal is mesenchymal, instructing ectoderm to form a hair germ. An ectodermal signal then initiates the formation of a mesenchymal dermal papilla, and finally a second dermal signal initiates proliferation and differentiation within the developing epithelial component to form the follicle and its sebaceous gland appendages (van Steensel MA *et al*., 2001). The inductive signals exchanged among epithelial components within the follicle and between follicular epithelia and mesenchymal are largely unknown. But several outstanding works have identified molecules expressed in the follicle that may play an important role in the development of hair follicle. In one instance, Wnt/β-catenin signaling, among other signaling

systems, is well implicated in the hair follicle morphogenesis (Widelitz RB *et al*., 2000). Many components of the Wnt signaling system have been identified. Extracellular Wnt ligands activate Frizzled receptors and induce the stabilization of cytoplasmic β-catenin by preventing its degradation in proteasome (Nusse R, 1999.). Upon accumulation, β-catenin interacts with Lef/Tcf family of DNA-biding proteins to generate a functional transcription factor complex. The important role of Lef-1 in hair follicle formation was previously demonstrated by Lef-1 null mice, which displayed a reduction in follicle numbers and lack of whiskers (van Genderen C *et al*., 1994). In accordance, Lef-1 is expressed in primitive ectoderm even before the dermal condensation is formed. And epithelial Lef-1 is maintained at the growing tip of the hair germ and induced in the adjacent mesenchyme, suggesting that it has an important role in establishing the placement of follicular primordial (Kratochwil K *et al*., 1996).

3. Hair growth cycle

Hair grows in cyclical manner that is categorized into anagen, catagen, and telogen phases by the growth characteristics such as active hair fiber production, regression, and resting, respectively (Hardy MH 1992). Hair follicles in different body sites produce hairs of different length, with the length proportional to the duration of the anagen phase. For

instance, scalp hair follicles stay in the anagen phase for 2 to 6 years and produce long hairs, whereas eyebrow hair follicles do so for only 2 to 3 months and produce short hairs. Individual follicles undergo growth cycle asynchronously although as much as 85 to 95% of the scalp hair follicles may be in the anagen phase at any given time. A few of scalp hair follicles are undergoing regression (less than 1%), and this phase is no longer than 2 weeks. The remaining population of 5 to 15% is in resting phase (Price VH, 1999). The telogen stage typically lasts for 2 to 3 months before the scalp follicles reenter the anagen phase. At the end of telogen, hair is released and shed, and the next cycle is initiated. It is estimated that the number of scalp hairs is approximately 100,000. Thus, about 50-150 hairs are normally shed from the scalp each day and about same number of follicles enter anagen. The cycle of active growth and rest is regulated by complex messages between the epithelium and the mesenchymal cells that are not yet well understood (Hardy MH, 1992). Cotsarelis *et al.* have found that follicular stem cells reside in the bulge area, and proposed bulge activation hypothesis to explain the importance of epithelial-mesenchymal interaction on hair growth. They have postulated that sometime during telogen and or early anagen, the normally slow cycling bulge cells are activated by the DP cells that are in close proximity to the bulge at that time. The nature of cell-cell interaction and the specific factors elaborated by the DP cells are not presently known. Activation results in the proliferation of the bulge

cells, which form down growth of epithelial cells that eventually give rise to new matrix. This hypothesis explains the nature of hair growth cycle well, since the regression is restricted to the lower part of hair follicle and the upper part above bulge area is permanently protected hair growth cycle as well as even physical trauma like plunking.

4. Molecular regulation of hair growth

4.1 Growth factors

Hair grows actively in anagen phase at which the matrix keratinocytes robustly proliferate and move upwardly, then become hair fiber. Although the precise regulatory mechanism of hair growth cycle is still unclear, it has been well known that interactions between the DP and overlying follicular epithelium are critical for hair growth control (Hardy MH, 1992). Especially, it is believed that DP-secreted molecules directly affect the matrix proliferation. One of important molecules that may have a direct effect on epithelial matrix proliferation is insulin-like growth factor (IGF-1). Evidences have shown that IGF-1 is expressed in the DP and their receptor has been characterized from the matrix cells of follicle bulbs (Hodak E *et al*., 1996). Moreover, IGF-1 receptor protein is differentially expressed in the matrix through the hair growth cycle (Rudman SM *et al*., 1997). Another important growth factor that exerts its important role in hair growth cycle is fibroblast growth factor-7 (FGF-7). It is produced

by DP and it receptor is found in the anagen follicle matrix (Rosenquist TA *et al*., 1996). The molecular nature governing the transition between each stages of hair growth cycle is poorly understood. However, it is suggested that fibroblast growth factor-5 has an important role in the cessation of anagen phase. Hebert *et al.*(Hébert JM *et al*., 1994) demonstrated that FGF-5 null mice had an extended anagen stage, resulting in the angora phenotype, with hair that is 50 percent longer than normal mice. Besides these factors, a lot of growth factors and cytokines have been implicated in the regulation of hair growth (Stenn KS *et al*., 1996). The precise roles of these molecules in hair growth, however, remain largely to be elucidated.

4.2 Androgen and 5α-reductase

The most striking alteration of skin in maturing from childhood to adult is the change of body hair. After puberty, the conversion of short and thin hair (vellus hair) into long and thick hair (terminal hair) takes place. Androgen stimulate hair growth in beard and pubis, but can cause regression and balding on the scalp (Stenn KS *et al*., 1996). It is commonly thought that the responses of hair follicle to androgens are very complex because there is familial and racial variation as well as the differences related body sites (Randall VA *et al*., 1992). The effect of androgens on the hair growth is not fully understood, but one strong possibility is that androgens alter the paracrine factor produced by the DP cells and hence alter the cellular activity of the follicles. This hypothesis has a great deal of experimental

support. Androgen receptors have been localized in the dermal papilla of hair follicle (Sawaya ME *et al*., 1997), and the cultured dermal papilla cells from androgen-dependent follicles showed higher androgen receptor levels than those from non-balding scalp cells (Randall VA *et al*., T 1992). Therefore it is reasonable that the dermal papilla is an actual target site for androgen action. Although many growth factors and cytokines have been implicated in hair growth, factors altered by androgens have been rarely studied. Several evidences indicated that some molecules were positively regulated by androgens in dermal papilla cells, including insulin-like growth factor-1 (IGF-1), stem cell factor (SCF) and vascular endothelial growth factor (VEGF) (Itami S *et al*., 1995; Hibberts NA *et al*., 1996; Lachgar S *et al*., 1999). It is believed that these growth factors stimulated the growth of hair follicle in androgen dependent site such as beard. In contrast, it has been also reported that androgens affect the DP cells negatively. For example, Androgen-inducible transforming growth factor-β1 (TGF-β1) from balding dermal papilla cells inhibited epithelial cell growth (Inui S *et al*., 2002) and the expression of the protease nexin-1 from balding follicles is inhibited by androgen. Protease nexin-1 is a potent inhibitor of serine proteases such thrombin, urokinase and plasmin, and it is believed that its levels are well correlated with the ability of dermal papilla cells to support hair growth (Yu DW *et al*., 1995). These could act as papacrine signals between the DP cells and other follicular components and may play an

important role, as alterations in the size of the DP are well known to correlate with the size of the hair produced by the follicles (Sonoda T *et al*., 1999).Taken together, androgen action on hair growth is strongly believed to be mediated via the DP cell. However, the regulation of target genes by androgens in the DP cells remains largely to be elucidated.

In androgen target cells, circulating testosterone (T) enters the cell, often undergoes reduction by 5α-reductase to dihydrotestosterone (DHT), and binds to the androgen receptor. The activated DHT-receptor complex binds to androgen responsive element of target genes and interacts with other transcription factors (Pratt WB and Toft DO, 1997). Testosterone has a two fold lower affinity than DHT for the androgen receptor, while the dissociation rate of testosterone from the receptor is five-fold faster than of DHT. Two isozymes of 5α-reductase, termed type Ⅰ and type Ⅱ 5α-reductase, are present depending on body sites (Andersson S *et al*., 1991.). Type Ⅰ 5α-reductase is a predominant form in the liver and skin, mostly in the sebaceous gland, while type Ⅱ 5α-reductase is abundant in the prostate and genital skin (Kaufman KD, 1996). It has not yet been clearly demonstrated which type of 5α-reductase is crucial for hair growth (Ando Y *et al*., 1999). Because subjects with an inherited lack of type Ⅱ 5α-reductase, but normal type Ⅰ5α-reductase isoenzyme neither go bald nor grow a beard, a key role of the type Ⅱ 5α-reductase in the local control of hair growth was proposed. This hypothesis is well supported by the effect of finasteride, which inhibits type Ⅱ 5α-

reductase, slows or reverse the progression of androgenetic apopecia (Kaufman KD *et al*., 1998). In addition, finasteride has been recently approved for oral-drug in men with androgenetic alopecia by the Food and Drug Administration (FDA), However, there is another explanation that serum DHT, originating from tissues with high 5α-reductase type Ⅱ levels such as prostate, could exert systemic effects on skin and its appendages. This hypothesis is supported by the finding that topical finasteride has no effect in preventing the expression of genetic hair loss in men. Since hair growth and balding are very slow processes, which spread over a period of months or years, possible differences in androgen metabolizing enzymes with physiological effects on hair growth could be very small and difficult to detect. Thus, it is still regarded that the development of effective inhibitors for both types of 5α-reductase would be beneficial for treatment of male pattern baldness.

5. Treatment of hair loss

So far, two drugs have been approved for hair loss treatment by the food and Drug Administration (FDA), including finasteride and minoxidil. As previously mentioned, finateride is a competitive inhibitor of type Ⅱ 5α-reductase. Finasteride rapidly lowers serum DHT level by more than 60 percent and shows no androgenic, estrogenic and other steroidal effects (Kaufman KD *et al*., 1998). Although the effect of finasteride on hair loss

treatment is prominent, the use of finasteride is strictly limited for men and not recommended for women because there are possible side effects of abnormal development of fetus. In men, it is known that finasteride may trigger slight effects decreasing libido, erectile and ejaculatory dysfunction (Price VH, 1999). Minoxidil is a potassium channel opener and vasodilator, which has been developed to treat hypertension. However, it has been reported that the side effects of this drug include hair growth. Consequently, topical minoxidil has been developed to try to improve hair growth in men with alopecia areata and androgenetic alopecia. The precise mechanism of minoxidil is not well understood, but Li *et al.* (Li M, 2001) reported that minoxidil-induced hair growth is mediated by opening of ATP-dependent potassium channel in human follicular dermal papillae. Human hair follicles contain two forms of ATP-sensitive potassium channels such as SUR1/Kir6.2 and SUR2B/Kir6.1. When hair bulb tissues were examined separately, epithelial matrix expressed SUR1 and Kir6.2, whereas both dermal papilla and sheath exhibited SUR2B and Kir6.1. Especially, they have suggested that dermal papilla cells can respond to minoxidil via a complex mechanism involving SUR2B channels (Shorter K *et al.*, 2008). Recently, Han *et al.* (Han JH, 2004) reported that minoxidil has proliferative and anti-apoptotic effects on dermal papilla cells.

7. *Magnolia officinalis* and 4-O-methylhonokiol

The stem bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae) has been used as a traditional medicine for the treatment of gastrointestinal disorders, cough, and allergic diseases including bronchial asthma, in Korea, mainland China, and Japan (Song WZ *et al*., 1989). Chemical studies have revealed a variety of neo-lignans and alkaloids as constituents of the plant. These compounds were shown to display muscle relaxation, (Watanabe K *et al*., 1975) a central depressant effect, (Watanabe K *et al*., 1983) and antigastric ulcer, (Watanabe K, 1986) vasorelaxant, (Teng CM *et al*., 1990) antiallergic, (Hamasaki Y *et al*., 1999) antibacterial, (Bae EA *et al*., 1998) and neurotrophic activities (Fukuyama *et al*., 1992). Among various constituents of the ethanol extract of *Magnolia officinalis*, we have isolated a major compound (16.6%) identified as 4-O-methylonokiol. It has been reported that 4-O-methylonokiol has anti-inflammatory effect through inhibition of NF-κB (Oh JH *et al*., 2009) and neurite outgrowth activity (Lee YK *et al*., 2009). In addition, 4-O-methylonokiol exhibited an acetylcholinesterase (AChE) inhibitor property (Lee YK *et al*., 2009). However, the promotion effect of *Magnolia officinalis* and 4-O-methylonokiol on hair growth has not yet been reported.

8. Purpose

In the present study, we examined the promotion effect of the ethanol extract of *Magnolia officinalis*, as well as 4-O-methylonokiol (isolated neolignan from *Magnolia officinalis)*, on the growth of hair. We also investigated how the 4-O-methylhonokiol could inhibit the TGF-β1-induced keratinocyte growth arrest using human keratinocyte HaCaT cell.

Ⅱ. MATERIALS AND METHODS

1. Reagents

The following reagents were obtained commercially: Recombinant Human TGF-β1 was from R&D systems (Minneapolis. MN, USA); Minoxidil, Minoxidil sulfate, Tolbutamide were from Sigma (St. Louis, MO, USA); 5% Minoxidil (MINOXYL^{TM}) was from Hyundi pharm Co. Ltd (Cheonan, Chungnam, Korea); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), ERK1/2, phospho-pERK1/2, p38, phospho-p38, SAPK/JNK and phospho-SAPK/JNK were from Cell signaling Technology; TGF-β1, TGF-β2, PCNA, Smad2/3, Smad4 and monoclonal β-actin were from Sigma (St. Louis, MO, USA); p21 was from BD Biosciences (San Diego, USA); Sp1 was from Millipore (Billerica, MA, USA);HRP-conjugated goat anti-rabbit and horse anti-mouse IgGs were from Vector (Vector Laboratories, Burlingame, USA); Aprotinin, leupeptin, Nonidet P-40 were from Roche (Roche Applied Science, Indianapolis, IN); West-zol enhanced chemilumin, Western blotting detection reagent was from Intron (Intron Biotechnology, Korea).

2. Isolation of 4-methylhonokiol from *Magnolia officinalis*

The bark of *Magnolia officinalis* was dried in the shade at room temperature and stored in a

dark, cold room until use. The air-dried bark of *Magnolia officinalis* (3 kg) was cut into pieces and extracted twice with 95% (v/v) ethanol (four times as much as the weight of the dried plants) for 3 days at room temperature. After filtration through the 400-mesh filter cloth, the filtrate was filtered again through filter paper (Whatman Grade No.5) and concentrated under reduced pressure. The combined extract (450 g) was suspended in H_2O and the aqueous suspension was extracted with n-hexane, ethyl acetate, and n-BuOH, respectively. The n-hexane layer was evaporated to dryness to give a residue (70 g), which was chromatographed on silica gel with n-hexane: ethyl acetate (9:1) gradient to yield a crude fraction that included 4-O-methylhonokiol. This fraction was repeatedly purified by silicagel chromatography using n-hexane: ethyl acetate as the eluent to obtain pure 4-O-methylhonokiol (Fig. 1). 4-Omethylhonokiol was identified by ^{1}H-NMR and ^{13}C-NMR. ^{1}H-NMR(400 MHz, CDCl^{3}): d 3.36 (2H, d, J = 7 Hz, H-7), 3.44 (2H, d, J = 7 Hz, 70-H), 3.89 (3H, s, OMe), 5.05–5.14 (5H, m, H-9, H-90, OH), 5.93–6.07 (2H, m, H-8, H-80), 6.92 (1H, d, J = 7 Hz, Ar-H), 6.97 (1H, d, J = 8 Hz, Ar-H), 7.04–7.08 (2H, m, Ar-H), 7.24–7.31 (2H, m, Ar-H). 13C-NMR (100 MHz, CDCl3): d 34.5 (C-7), 39.6 (C-70), 55.8 (OMe), 111.2 (C-30), 115.7 (C-40), 115.8 (C-9), 116.1 (C-90), 128.0 (C-10), 128.1 (C-6), 129.0 (C-3), 129.2 (C-1), 130.0 (C-5), 130.4 (C-60), 130.7 (C-2), 132.4 (C-50), 136.7 (C-8), 138.0 (C-80), 151.0 (C-20), 157.2 (C-4). The ethanol extract of *Magnolia*

officinalis contained 16.6% 4-O-methylhonokiol, followed by 16.5% honokiol and 12.9% magnolol, and 42–45% others.

3. Animals

Male Wistar rats (3 weeks of age) were supplied from Orient Bio (Seongnam, Gyeonggi, Korea). 6-Week-old female C57BL/6 mice and Male Spargue-Dawley rats (8 weeks) were purchased from Dae-Han Biolink (Eumsung, Chungbuk, Korea) and provided with a standard laboratory diet and water *ad libitum*. All animals were cared for by using protocols (20070002) approved by the Institutional Animal Care and Use Committee (IACUC) of the Jeju National University.

4. Cell cultures

Rat vibrissa immortalized dermal papilla cell line (Filsell W *et al*., 1994) was donated by the Skin Research Institute, Amore Pacific Corporation R&D Center, South Korea. The dermal papilla cells and HaCaT cells were cultured in DMEM (Hyclone Inc, UT, USA) supplemented with 10% fetal bovine serum (Gibco BRL, NY, USA) and penicillin/streptomycin (100 unit/㎖ and 100 ㎍/㎖, respectively) at 37°C in a humidified atmosphere under 5% CO_2. On the other hand, NIH3T3 fibroblasts were obtained from the

American Type Culture Collection (ATCC, Manassaa, VA, USA). NIH3T3 were incubated in DMEM (ATCC, Manassaa, VA, USA) supplemented with 10% bovine calf serum (Hyclone, Logan, UT, U.S.A), 100 unit/㎖ penicillin and maintained at same conditions adopted for maintaining of dermal papilla cells.

5. Isolation and culture of rat vibrissa follicles

Isolation of rat vibrissa follicles was performed as described previously (Philpott MP and Kealey T, 2000). Briefly, rat vibrissa follicles were harvested from male Wistar rats that were 23 days old. To accomplish this, the rats were sacrificed under diethyl ether. Next, both the left and right mystacial pads were removed from the rats and placed in a 1:1 (vol/vol) solution of Earle's balanced salts solution (EBSS, Sigma, MO, USA) and phosphate buffered saline (PBS, Sigma, MO, USA) that contained 100 unit/㎖ of penicillin and 100 μg/㎖ of streptomycin. Anagen vibrissa follicles were then carefully dissected under a stereomicroscope (Olympus, Tokyo, Japan) from posterior parts of the mystacial pads with considerable care being taken to remove the surrounding connective tissue without damaging the vibrissa follicle. Using this method we were able to routinely isolate more than 40 follicles from each animal. The isolated follicles were then placed in separate wells in 24-well plates that contained 500 μL of Williams medium E (Gibco Inc, NY, USA)

supplemented with 2 mM L-glutamine (Gibco Inc, NY, USA), 10 μg/㎖ insulin (Sigma, MO, USA), 50 nM hydrocortisone (Sigma, MO, USA), 100 unit/㎖ penicillin and 100 μg/㎖ streptomycin at 37°C and cultivated in an atmosphere comprised of 5% CO_2 and 95% air. The isolated follicles were then treated with vehicle (DMSO diluted 1:1000 in Williams medium E) as a control, *M. officinalis* extract (0.01, 0.1 and 1μg/㎖) and 4-O-methylhonokiol (3, 30 and 300 nM). Minoxidil sulfate (Sigma, MO, USA) was used as a positive control in the culture systems (Buhl AE *et al.*, 1990). The culture medium was changed every 3 days and photographs of the cultured rat vibrissa follicles were taken using a stereomicroscope for 3 weeks. The length of the hair follicles was measured using a DP controller (Olympus, Tokyo, Japan).

6. Hair growth activity in vivo

Anagen was induced on the back skin of C57BL/6 mice that were in the telogen phase of the cycle by depilation, as described previously (Müller-Röver S *et al.*, 2001). Briefly, 6 week old female C57BL/6 mice were allowed to adapt to their new environment for one week. The anagen was then induced in the back skin of the seven week old female C57BL/6 mice by shaving, which led to synchronized development of anagen hair follicles. From the following day (day 1), 0.2 ㎖ of a 30 nM of 4-O-methylhonokiol in 50% ethanol was

topically applied on every day for 20 days. 5% Minoxidil (MINOXYL™; Hyundai Pharm. Co. Ltd., Cheonan, Chungnam, Korea) was used as a positive control. The back skin of the mice was then observed and photographed at 0, 10, and 20 days after shaving.

7. Hematoxylin and eosin staining

Back skins were collected from each group on days 0 and 20 of treatment. The Back skins were fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA), and the tissues were then dehydrated and embedded in paraffin, and sectioned at 3 μm for hematoxylin and eosin (H-E) staining. H-E staining methods are as follows: briefly, the slides were dipped into hematoxylin (Merk & Co., NJ, USA) for 5 min, then rising them with tap water, dipping them in eosin (Sigma-Aldrich, MO, USA) for 5 min, and again rinsing them with water. The slides were dipped in 95% ethanol for 30 sec, twice in 100% ethanol for 60 sec, and four times in 100% xylene for 5 min. The coverslips were finally mounted using Permount® (Fisher Scientific, PA, USA). The stained section were observed and photographed under a stereomicroscope (Olympus, Tokyo, Japan).

8. Immunohistochemistry

For immunohistochemistry, vibrissa follicles were collected from each group on days 0 and

7 of treatment. The vibrissa follicles were fixed in 4% paraformaldehyde (Sigma-Aldrich, MO, USA), and the tissues were then dehydrated and embedded in paraffin.

Immunohistochemistry was then performed according to the manufacturer's instructions (Santa Cruz Biotechnology, CA, USA). The following primary antibodies were used at the indicated concentrations: PCNA, TGF-β1, and TGF-β2 (1:200; Santa Cruz Biotechnology, CA, USA). In addition, the relevant goat secondary antibodies (1:200; Santa Cruz Biotechnology, CA, USA) were used for detection of the primary antibodies. The stained section were observed and photographed under a stereomicroscope (Olympus, Tokyo, Japan).

9. MTT Assay

The proliferation of dermal papilla cells was evaluated by measuring the metabolic activity using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay (Carmichael J *et al*., 1987). Briefly, dermal papilla at 1.0×10^4 cells/㎖ were seeded into 96-well plate, cultured 24 h in serum-free DMEM, and then treated with vehicle (DMSO diluted 1:1000 in serum-free DMEM) as a control, *M. officinalis* extract (0.01, 0,1 and 1 μg/㎖) and 4-O-methylhonokiol (3, 30 and 300 nM) for 4 days. NIH 3T3 cells plated to give 10^4 cells per ㎖ in 96-well plates. NIH 3T3 cells were incubated for 24 h with DMEM supplemented with 10% BCS to aid attachment, then washed 3 times with PBS and the medium containing

1.5% BCS was added, and then treated with vehicle (DMSO diluted 1:1000 in serum-free DMEM) as a control, 4-O-methylhonokiol (3, 30 and 300 nM), 75 uM Minoxidil, or 4-O-methylhonokiol (3, 30 and 300 nM) plus 5 mM Tolbutamide, 75 uM Minoxidil plus 5 mM Tolbutamide for 4 days. After incubation, 0.1 mg (50 μL of a 2 mg/㎖ solution) of MTT (Sigma, MO, USA) was added to each well, and the cells were then incubated at 37°C for 4 h. Next, the plates were centrifuged at 1000 rpm for 5 min at room temperature and the media was then carefully aspirated. 200 μL of dimethyl sulfoxide was then added to each well to dissolve the formazan crystals and the absorbance of the plates at 540 nm was then read immediately on a microplate reader (BioTek Instrument, Inc., VT, USA). All experiments were performed three times and the mean absorbance values were calculated. The results are expressed as the percentage reduction in the absorbance caused by treatment with the extract or the active component compared to that of the untreated controls.

10. RNA preparation and RT-PCR

Total RNA was extracted from cells by the Tri-Reagent (MRC, Cincinnati, USA) method following the manufacturer's instructions. The RNA extraction was carried out in an RNase-free environment. The reverse transcription of 1 ㎍ RNA was carried out using M-MuLV reverse transcriptase (Promega, WI, USA), oligo $(dT)_{15}$ primer, dNTP (0.5 μM) and 1 U

RNase inhibitor. After incubation at 70℃ for 5 min, 25℃ for 5 min, 37℃ for 60 min, and M-MuLv reverse transcriptase was inactivated by heating at 70℃ for 15 min. The polymerase chain reaction (PCR) was performed in a reaction buffer [cDNA, 1.25 U Taq DNA polymerase (Promega, WI, USA), 3'and 5' primer 50 μM each and 200 mM dNTP in 20 mM Tris-HCl buffer, pH8.4, containing 500 mM KCl and 1-4 mM $MgCl_2$] The PCR was performed with a C1000^{TM} Thermal cycler (BIO-RAD, HC, USA), and the amplification was followed by 35 cycles of 94℃ for 30 sec (denaturing), 60℃ for 30 sec for (annealing) and 72℃ for 30 sec (extension). The PCR products were electrophoresed on a 1.2% agarose gel. The nucleotide sequence of each primers and the size of product were shown in Table 2.

11. Cell cycle analysis

HaCaT keratinocytes (1.0×10^5 cells/㎖) were pre-incubated for 24 h, and then washed 3 times with PBS, cultured 48 h in serum-free DMEM. Serum starvation-arrested cells were stimulated by 10% FBS without or with TGF-β1 (10 ng/㎖) and in the absence or presence of 4-O-methylhonokiol. For the flow cytometric analysis to determine cell cycle phase distribution, the treated cell were harvested, washed twice with PBS, and fixed in 70% ethanol for 30 min at 4℃. Cells were then with PBS and incubated in propidium iodide at a final concentration of 50 ug/㎖ and RNase A at a final concentration of 50 ug/㎖ in the dark

30 min at 37℃. Flow cytometry analysis was performed using a FACScan Flow Cytometer (Becton-Dickinson, San Jose, USA). Histograms were analyzed with the software program Cell Quest (Becton-Dickinson, San Jose, USA) (Krishan A, 1975).

12. Measurement of intracellular reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) were analyzed by flow cytometry using the oxidation-sensitive probes 2',7'-dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE). HaCaT keratinocytes (1.0×10^5 cells/㎖) were seeded on 35-mm dishes and incubated for 24 h, then washed 3 times with PBS, cultured 24 h in serum-free DMEM. After serum starvation for 24 h, HaCaT cells treated with TGF β1 (10 ng/㎖) for different times. Specially, cells were preincubated with 4-O-methylhonokiol (3, 30 and 300 nM) for 60 min before treatment with TGF-β1 (10 ng/㎖). After stimulation for 1 h, the cells were washed with PBS and incubated in the medium containing DCFH-DA (10 μM) or DHE (10 μM) for 30 min at 37 °C with 5% CO_2 in the dark. HaCaT cells were washed twice with PBS and Intracellular accumulation of fluorescence DCF-DA or DHE were measured (10,000 cells each) using a FACScan Flow Cytometer (Becton-Dickinson, San Jose, USA). For DCF-DA assay, excitation was at 488 nm with an emission at 540 nm. For DHE assay, excitation was at 535 nm with an emission at 610 nm. Histograms were analyzed with the

software program Cell Quest (Becton-Dickinson, San Jose, USA).

13. Superoxide Dismutase (SOD) Activity

The HaCaT cells were seeded at 1×10^5 cells/㎖, and sixteen hours after plating, the cells were treated with various concentrations of 4-O-methylhonokiol for 1 h. The harvested cells were suspended in 10 mM potassium phosphate buffer (pH 7.5) and then lysed on ice by sonication twice for 15 sec. Triton X-100 (0.1%) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifugated at 5000×g for 30 min at 4°C to remove the cellular debris. The protein content of the supernatant was determined by Bradford method (Bradford LW, 1976), with bovine serum albumin as the standard. The SOD activity was used to detect the inhibited level of auto-oxidation of epinephrine. (Misra HP *et al.*, 1972). Fifty ug of the protein was added to 50 mM potassium phosphate buffer (pH 10.2) containing 0.1 mM EDTA and 0.4 mM epinephrine. Epinephrine rapidly undergoes auto-oxidation at pH 10 to produce adrenochrome, a pink colored product, which can be measured at 480 nm using a UV/VIS spectrophotometer in kinetic mode. SOD inhibits the auto-oxidation of epinephrine. The rate of inhibition was monitored at 480 nm. The SOD activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required for 50% inhibition of auto-oxidation of

epinephrine.

14. Catalase (CAT) Activity

Fifty ug of protein was added to 50 mM potassium phosphate buffer (pH 7) containing 100 mM (v/v) H_2O_2. The reaction mixture was incubated for 2 min at 37°C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of H_2O_2 (Carrillo MC *et al.*, 1991). The CAT activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required to breakdown of 1 mM H_2O_2.

15. Assay of rat prostatic 5α-reductase

Male SD rats (8 weeks) were killed by diethyl ether. The ventral prostates of rats were dissected free of their capsules, washed with saline, and stored at -80℃. Frozen tissues were thawed on ice and procedures were carried out at 4℃. The tissues were homogenized with Polytron homogenizer (Brinkman instruments, Wesrbury, NY, USA) in 5-6 tissue volumes of medium A (0.32 M sucrose, 1 mM DTT, 0.2 mM PMSF, and 20 mM potassium phosphate buffer pH 6.6). The homogenates were centrifuged at 1500 g for 20 min, The pellets were separated, washed with three tissue volumes of medium A and centrifuged two additional

times at 400 g at 0℃ for 10 min. The washed pellets were suspended in medium A and stored at -80℃ until use. The suspension (2.5 mg protein/㎖ for Rat prostates, determined by the Bradford method) was used as source of 5α-reductase.

5α-reductase activities were assayed as previously described (Hirosumi J *et al.,* 1995). The reaction mixture contained a final volume of 500 μL: 1mM DTT, 40 mM potassium phosphate buffers, 2 mM NADPH, Testosterone including 120 n Ci [1,2,6,7-^{3}H]. The reaction in triplicate was started when it was added to the rat prostatic enzyme fraction (250 ug protein), 0.2% DMSO as a control, 4-O-methylhonokiol (3, 30 and 300 nM), and Finasteride (MERCK SHARP & DOHME, Australia) was used as a positive control. The mixture was incubated at 37℃ for 60 min, and the reaction was stopped by the addition of 1 ㎖ of ethyl acetate and mixing for 1 min. After centrifugation (1000 g for 5 min at 22℃), the organic phase was removed, dried under heating plate, dissolved in 50 μL of ethyl acetate containing 500 ug/㎖ of testosterone and 500 ug/㎖ DHT and applied to a silica gel 60 F254 TLC plate (Merck, Darmstadt, Germany), which was developed in a solvent system consisting of ethyl acetate: cyclohexane (1:1). The plate was air dried. The testosterone was visualized under the UV lights (254 nm) and DHT was detected using 10% H_2SO_4 solution with a posterior heating of the plate. DHT develops a classical dark yellow color. Androgen containing areas were cut off and the strips were soaked in the 10 ㎖ of ULTIMA GOLD™

Cocktails (PerkinElmer, Massachusetts, USA) and radioactivities were counted in the Liquid scintillation analyzer (Packard Bioscience, Meriden, USA). The conversion activity of 5α-reductase was expressed as the ratio [DHT/(T+DHT)] x 100

16. Western blot analyses

After incubation, the cells were harvested and washed twice with cold-PBS. The cells were lysed in a lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM $NaVO_3$, 10 mM NaF, 1 mM dithiothreitol, 1 mM Phenylmethylsulfonylfluoride, 25 ug/㎖ aprotinin, 25 ug/㎖ leupeptin, 1 mM DTT, 1% Nonidet P-40) to obtain whole cell protein and kept on ice for 30 min. The cell lysates were centrifuged at 15,000 rpm at 4°C for 15 min. Supernatants were stored at -20°C until analysis. In some experiments, nuclear fractions were prepared from the cells, using NE-PER nuclear extraction reagents (Pierce Biotechnology, Rockford, USA) by following the manufacture's instructions. Protein concentration was determined by Bradford method (Bradford LW, 1976). Equal amounts of protein were loaded onto a SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) gel. After electrophoretic separation, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-rad, HC, USA) with a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.8), 20% MeOH (v/v)) at 120 V for 1.5 h. After blocking with 1% bovine serum albumin (BSA) in TBS-Tween (TBS-T) (50 mM Tris, pH

7.6, 150 mM NaCl, 0.1% Tween-20), the membrane was incubated with specific primary antibodies. The primary antibodies used in this study were as follows (see Table 2). Primary antibody incubation was followed by washing in 0.1% Tween-20 TBS solution and then incubating with a secondary HRP antibody (1:5000; Vector Laboratories, Burlingame, USA) at room temperature. A chemiluminescence reaction (ECL, Intron Biotechnology, Korea) was used to visualize protein bands on X-ray films (AGFA, Belgium). All blots were probed with β-actin to confirm that equal amounts of protein were loaded.

17. Statistical analyses

The hair growth data are expressed as the mean of the follicle lengths ± the standard errors (S.E.) of at least three independent experiments performed in triplicate. The Student's t-test was used to determine the statistical significance (P-value<0.05) of the differences between the values for the various experimental and control groups.

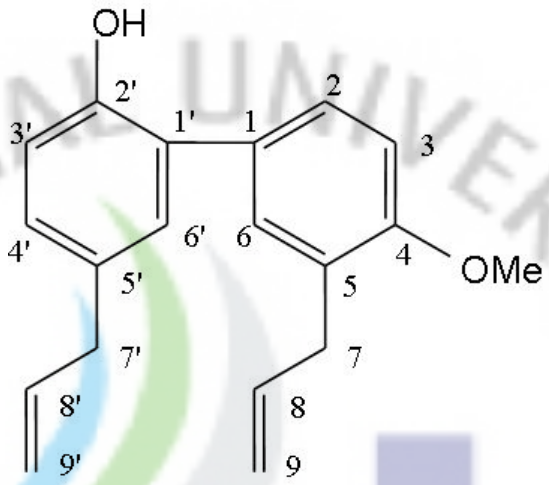


Figure 1. The Structure of 4-O-methylhonokiol isolated from *M. officinalis*

Table 1. Antibodies used in Western blot analyses.

Antibody	Origin	Company
TGF-β1	rabbit polyclonal	Santa Cruz Biotechnology
TGF-β2	〃	〃
PCNA	〃	〃
p21	mouse monoclonal	BD Biosciences
ERK1/2	rabbit polyclonal	Cell signaling Technology
phospho-ERK1/2	〃	〃
p38/MAPK	〃	〃
phospho-p38/MAPK	〃	〃
SAPK/JNK	〃	〃
phospho-SAPK/JNK	mouse monoclonal	〃
Sp1	rabbit polyclonal	Millipore
Smad2/3	goat polyclonal	Santa Cruz Biotechnology
Smad4	mouse monoclonal	Santa Cruz Biotechnology
β-actin	〃	Sigma

Table 2.The sequences of primers used in RT-PCR analysis and the sizes of RT-PCR products

Gene (type)	Primer sequences	Fragment Size (bp)
NOX1	F 5'-GTACAAATTCAGTGTGCAGACCAC-3'	420
	R 5'-CAGACTGCAATATCGGTGACAGCA-3'	
NOX2	F 5'-GGAGTTTCAAGATGCGTGGAAACTA-3'	550
	R 5'-CAGACTGCAATATCGGTGACAGCA-3'	
NOX4	F 5'-CTCAGCGGAATCAATCAGCTGTG-3'	251
	R 5'- AGAGGAACACGACAATCAGCCTTAG-3'	

Ⅲ. RESULTS

1. The Hair Growth Promoting Effect of 4-O-methylhonokiol from *Magnolia officinalis*

1.1 The effect of *M. officinalis* extract on rat vibrissa follicle elongation.

To determine if *M. officinalis* induced hair growth, we examined the activity of *M. officinalis* extract using an organ culture of the rat vibrissa follicle. When rat vibrissa follicles were treated with various concentrations of *M. officinalis* extract for 2 weeks, the hair-fiber length of the vibrissa follicles was significantly increased in a dose-dependent manner with respect to the control (Figure 2). In particular, in the vibrissa follicle that was treated with 0.1 μg/㎖ of *M. officinalis* extract for 14 days, the vibrissa follicles were 211.5±37.3% longer (P<0.05) than those in the control group. These results indicate that *M. officinalis* extract is capable of promoting hair growth.

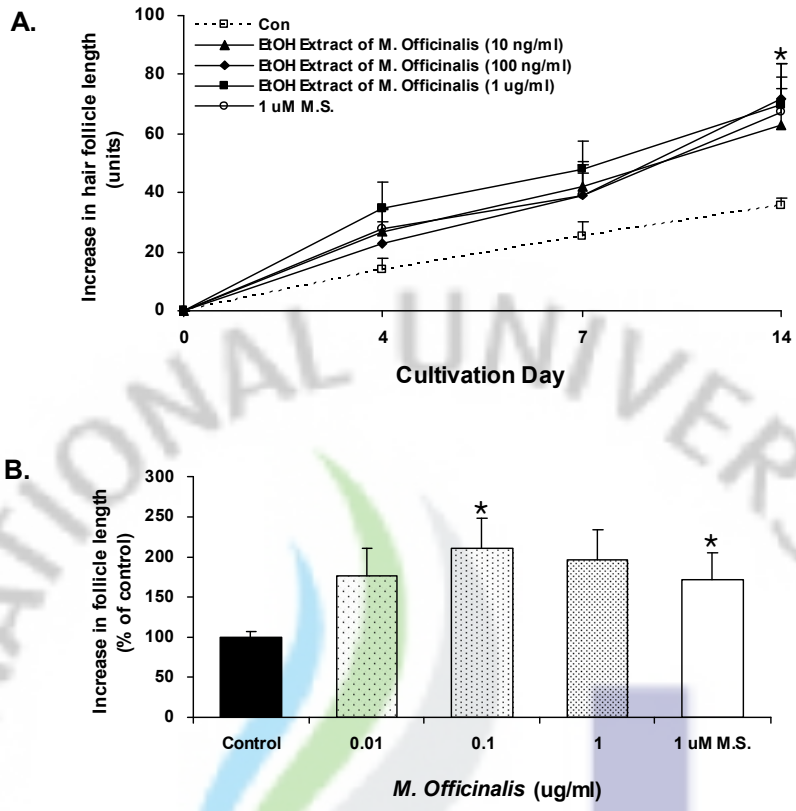


Figure 2. Hair growth effect of 95% EtOH extract from *M. officinalis* on rat vibrissa follicles.

Individual vibrissa follicles from Wistar rats were microdissected and then cultured in William's E medium at 37°C under 5% CO_2. Vibrissa follicles were then treated with 0.01, 0.1 and 1 ug/㎖ of 95% EtOH extract from *M. officinalis* for 14 days. Stimulation with minoxidil sulfate (M.S.) served as a positive control. All experiments were performed in triplicate. (A) The mean length of follicles in the control and treated groups on day 0 were taken to be 0 units. Data are presented as unites of the length of follicles on day 4, 7 and 14

based on the length of the follicles on the day 0 ± the S.E. (B) The difference in the length of vibrissa follicles of the control group on day 14 was taken to be 100%. Data are presented as the percentage of the length of the treated follicles based on the mean length of the control follicles ± the S.E. Vibrissa follicles treated with *M. officinalis* showed a significant increase in hair-fiber length when compared with the control. The hair-fiber length of vibrissa follicles that were treated with *M. officinalis* was greater than those of the positive control. *$P<0.05$ vs. Control.

1.2. The effect of 4-O-methylhonokiol from *M. officinalis* on the promotion of hair-growth

We examined whether 4-O-methylhonokiol, a main component of *M. officinalis,* has a hair growth promoting activity. As shown in Figure 3, when rat vibrissa follicles were treated with 4-O-methylhonokiol at 3, 30 and 300 nM for 14 days, 4-O-methylhonokiol significantly increased the growth of hair fiber by 142.9±39.5% (*P<0.05)* at a dose of 3 nM, 253.5±43.1% (*P<0.05*) at a dose of 30 nM and 154.2±25.9% at a dose of 300 nM compared with the control group. These results indicated that 4-O-methylhonokiol was capable of promoting hair growth.

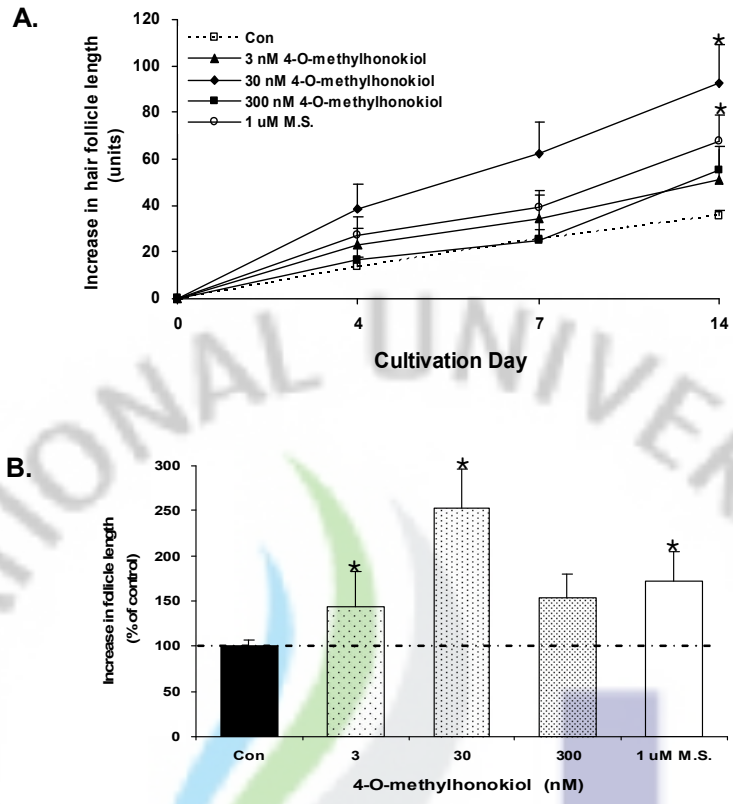


Figure 3. Hair growth effect of 4-O-methylonokiol from *M. officinalis* on rat vibrissa follicles.

Individual vibrissa follicles from Wistar rats were microdissected and then cultured in William's E medium at 37°C under 5% CO_2. Vibrissa follicles were then treated with 3, 30 and 300 nM of 4-O-methylhonokiol for 14 days. Stimulation with minoxidil sulfate (M.S.) served as a positive control. All experiments were performed in triplicate. (A) The mean length of follicles in the control and treated groups on day 0 were taken to be 0 units. Data are presented as unites of the length of follicles on day 4, 7 and 14 based on the length of the

follicles on the day 0 ± the S.E. (B) The difference in the length of vibrissa follicles of the control group on day 14 was taken to be 100%. Data are presented as the percentage of the length of the treated follicles based on the mean length of the control follicles ± the S.E. Vibrissa follicles treated with 4-O-methylhonokiol showed a significant increase in hair-fiber length when compared with the control. The hair-fiber length of vibrissa follicles that were treated with 4-O-methylhonokiol was greater than those of the positive control. *$P<0.05$ vs. Control.

1.3. The effect of 4-O-methylhonokiol on anagen induction in C57BL/6 mice.

To investigate whether anagen induction was promoted by 4-O-methylhonokiol, we used C57BL/6 mice. C57BL/6 mouse dorsal hair is known to have a time-synchronized hair growth cycle. Shaved skin of telogen mice is pink and darkens along with anagen initiation. After being shaved, the skin color of the mice was observed to be pink. As shown in Figure 4, 5% MINOXYL™ almost exhibited gray skin at 8 days (data not shown) post-hair growth induction and their hair shafts were visible at 10 days. 4-O-methylhonokiol groups remained pink until day 10 and exhibited gray skin by day 12 (data not shown). The hair shafts of 4-O-methylhonokiol groups were first visible on days 15 (data not shown) to 17. On the 20th day, the back skin was in anagen phase in the mice, whereas the hair that grew back on the back skin of the control groups was clearly less pigmented. Histologic studies showed that 4-O-methylhonokiol markedly increased the depth and size of hair follicles as compared with control group (Figure 5). Overall, these results indicate that 4-O-methylhonokiol induced early telogen-to-anagen conversion of hair follicles in the C57BL/6 mice.

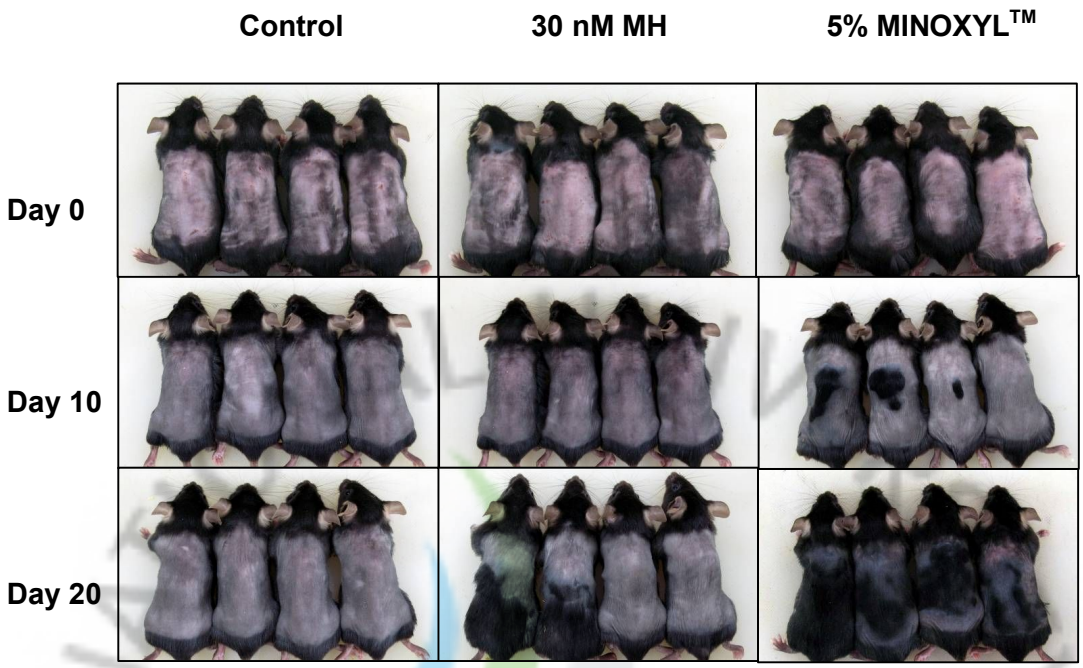


Figure 4. The effect of 4-O-methylhonokiol on anagen induction in C57BL/6 mice.

C57BL/6 mice were shaved when 7 weeks old (Day 0), then 30 nM of 4-O-methylhonokiol or 5% MINOXYL™ as a positive control were topically applied daily for 20 days. The back skins were photographed at 0, 10 and 20 days after shaving. Skin darkness was increased in 4-O-methylhonokiol treated mice, whereas that of control mice was less affected.

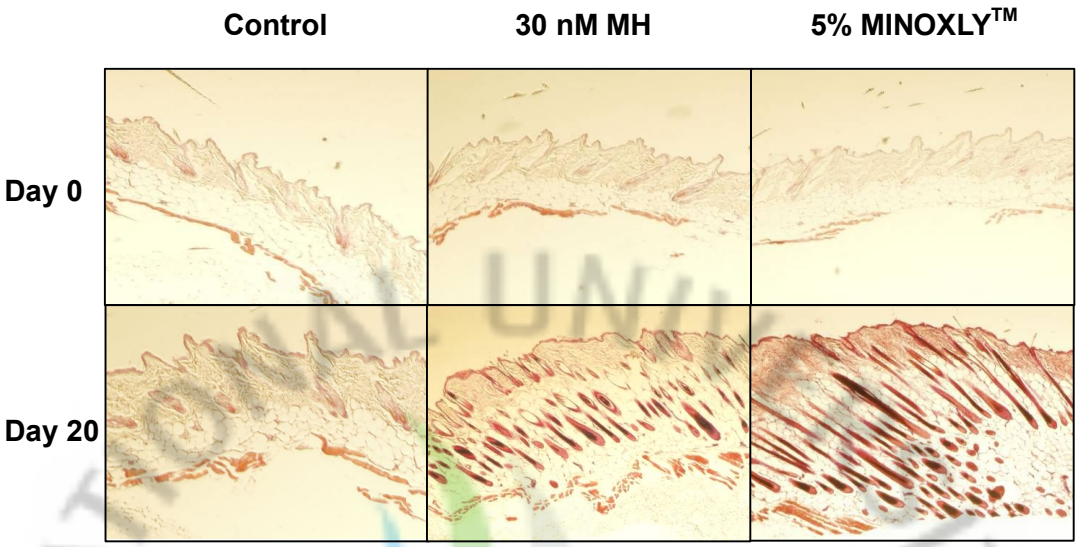


Figure 5. Histologic features of hair follicles resulting from treatment with 4-O-methylhonokiol.

C57BL/6 mice were shaved when 7 weeks old (Day 0), then 30 nM of 4-O-methylhonokiol or 5% MINOXYL™ as a positive control were topically applied daily for 20 days. Tissue sections were stained with haematoxylin and eosin staining. In 4-O-methylhonokiol-treated mouse, whole skin thickness and size of hair follicles are markedly increased. In addition, the location of hair follicles is deeper than that of control mouse.

1.4. The effect of 4-O-methylhonokiol on cell proliferation of hair follicles

To evaluate the effect of 4-O-methylhonokiol on cell proliferation of hair follicles, proliferation of dermal papilla cells and the expression of PCNA were examined. Immortalized rat vibrissa dermal papilla cells were treated with various concentrations of 4-O-methylhonokiol and the mitogenic effect on the dermal papilla cells was examined. 4-O-methylhonokiol promoted the proliferation of dermal papilla cells at a concentration of 0.03 uM ($P<0.05$) compared with the vehicle-treated control. However, 0.3 and 3 uM of 4-O-methylhonokiol did not affect the proliferation of dermal papilla cells (Figure 6). These results suggest that the hair growth promoting effect of 4-O-methylhonokiol may be mediated through a mitogenic effect on dermal papilla cells.

The isolated rat vibrissa follicles were treated with 4-O-methylhonokiol and then examined for activation of PCNA (Figure 7). In the anagen vibrissa follicles (0 day), the expression of PCNA was positively stained in the bulb region, whereas the 7-day cultured vibrissa follicles, which were expected to be in the anagen-catagen transition phase, were negatively stained. The vibrissa follicles treated with 30 nM of 4-O-methylhonokiol for 7 days were positively stained in the bulb regions. In addition, the bulb regions of the vibrissa follicles that were treated with 1 μM of minoxidil sulfate for 7 days were positive for PCNA. These results indicate that the cells in the bulb regions of follicles treated with 4-O-methylhonokiol or

minoxidil sulfate were induced to grow (Figure 7).

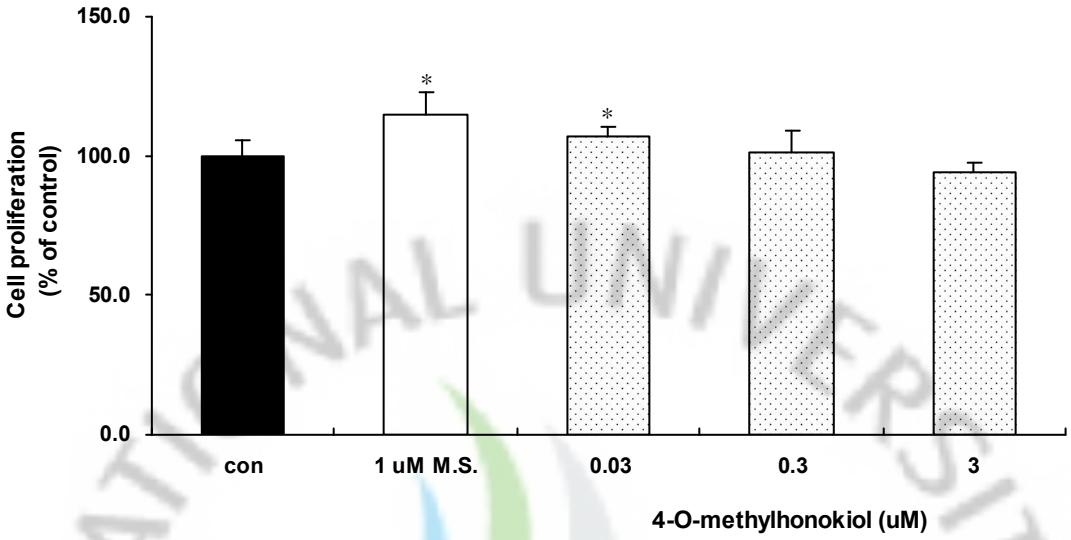


Figure 6. Proliferation effect of 4-O-methylhonokiol on cultured dermal papilla cells.

Rat dermal papilla cells (1.0×10^4 cells/㎖) were plated in 96 well plates. Dermal papilla cells were treated with various concentration of 4-O-methylhonokiol or 1uM minoxidil sulfate, as indicated. Cell proliferation was measured using a MTT assay for 5 days. All experiments were performed in triplicate. Data are presented as the mean ± the S.D. *P<0.05 vs. control.

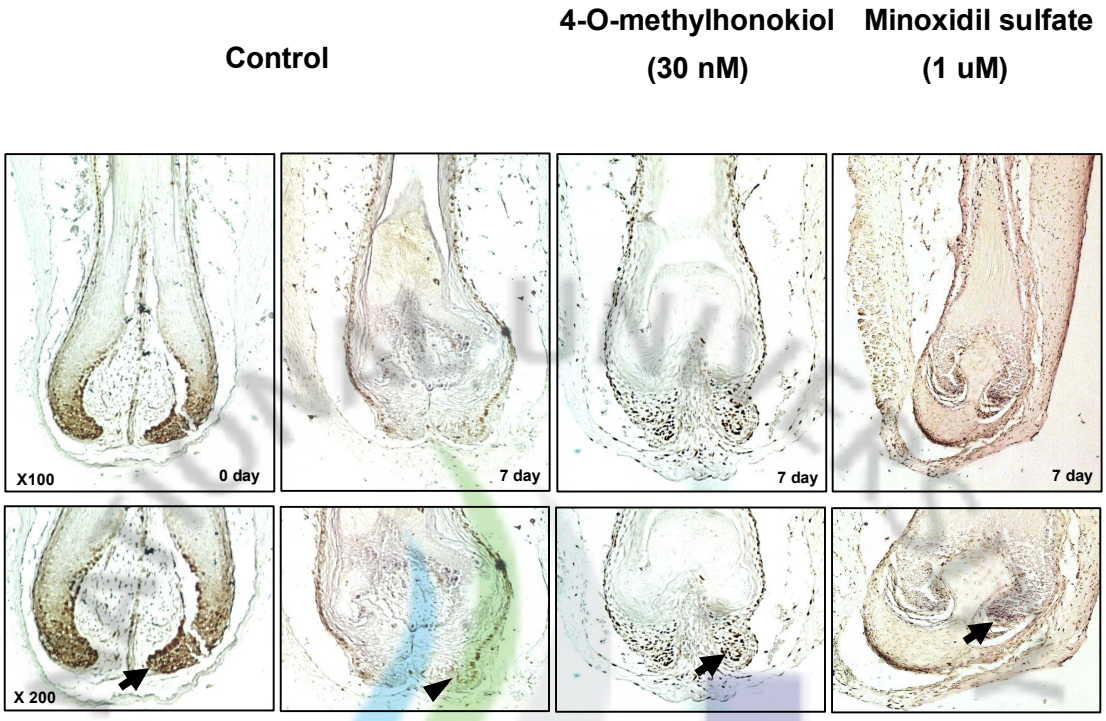


Figure 7. The effect of 4-O-methylhonokiol on PCNA expression in vibrissa follicles.

Vibrissa follicles were treated with 4-O-methylhonokiol or minoxidil sulfate for 7 days. Sections of the vibrissa follicles were stained with anti-PCNA antibody. In anagen vibrissa follicles (0 day), the expression of PCNA was positively stained in the bulb region (indicated by *arrow*). The 7-day cultured vibrissa follicles that were expected to be in the anagen-catagen transition phase were negatively stained (indicated by *arrowhead*), whereas the vibrissa follicles treated with 30 nM of 4-O-methylhonokiol for 7 days were positively stained in the bulb region (indicated by *arrow*). In addition, the bulb regions of the vibrissa follicles treated with 1 μM of minoxidil sulfate (M.S.) for 7 days were positive for PCNA (indicated by *arrow*).

1.5. Inhibitory effect of 4-O-methylhonokiol on the activity of prostatic 5α-reductase

The steroid 5α-reductase is considered to be one of the most important targets for developing drugs for treatment of hair loss. Therefore, we examined whether 4-O-methylhonokiol has an inhibitory activity on steroid 5α-reductase. As shown in Figure 8, 4-O-methylhonokiol inhibited steroid 5α-reductase activity by 6.1% at 0.3 nM, 10.8% at 3 nM, 23.7% at 30 nM and 10.7% at 300 nM compared with the control group.

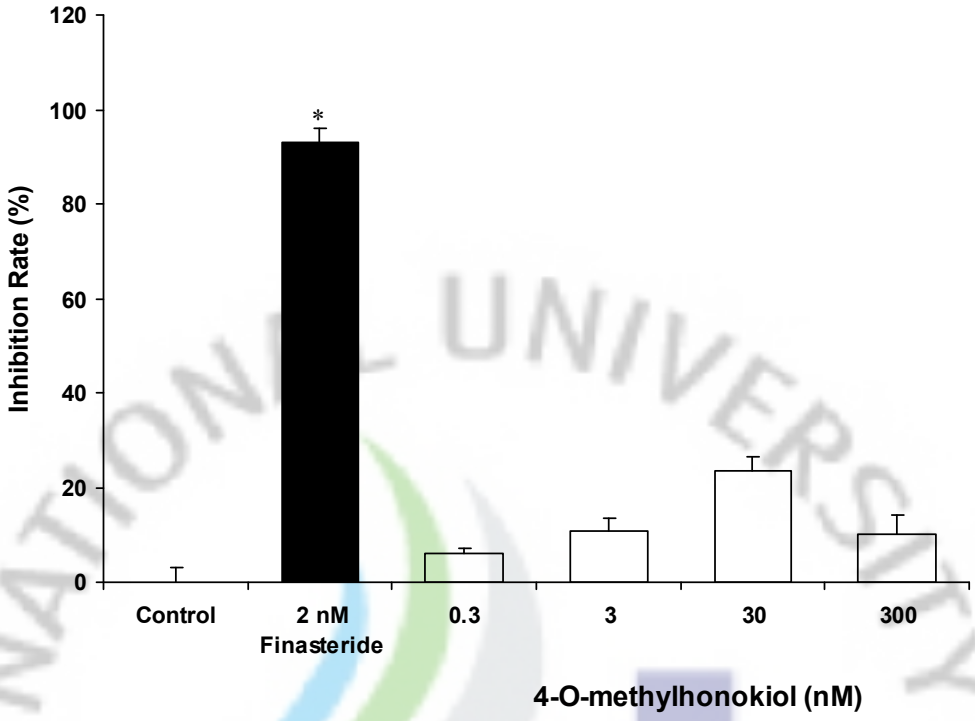


Figure 8. Effect of 4-O-methylhonokiol on prostatic 5α-reductase activity.

Steroid 5α-reductase inhibition was measured using prostatic homogenate of Sprague-Dawley rat. After enzyme reaction finished, steroids were extracted, separated on TLC plastic sheet, and then developed in ethyl acetate/cyclohexane. Inhibition ratio in the conversion of testosterone to dihydrotestosterone (DHT) was calculated and expressed as a percentage of control. Each column represents the average of three experiments.

1.6. Proliferation effect of 4-O-methylhonokiol on cultured NIH3T3 fibroblast cells

Several studies have examined the effect of minoxidil on cell proliferation *in vitro*. Sanders *et al*. proposed that the variable results of cell culture experiments may be explained by the potassium channel-blocking activity of aminoglycoside antibiotics, routinely incorporated into cell culture media. Minoxidil stimulated growth of NIH 3T3 fibroblasts cultured in the absence of aminoglycosides but not in their presence, and the proliferative response of 3T3 cells to minoxidil was prevented by the potassium channel blockers such as tolbutamide and tetraethylammonium. We investigated whether 4-O-methylhonokiol could stimulate NIH3T3 fibroblasts cell growth by opening ATP-dependent potassium channels. When NIH3T3 fibroblasts cell were treated with 4-O-methylhonokiol at 3, 30 and 300 nM, 4-O-methylhonokiol did not affect the proliferation of NIH3T3 fibroblasts (Figure 9.).

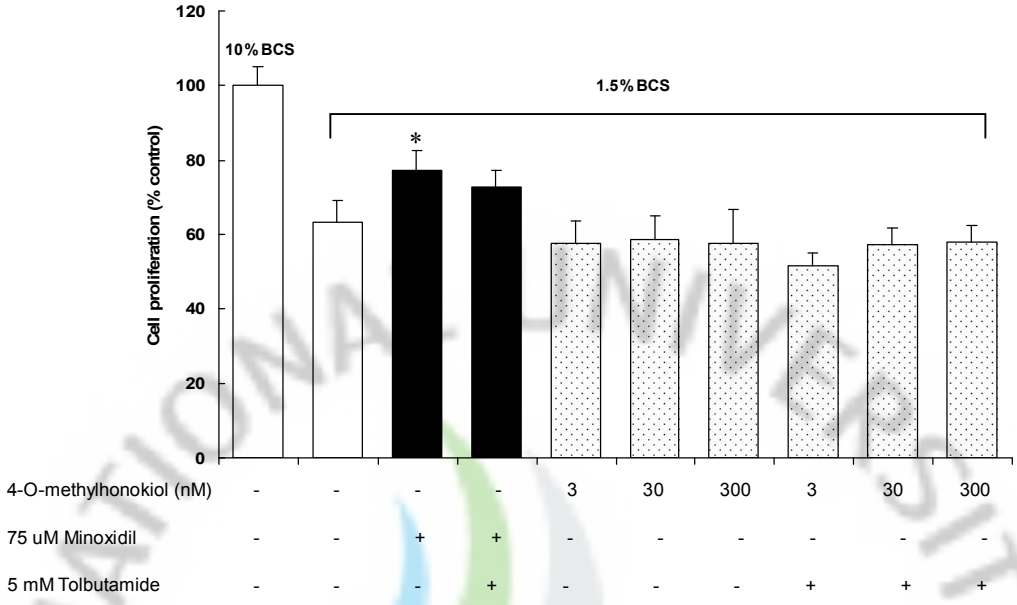


Figure 9. Proliferation effect of 4-O-methylhonokiol on cultured NIH 3T3 fibroblast cells.

NIH 3T3 fibroblasts (1.0×10^4 cells/㎖) were grown in medium containing 1.5% BCS in the absence of streptomycin. NIH 3T3 fibroblasts were treated with various concentration of 4-O-methylhonokiol, 75 uM minoxidil, or 5 mM tolbutamide. Cell proliferation was measured using a MTT assay for 4 days. Data are expressed as a percentage the control growth in 10% BCS after 4-d culture and are show as mean ± the S.D. of three experiments performed in triplicate. Statistical significance was determined using the Student t test and is compared to both growth in 1.5% BCS alone and growth in 1.5% BCS puls minoxidil. *P<0.05 significance compared to growth in 1.5% BCS.

1.7. The effect of 4-O-methylhonokiol on TGF-β1 and TGF-β2 expression in rat vibrissa follicles

In order to determine the mechanism by which 4-O-methylhonokiol promotes hair growth, we examined the expression levels of transforming growth factor-β1 (TGF-β1) and TGF-β2 which have been known to play an important role in anagen to catagen transition via the induction of keratinocyte apoptosis. When the vibrissa follicles in the anagen phase were treated with 4-O-methylhonokiol for 7 days, the expressions of TGF-β1 in cells of outer root sheath (ORS) and epithelial strand, i.e., in the epithelial residue of the regressing hair bulb during catagen were found to be lower than those of the control follicles that were expected to be in the anagen-catagen transition phase. 4-O-methylhonokiol also decreased the expression of TGF-β2 in the bulb matrix region of the 7-day cultured follicles. These results suggest that 4-O-Methylhonokiol has the potential to promote hair growth via down regulation of TGF-β1 and TGF-β2 (Figure 10, 11).

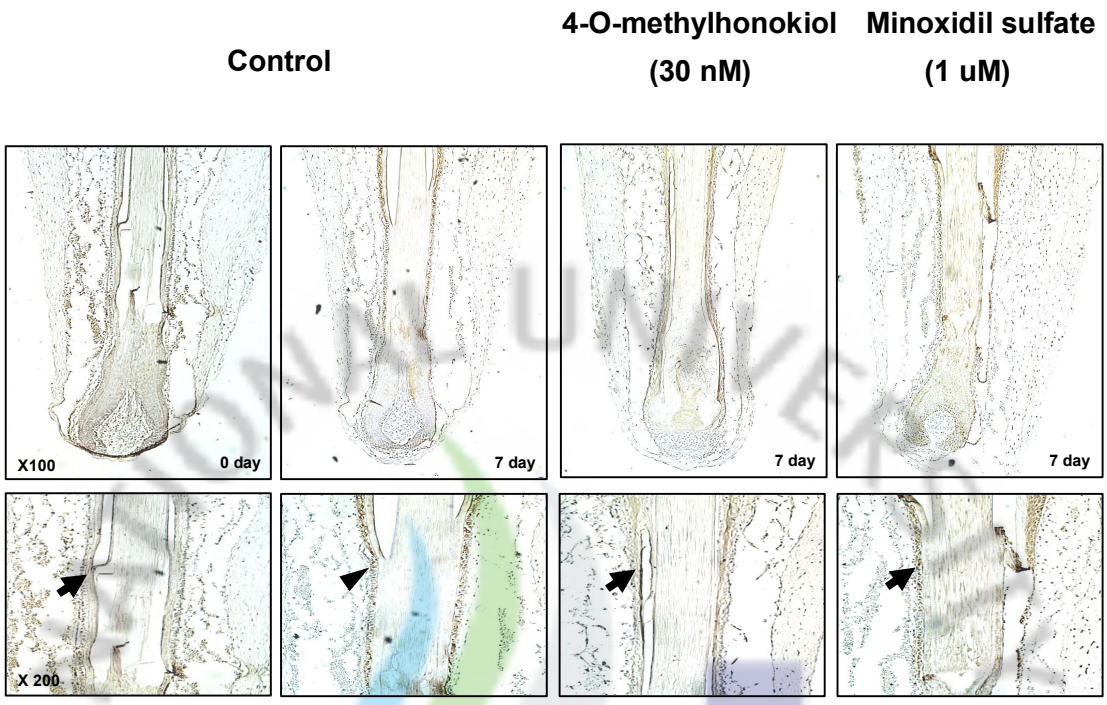


Figure 10. The effect of 4-O-methylhonokiol on TGF-β1 expression in vibrissa follicles.

The vibrissa follicles were treated with 4-O-methylhonokiol or minoxidil sulfate for 7 days. Sections of the vibrissa follicles were then stained with anti-TGF-β1 antibody. In the vibrissa follicles on day 0, anagen follicle, most cells were negative for TGF-β1 in the outer root sheath (ORS) and epithelial strand, i.e., in the epithelial residue of the regressing hair bulb (indicated by *arrow*). After 7 days, the cultured vibrissa follicles, which were expected to be in catagen phase, were positive for TGF- β1 (indicated by *arrowhead*), whereas the vibrissa follicles treated with 30 nM of 4-O-methylhonokiol for 7 days were negative for TGF-β1 in the outer root sheath (ORS) and epithelial strand (indicated by *arrow*). In addition, the outer

root sheath (ORS) and epithelial strand of the vibrissa follicles treated with 1 μM of minoxidil sulfate for 7 days were negative for TGF-β1 (indicated by *arrow)*.

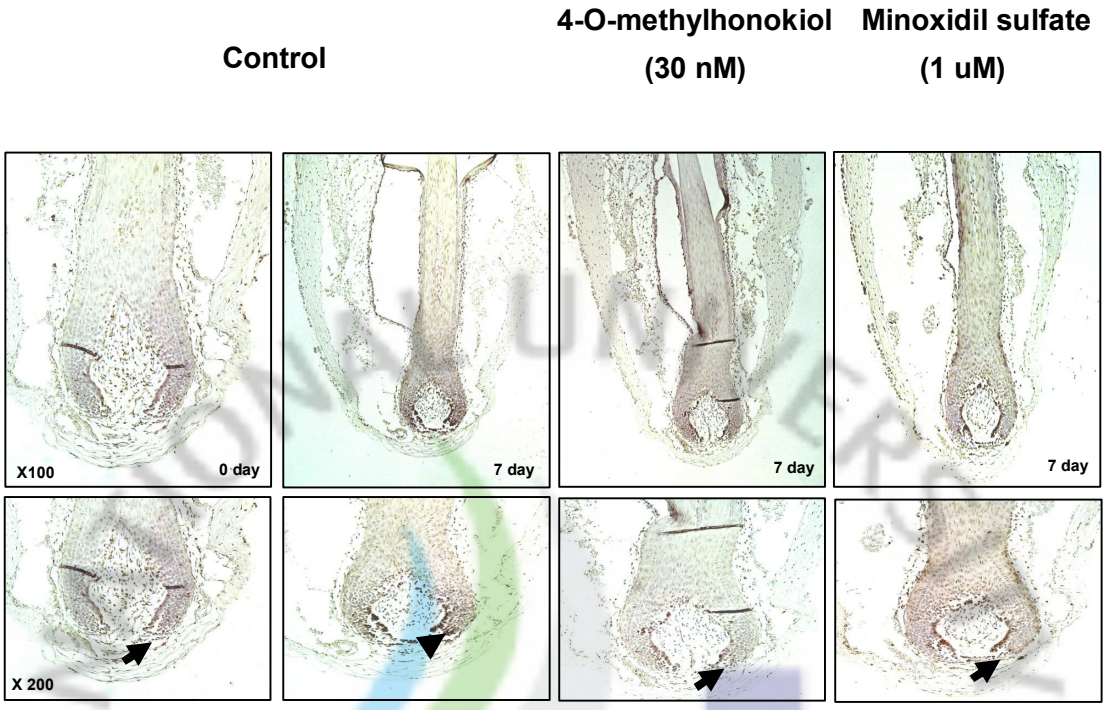


Figure 11. The effect of 4-O-metylhonokiol on TGF-β2 expression in vibrissa follicles. The vibrissa follicles were treated with 4-O-metylhonokiol or minoxidil sulfate for 7 days. Sections of the vibrissa follicles were then stained with anti-TGF-β2 antibody. In the vibrissa follicles on day 0, anagen follicle, most cells were negative for TGF-β2 in the bulb region (indicated by *arrow)*. After 7 days, the cultured vibrissa follicles, which were expected to be in catagen phase, were positive for TGF- β2 (indicated by *arrowhead*), whereas the vibrissa follicles treated with 30 nM of 4-O-metylhonokiol for 7 days were negative for TGF-β2 in the bulb region (indicated by *arrow*). In addition, the bulb regions of the vibrissa follicles treated with 1 μM of minoxidil sulfate for 7 days were negative for TGF-β2 (indicated by *arrow*).

2. Inhibitory Effect of 4-O-methylhonokiol from *Magnolia Officinalis* on TGF-β1-induced Cell Cycle Arrest in a Human Keratinocyte Cell Line (HaCaT)

2.1. 4-O-methylhonokiol blocked TGF-β1-induced G_1 arrest in HaCaT cell.

To examine whether 4-O-methylhonokiol promotes cells from G_0-G_1 phase to S phase by blocking TGF-β1-induced G_1 arrest, we performed by flowcytometric analysis by propidium iodide staining of HaCaT cells. As shown in Figure 12D, TGF-β1 (10 ng/㎖) arrested cells in the G_0-G_1 phase (74.2%) of the cell cycle. Cell treated with 4-O-methylhonokiol at 3, 30 and 300 nM before TGF-β1 (10 ng/㎖) treatment resulted in a decrease the percentage of G_1 phase cells to 68.3%, 64.5% and 58.2%, respectively.

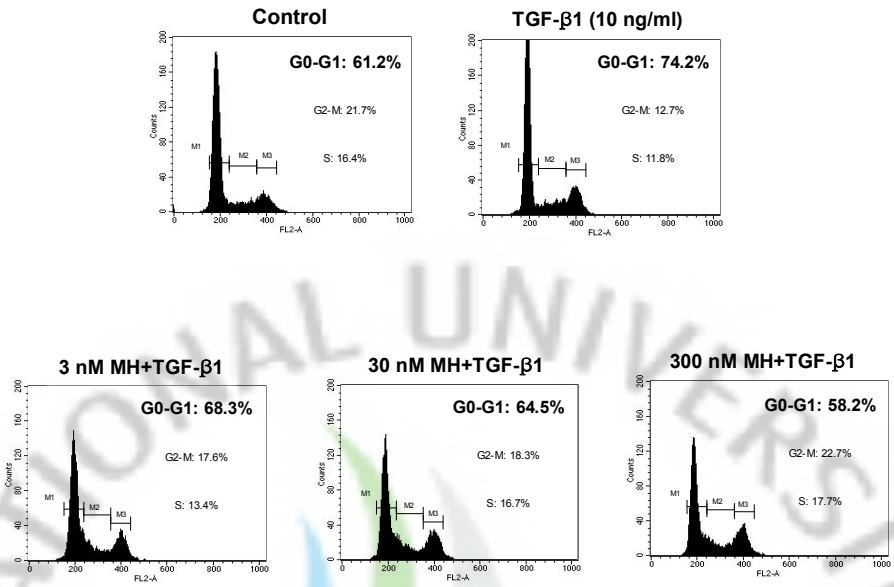


Figure 12. The effect of 4-O-methylhonokiol on TGF-β1-induced G_1 arrest in HaCaT cell.

After serum starvation for 48 h, HaCaT were pretreated with 4-O-methylhonokiol at final concentrations of 3, 30 and 300 nM for 1h, and then treated with TGF-β1 (10 ng/㎖) for 24 h. cellular DNA was stained with PI staining solution and analyzed by flowcytometry. Each experiment was repeated three times.

2.2. 4-O-methylhonokiol inhibited TGF-β1-induced p21 expression in HaCaT cell.

Previous studies have shown that p21 is an important cell cycle regulator and is induced by TGF-β1. The induction of p21 by TGF-β1 plays a causative role in TGF-β1-mediatied inhibition of cell growth (Moustakas A *et al*., 2002). We confirmed that TGF-β1 treatment led to an increase in the expression level of p21 in human keratinocytes HaCaT cells in a time-dependent manner (Figure 13A.). We examined 4-O-methylhonokiol effect on TGF-β1-induced p21 expression. HaCaT cells were pretreated with 4-O-methylhonokiol at final concentrations of 3, 30 and 300 nM for 60 min, and then treated with 10 ng/㎖ of TGF-β1 for 24h h. As shown in Figure 13B, 4-O-methylhonokiol treatment dose-dependently decreased p21 expression.

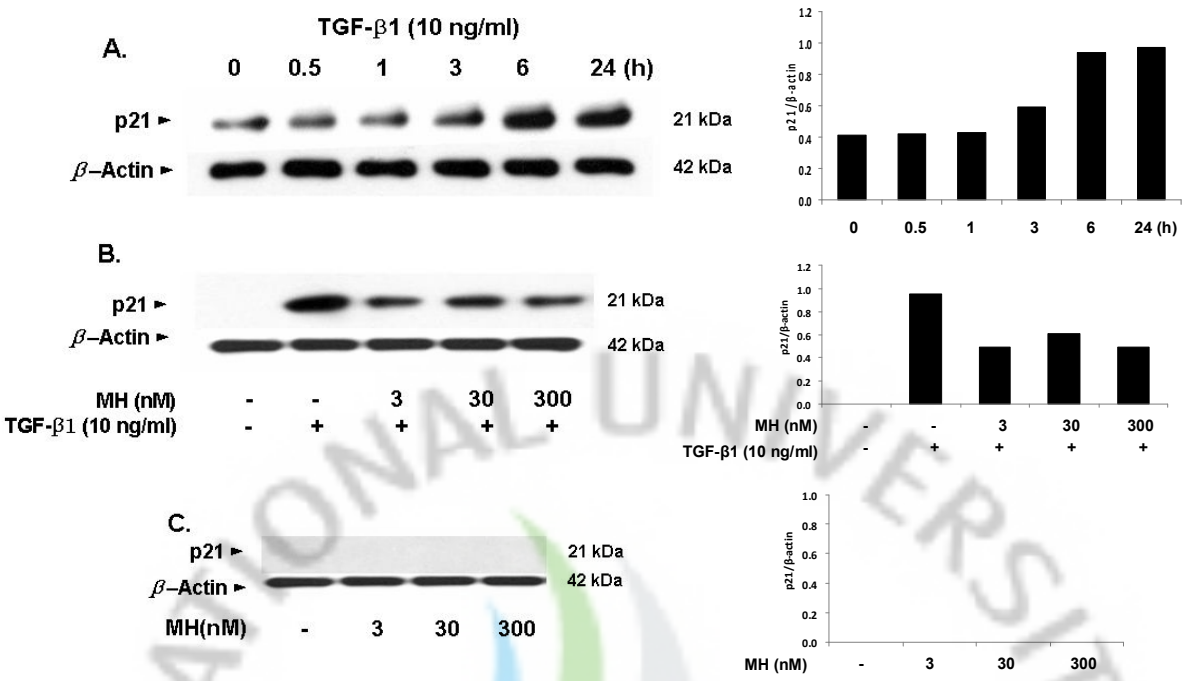


Figure 13. The effect of 4-O-methylhonokiol on TGF-β1-induced p21 expression in HaCaT cell.

(A) HaCaT cells were treated with TGF-β1 (10 ng/㎖) for the indicated times. Whole lysates (30 ug proteins) were examined by 10% SDS-PAGE and analyzed with Western blotting using for $p21^{WAF1/Cip1}$. (B) After pretreatment with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, HaCaT cells were treated with TGF-β1 (10 ng/㎖) for another 24 h. The expression level of p21 was analyzed by Western blotting. (C) HaCaT cells were incubated with 4-O-methylhonokiol (3, 30 and 300 nM) for 24 h, and the expression level of p21 was analyzed. Blots were then probed with the corresponding antibodies after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Each experiment was repeated three times.

2.3. 4-O-methylhonokiol inhibited nuclear translocation of Smads by TGF-β1.

It has been previously reported that Smads were translocated into the nucleus compartment in response to TGF-β1 stimulation, which was essential for induction of TGF-β1-target gene (Massague J *et al.*, 2000). To determine the effect of 4-O-methylhonokiol on TGF-β1-mediated nuclear translocation of Smad proteins, we analyzed the cytoplasmic and nuclear fractions after treating HaCaT cells with TGF-β1 and/or 4-O-methylhonokiol. As shown in Figure 14, TGF-β1 induced nuclear translocation of Smad2/3, and Smad4 within 60 min, which was blocked by 4-O-methylhonokiol. 4-O-methylhonokiol alone did not show any effect on the localization of Smad proteins. These results demonstrated that 4-O-methylhonokiol blocked the nuclear translocation of Smad2/3, and Smad4.

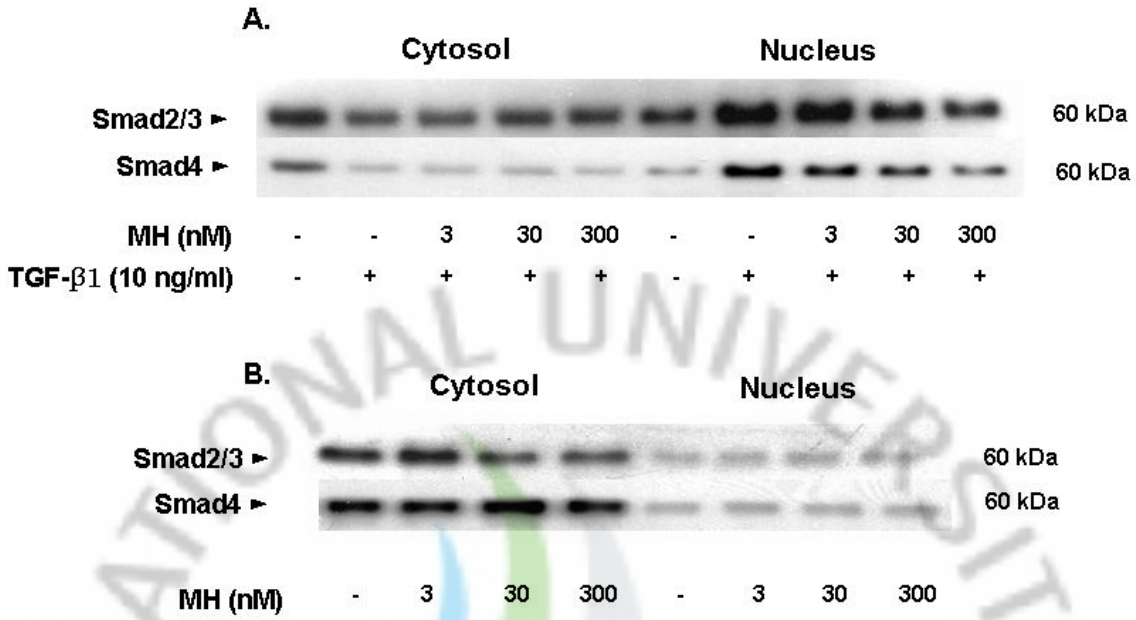


Figure 14. The effect of 4-O-methylhonokiol on TGF-β1-induced Smads translocation in HaCaT cell.

Nuclear translocation of Smad proteins was analyzed by separating cytoplasmic and nuclear fractions after treating HaCaT cells with TGF-β1 and/or 4-O-methylhonokiol for 1 h. Smad proteins were detected by Western blotting using antibodies against Smad2/3, Smad4, respectively.

2.4. 4-O-methylhonokiol inhibited TGF-β1-induced Sp1 activation in HaCaT cell.

It has been demonstrated that $p21^{WAF1/Cip1}$ induction by TGF-β1 was mediated through binding of Sp1 transcription factor to Sp1 sites in the region of promoter (Datto MB *et al*., 1995), and interaction between Sp1 transcription factor and Smad proteins was critical for the induction of TGF-β1 target genes (Pardali K *et al*., 2000). To determine if there was any effect of 4-O-methylhonokiol on the TGF-β1-induced Sp1 activation, HaCaT cells were pretreated with 4-O-methylhonokiol at final concentrations of 3, 30 and 300 nM for 1 h, and then treated with TGF-β1 (10 ng/㎖) for 1 h. Treatment of HaCaT cells with TGF-β1 (10 ng/㎖) increased the activation of Sp1 (Fig. 15B), while pretreatment with 4-O-methylhonokiol decreased the activation of Sp1 in a dose-dependent manner (Fig. 15B). However, 4-O-methylhonokiol alone did not affect TGF-β -induced Sp1 activation (Fig. 15C).

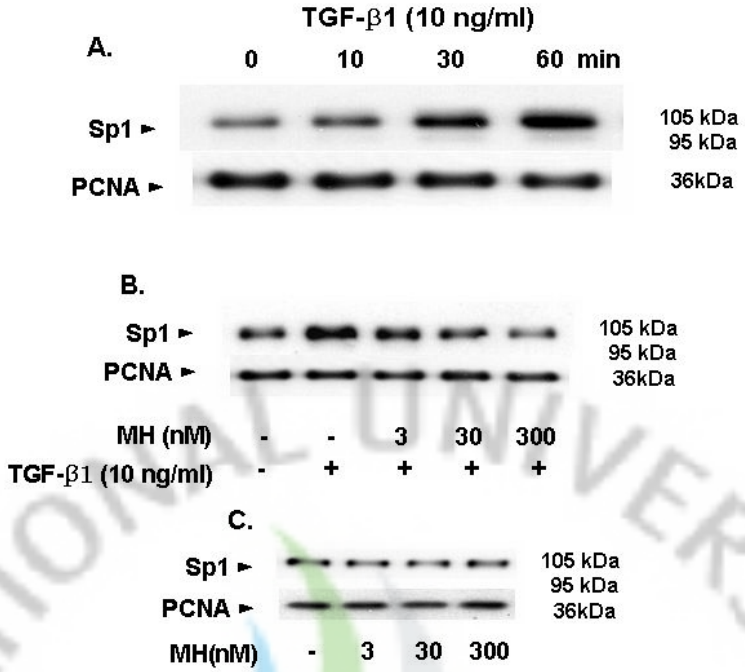


Figure 15. The effect of 4-O-methylhonokiol on TGF-β1-induced Sp1 activation in HaCaT cell.

(A) HaCaT cells were treated with TGF-β1 (10 ng/㎖) for the indicated times. Nuclear fractions were examined by 8% SDS-PAGE and analyzed with Western blotting using antibodies for Sp1. (B) After pretreatment with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, HaCaT cells were treated with TGF-β1 (10 ng/㎖) for another 1 h. The activation of Sp1 was analyzed by Western blotting. (C) HaCaT cells were incubated with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, and t the activation of Sp1 was analyzed. Blots were then probed with the corresponding antibodies after which they were stripped and reprobed with an antibody to PCNA to ensure equivalent loading and transfer. Each experiment was repeated three times.

2.5. Effect of MAPK inhibitors on TGF-β1-induced p21 expression in HaCaT cells.

It has been demonstrated that the MEK pathway is required for stimulation $p21^{WAF1/Cip1}$ induction by TGF-β1 (Hu pp *et al*, 1999). We examined the effect of U0126, a pharmacological inhibitor of MEK, on the induction of p21WAF1/Cip1 by TGF-β1. As shown in Figure. 16, pretreatment of human keratinocytes HaCaT cells with U0126 dramatically inhibited induction of $p21^{WAF1/Cip1}$ by TGF-β1. To investigate whether the other ERK family members, p38 and JNK, are involved in induction of $p21^{WAF1/Cip1}$ by TGF-β1, we next examined the effect of SB203580 and SP600125 which are specific inhibitor of p38 and JNK, respectively. Pretreatment of cells with these inhibitors did not affect the induction of $p21^{WAF1/Cip1}$ by TGF-β1 (Fig. 16). These data indicated that induction of $p21^{WAF1/Cip1}$ by TGF-β1 required the activation of ERK signaling pathway, but not of p38 or JNK signaling pathway.

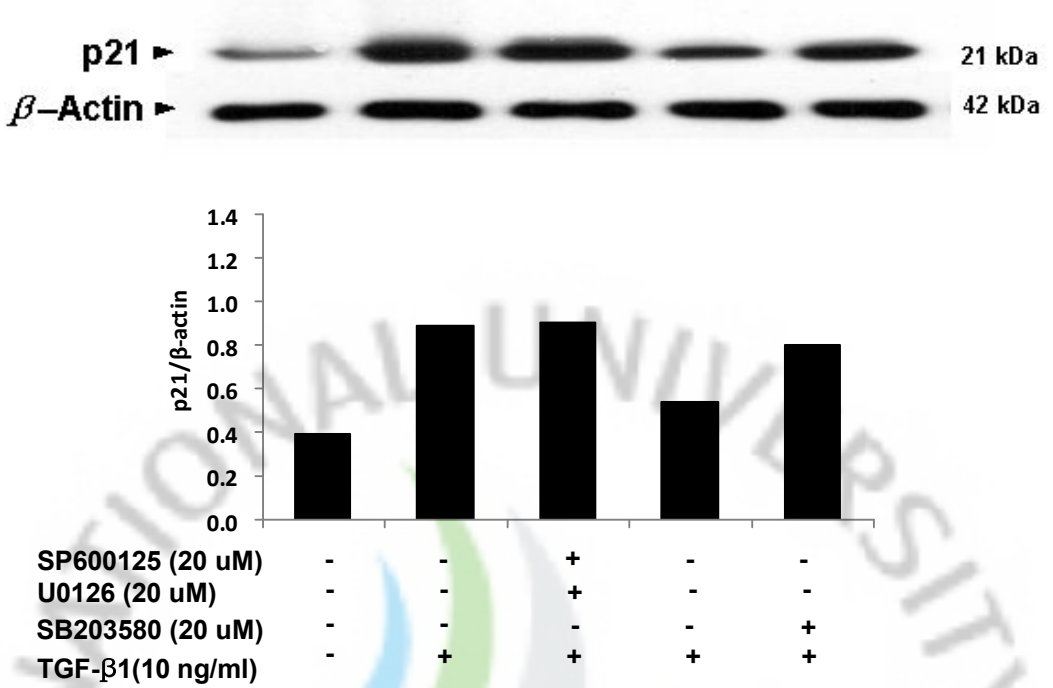


Figure 16. Effect of MAPK inhibitors on TGF-β1-induced p21 expression in HaCaT cells.

(A) HaCaT cells were pretreated with U0126 (20 uM), SB203580 (20 uM), and SP600125 (20 uM) for 1 h, and further incubated in the absence or presence of TGF-β1 (10 ng/ml) for 24 h. Whole lysates (30 ug proteins) of the HaCaT cells were examined by 10% SDS-PAGE and analyzed with Western blotting using antibodies for $p21^{WAF1/Cip1}$.

2.6. Effect of 4-O-methylhonokiol on TGF-β1-induced MAPKs activation in HaCaT cells.

Next, we determined if there were any effects of 4-O-methylhonokiol on the TGF-β1-induced activation of MAPK pathways. The increase in the phosphoryation of ERK following TGF-β1 treatment was apparent with maximum activity at 60 min, which was sustained longer than 120 min (Fig. 17A). However, TGF-β1 did not affect the phosphoryation of p38 and JNK (Fig.16B, C). 4-O-methylhonokiol decreased the phosphorylations of ERK (Fig. 17A).

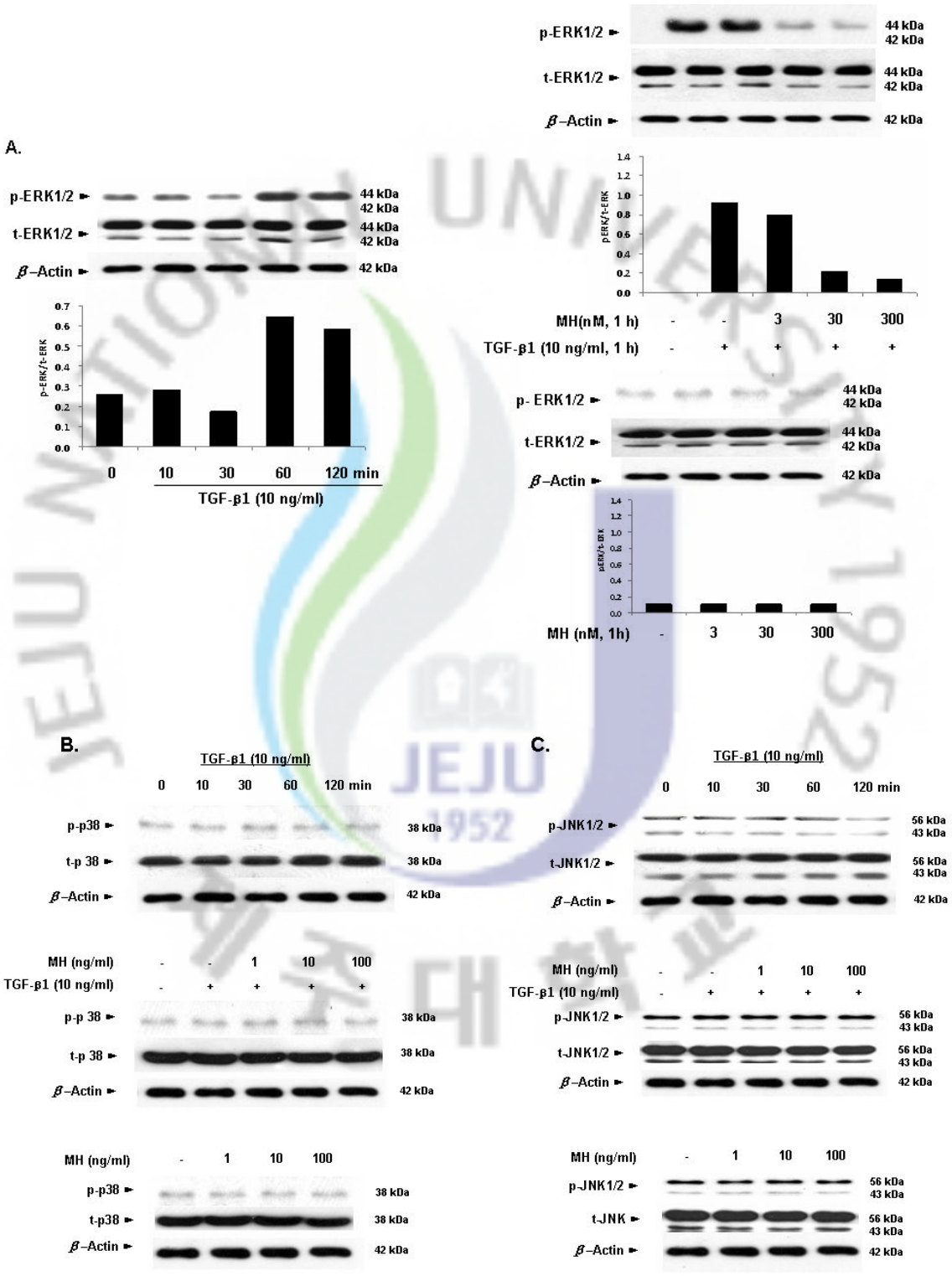


Figure 17. Effect of 4-O-methylhonokiol on TGF-β1-induced MAPKs activation in HaCaT cells.

After serum starvation for 24 h, HaCaT cells were stimulated with TGF-β1 (10 ng/㎖) for the indicated times. Whole lysates (30 ug proteins) were examined by 10% SDS-PAGE and analyzed with Western blotting using antibodies for phospho-ERK1/2 or ERK1/2 (A), phospho-p38 or p38 (B), phospho-JNK or JNK (C). After pretreatment with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, HaCaT cells were stimulated with TGF-β1 (10 ng/㎖) for another 1 h. Phosphorylations of ERK1/2, p38, and pJNK were analyzed by Western blotting.

2.7. Effect of 4-O-methylhonokiol on the TGF-β1-induced ROS production in HaCaT cells.

Recent accumulating evidences suggest that ROS is an important intracellular messenger to mediate the activation of various signaling molecules including ERK in various cells (Yoon Jiang Z *et al*, 2003; YS *et al*, 2005). We examined the effect of 4-O-methylhonokiol on the TGF-β1-induced ROS production. When HaCaT cells were incubated with TGF-β1 (10 ng/㎖) for 15, 30, 60 and 120 min, The increase of DCF fluorescence appeared from 30 min, with at maximal production at 60 min, which was slightly decreased at 120 min (Fig. 18A). However, when HaCaT cells were treated with TGF-β1 (10 ng/ml) for 60 min after pretreatment with 4-O-methylhonokiol, pretreatment of 4-O-methylhonokiol significantly reduced the TGF-β1-induced ROS generation (Fig. 18B). In addition, we also evaluated whether 4-O-methylhonokiol could block production of superoxide by TGF-β1. Figure 18C showed that the DHE fluorescence increased in TGF-β1-treated HaCaT cells. However, pretreatment of 4-O-methylhonokiol reduced the TGF-β1-induced ROS generation (included superoxide form) (Fig. 18D).

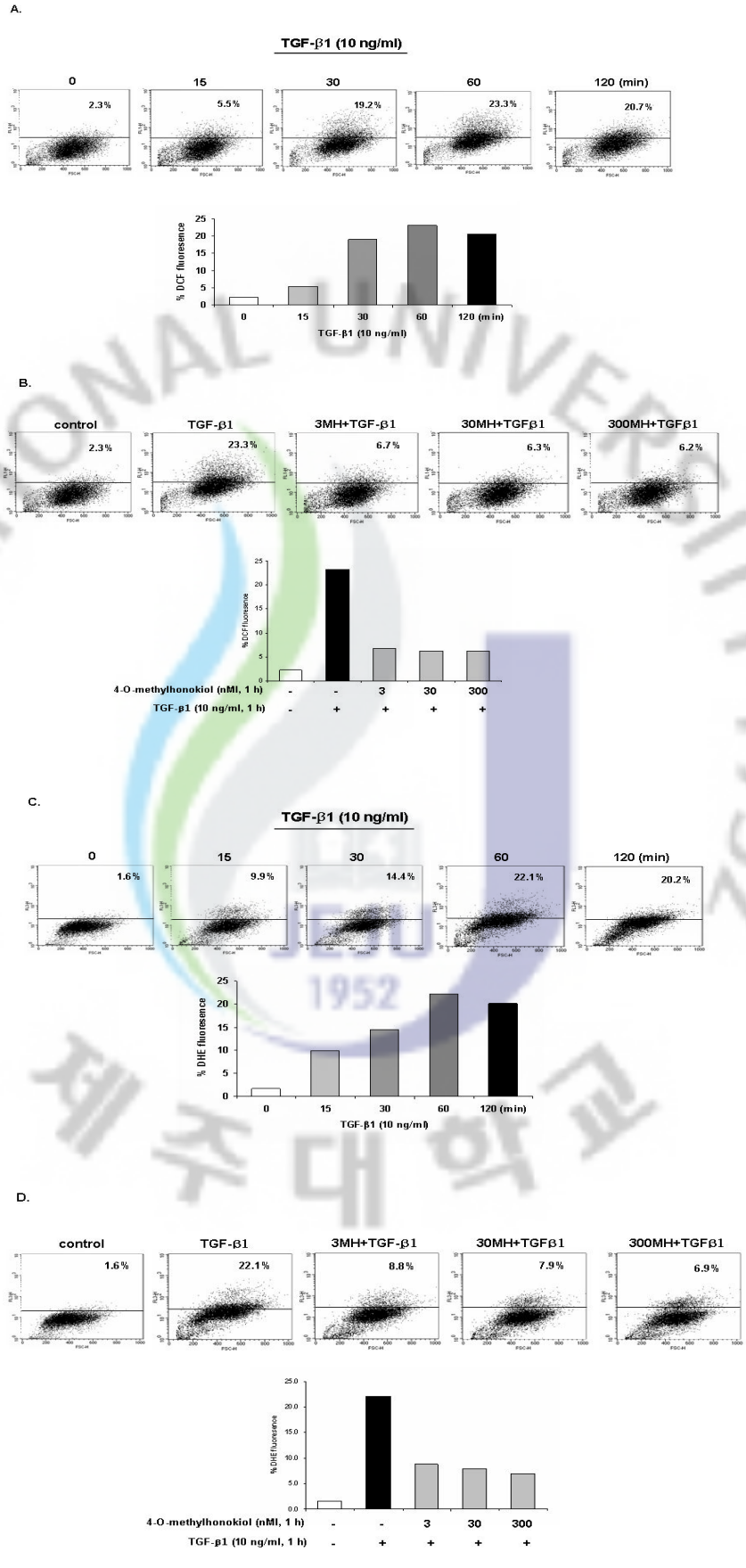


Figure 18. Effect of 4-O-methylhonokiol on the TGF-β1-induced ROS production in HaCaT cells.

(A) After incubation of serum-starved HaCaT cells in the presence of TGF-β1 (10 ng/㎖) for the indicated times, DCF fluorescence was measured with flow cytometry. (B) HaCaT cells were pretreated with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, and further incubated following treatment with TGF-β1 (10 ng/㎖) for 1 h. DCF fluorescence was measured with flow cytometry. (C) After incubation of serum-starved HaCaT cells in the presence of TGF-β1 (10 ng/㎖) for the indicated times, DCF fluorescence was measured with flow cytometry. (D) HaCaT cells were pretreated with 4-O-methylhonokiol (3, 30 and 300 nM) for 1 h, and further incubated following treatment with TGF-β1 (10 ng/㎖) for 1 h. DHE fluorescence was measured with flow cytometry.

2.8. Effect of 4-O-methylhonokiol on enzymatic and non-enzymatic antioxidant systems.

To examine whether 4-O-methylhonokiol could eliminate TGF-β1-induced ROS, we measured the DPPH free radical scavenging activities, the SOD and CAT activities. As shown in Figure 19, 4-O-methylhonokiol did not affect the DPPH free radical scavenging activities, the SOD and CAT activities.

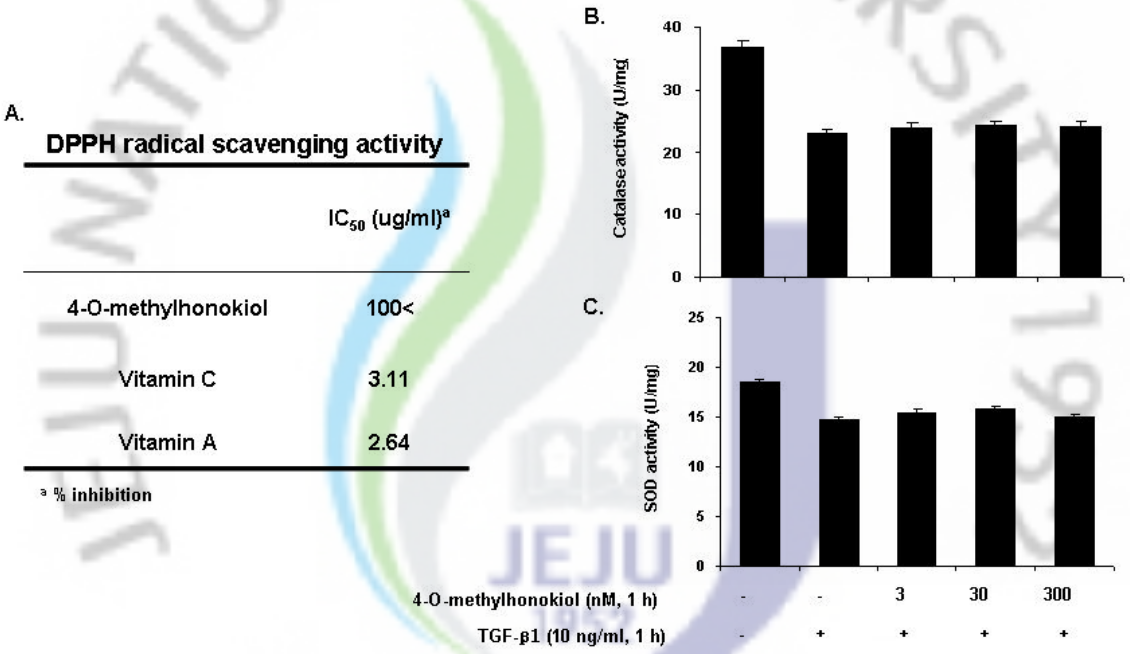
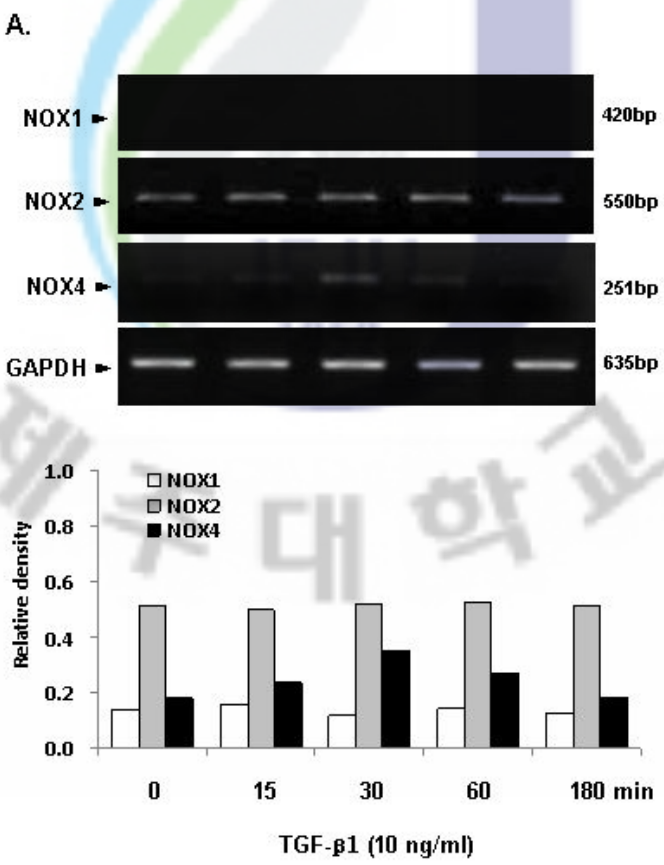


Figure 19. Effect of 4-O-methylhonokiol on enzymatic and non-enzymatic antioxidant systems.

(A) DPPH radical scavenging activity. Data are expressed the concentration necessary to scavenge 50% of DPPH radical. (B) Catalase and (C) SOD activity in HaCaT cells. The enzyme activities were expressed as average enzyme unit per milligram protein.

2.9. Effect of 4-O-methylhonokiol on the TGF-β1-induced NADPH oxidase NOX4 in HaCaT cells.

It has been previously reported that NOX4 mediate TGF-β1-induced ROS production (Carmona-Cuenca I *et al*, 2008). We examined the effect of 4-O-methylhonokiol on TGF-β1-induced NOXs expressions. As shown in Figure 20A, NOX4 mRNA expression increased at 30 min after TGF-β1 treatment. TGF-β1 did not affect the expression of NOX1 and NOX2. However, the expression of NOX4 was reduced by treatment of 4-O-methylhonokiol (Fig. 20B).



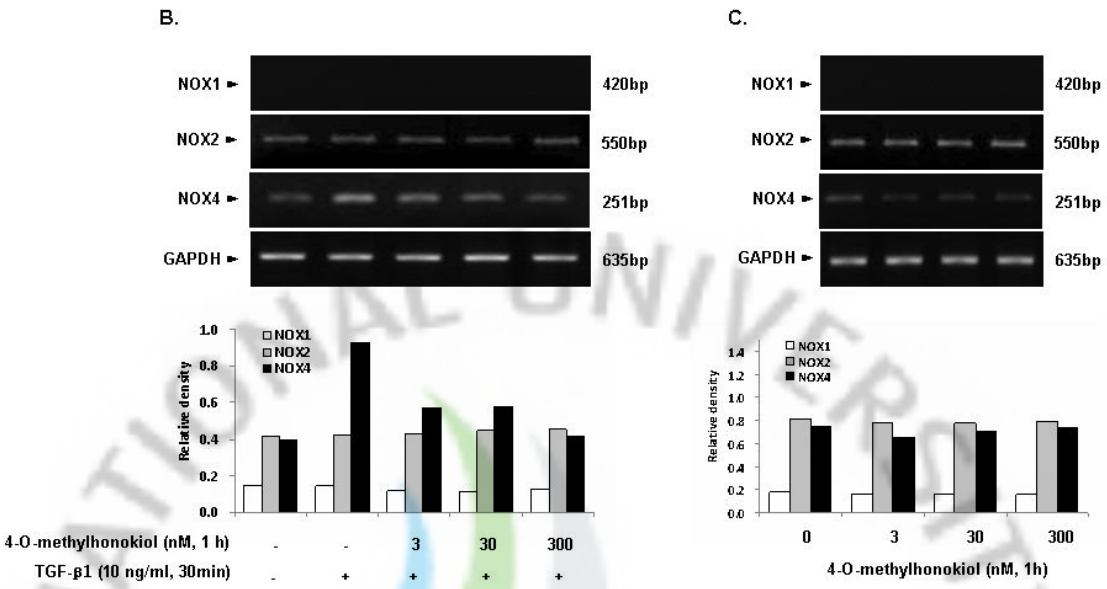


Figure 20. Effect of 4-O-methylhonokiol on the TGF-β1-induced NADPH oxidase NOX4 in HaCaT cells.

(A) After serum starvation for 24h, HaCaT cells were treated with TGF-β1 (10 ng/㎖l) for the indicated times. RNA extraction was carried out in RNase-free environment and the mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR. (B) After pretreated with 4-O-methylhonokiol (3, 30, 300 nM) for 1 h, HaCaT cells were incubated with TGF-β1 (10 ng/㎖) for 30 nim. The mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR as described above. (C) HaCaT cells were treated with 4-O-methylhonokiol (3, 30, 300 nM) for 1 h, and the mRNA expression of NOX1, NOX2 and NOX4 were determined by RT-PCR as described above.

Ⅳ. DISCUSSION

In this study, the hair growth promoting effect of *M. officinalis in vitro* was investigated. To the best of our knowledge, this study is the first to demonstrate that *M. officinalis* and 4-O-methylhonokiol, a main principal of *M. officinalis,* have the potential to promote hair growth via down regulation of TGF-β1 and TGF-β2, as well as the proliferation of dermal papilla. In addition, we investigate how the 4-O-methylhonokiol could inhibit the TGF-β1-induced keratinocyte grow arrest using human keratinocyte HaCaT cell.

The difficulties in developing the effective therapies for hair growth lie in the fact that a single proper evaluation method has not yet been established. In 1990, Philpott *et al*. demonstrated that a human hair follicle could be cultured organo-typically *in vitro* (Philpott MP *et al*., 1990). Using a similar experimental technique, the culture of hair follicle from multiple other species, such as rat, sheep and horse, has also been successfully established (Philpott MP *et al*., 1996). Many investigators have adopted hair follicle culture models to evaluate the effects of several compounds (Buhl AE *et al*., 1992; Taylor M *et al*., 1993). Moreover, vibrissa follicles from rats are much larger than pelage follicles and can be successfully cultured *in vitro* (Buhl AE *et al*., 1990). In particular, the hair growth cycles of the rat vibrissa follicles have been reported to be synchronized according to their age

(Ibrahim L *et al*., 1975) and large posterior vibrissae of 21 d rats have been known to be always in anagen and show no sign of catagen (Philpott MP and Kealey T, 2000). The isolated rat vibrissa follicles could be maintained *in vitro* up to 23 days and then the follicles may enter into catagen phase (Philpott MP and Kealey T, 2000). In recent, *in vitro* culture system for murine vibrissae to reinitiate anagen was developed (Lee J *et al*., 2008). In this study we have isolated large posterior vibrissa follicles from 23 d old rats and maintained them for up to 21 d *in vitro*.

The results of this study showed that the *M. officinalis* extract increased hair-fiber length in cultured rat vibrissa follicles. Specifically, 0.1 μg/㎖ of *M. officinalis* extract was found to induce a greater increase in hair-fiber length than minoxidil sulfate, a positive control (Fig. 2). We next examined which compounds are responsible for hair growth promoting activity of *M. officinalis*. As shown in Figure 3, when rat vibrissa follicles were treated with 4-O-methylhonokiol (the main component of *M. officinalis*), 4-O-methylhonokiol significantly increased hair growth promoting activity by 253.5±43.1% ($P<0.05$) at a dose of 30 nM compared with the control group. Therefore, 4-O-methylhonokiol is very likely responsible for the hair growth promoting activity of *M. officinalis*.

The use of organ culture methods to evaluate hair follicle growth is thought to be correlated with *in vivo* systems because the extent of hair growth can be observed as the sum

of the function of each cell (Philpott MP *et al.*, 1990). The hair growth promoting *in vitro* effect of 4-O-methylhonokiol was also observed *in vivo* using C57BL/6 mice (Fig. 4). The topical application of 5% MINOXYL^{TM} promoted hair growth faster than 4-O-methtylhonokiol. This suggests at least the following possibilities: 1) The active component of 4-O-methtylhonokiol was absorbed into skin much less than minoxidil; 2) After absorbed into skin, the active component of 4-O-methtylhonokiol was metabolized to inactive metabolites faster than the minoxidil; 3) Another unexpected factors were involved in the *in vivo* activity.

The mesenchyme-derived dermal papilla cells play a pivotal role in hair growth regulation. The morphology of dermal papilla cells can be altered through the hair growth cycle, being maximal in volume in the growing phase (anagen) and least in the resting phase (telogen). Evidence has shown that the size of dermal papilla cells is well correlated with hair growth, and the number of dermal papilla cells is increased in the growing phase of hair cycle (Jahoda CAB *et al.*, 1984; Elliott K *et al.*, 1999). To investigate the effect of 4-O-methylhonokiol on cell growth in the hair follicles, we examined the proliferation of dermal papilla cells and the expression of PCNA, as an index of cell proliferation (Hall PA *et al.*, 1990). As shown in Figure 6, 4-O-methylhonokiol was found to increase the growth of dermal papilla cells and the expression of PCNA in the bulb region of the 7 day-cultured

follicles (Fig. 7). Taken together, the results of this study indicated that that the hair growth induced by 4-O-methylhonokiol may be mediated through mitogenic effects that occur in the dermal papilla region.

The important role of 5a-reductase in the hair growth regulation is well recognized. It has been previously reported that metabolism of testosterone in hair follicles is different within body sites depending on their androgen sensitivity, and the type II 5a-reductase in the DP plays a central role by the intrafollicular conversion of testosterone to dihydrotestosterone (Hoffmann R and Happle R, 2000). Thus, type II 5a-reductase is considered to be one of the most important targets for developing drugs for treatment of hair loss. Moreover, several studies have reported that potassium channel openers, e.g., minoxidil, are able to stimulate hair growth (Davies GC *et al*., 2005). Therefore, we examined whether the hair growth promoting activity of 4-O-methylhonokiol was mediated by inhibition of 5α-reductase activity or opening of ATP-sensitive potassium (K_{ATP}) channels. As shown in Figure 8 and 9, 4-O-methylhonokiol did not affect both 5α-reductase activity and ATP-sensitive potassium opening.

In order to determine the mechanism by which 4-O-methylhonokiol promotes hair growth, we examined the expression levels of transforming growth factor-β1 (TGF-β1) and TGF-β2 which have been known to play an important role in anagen to catagen transition via the

induction of keratinocyte apoptosis. When the vibrissa follicles in the anagen phase were treated with 4-O-methylhonokiol for 7 days, the expressions of TGF-β1 in cells of outer root sheath (ORS) and epithelial strand, i.e., in the epithelial residue of the regressing hair bulb during catagen were found to be lower than those of the control follicles that were expected to be in the anagen-catagen transition phase (Fig. 10). 4-O-methylhonokiol also decreased the expression of TGF-β2 in the bulb matrix region of the 7-day cultured follicles (Fig. 11). Recently, several growth factors have been found to play important regulatory roles in the growth of hair (Jindo T *et al*., 1994; Danilenko DM *et al*., 1996; Ozeki M and Tabata Y 2003) and TGF-β has been shown to play a critical role in the growth of hair follicles as well as their morphogenesis (Paus R *et al*.. 1997; Foitzik K *et al*., 1999). The localization of the TGF-β isoforms in hair follicles has also been observed. TGF-β1 and TGF-β3 were strongly detected in the hair cuticle, hair cortex and connective tissue sheath cells of the anagen hair follicle. Conversely, when the anagen-catagen transition was evaluated, the expression of TGF-β2 markedly increased in bulb matrix cells (Soma T *et al*., 2002). Moreover, TGF-β is believed to inhibit hair growth and contribute to the promotion of the regression phase of the hair cycle in human hair follicles (Soma T *et al*., 1998; Soma T *et al*., 2003). Hair follicles treated with TGF-β2 have been found to exhibit decreased hair growth and accelerated categen-like morphology (Soma T *et al*., 1998). TGF-β2 and TUNEL positive cells were

found in a similar area of the catagen hair follicle, which indicates that TGF-β2 expression in the bulb region may be associated with catagen induction (Soma T *et al*., 1998; Soma T *et al*., 2003). Moreover, the results of another study suggest that TGF-β2 contributes to a decrease in the length of the hair cycle via activation of the caspase network (Hibino T and Nishiyama T, 2004). TGF-β1 has been considered to be a potent triggering factor of catagen induction in the murine hair cycle (Foitzik K *et al*., 2000). In addition, TGF-β1 is involved in the regulation of hair follicle regression, and is capable of inducing premature catagen *in vivo* via the induction of apoptosis and inhibition of keratinocyte proliferation (Seiberg M *et al*., 1995; Paus R *et al*., 1997; Welker P *et al*., 1997). Furthermore, androgen-inducible TGF-β1 derived from dermal papilla cells (DPCs) has been shown to mediate the suppression of hair growth and epithelial cell growth in androgenetic alopecia (AGA) (Inui S *et al*., 2002). Collectively, TGF-β1 operates as a catagen inducer and indirectly suppresses hair growth. These results suggest that 4-O-Methylhonokiol has the potential to promote hair growth via down regulation of TGF-β1 and TGF-β2, as well as the proliferation of dermal papilla.

The possible action mechanism of 4-O-methylhonokiol on inhibiting TGF-β1-induced keratinocyte growth arrest was investigated using a human keratinocyte HaCaT cells.

TGF-β1 is a multifunctional cytokine that has the ability to regulate cell differentiation, survival, and death. In many cell types, TGF-β1 inhibits cellular proliferation by causing

growth arrest in the G1 phase of the cell cycle (Moustakas A *et al*., 2002). The TGF-β1–induced G1 cell cycle arrest has been attributed to the regulatory effects of TGF-β1 on both the levels and activities of G1 cyclins and CDKs, which include p21, $p27^{kip1}$, p18, p16, and p15 (Peter M *et al*., 1994). As shown in Figure 12, we found that 4-O-methylhonokiol prevented TGF-β1–induced keratinocyte growth arrest in G_1 phase. These effects were examined to be associated with decreased expression of downstream effectors of TGF-β1 pathway, $p21^{WAF1/Cip1}$. Smad proteins may play a role in regulation of cdk inhibitor p21 gene expression by TGF-β1, because TGF-β1-induced p21 gene expression is further enhanced by overexpression of Smad3 and Smad4. In contrast, dominant negative mutants of Smad3 and Smad4 inhibit TGF-β1-induced p21 gene expression (Pardali K *et al*., 2000). In the present study, Smad2/3 and Samd4 were significantly translocated into the nucleus following treatment with TGF-β1, however, this was blocked by 4-O-methylhonokiol. 4-O-methylhonokiol alone did not show any effect on the localization of Smad proteins (Fig. 14). It has been demonstrated that the ability of Smads to induce specific transcription programs in response to TGF-β1 resulted from a functional cooperativity with other transcription factors in multiprotein complexes in nucleus (Massague J *et al*., 2000; Datta PK *et al*., 2000). In addition, the requirement of functional and physical interaction between Smad proteins and Sp1 transcription factors for the induction of $p21^{WAF1/Cip1}$ in response to TGF-β1 has

been reported (Pardali K *et al*., 2000). As shown in Figure 15, Sp1 phosphorylation by TGF-β1 was also inhibited by treatment with 4-O-methylhonokiol. Collectively, this result indicates that 4-O-mthtylhonokiol might inhibit TGF-β1-induced growth arrest through down regulation of smad2/3, smad4 in Human keratinocyte HaCaT cell.

Although the Smad pathway is the main mediator of TGF-β signaling, recent studies have implicated other pathways such as extracellular signal-regulated kinase (ERK)/p38 mitogen-activated protein (MAP) kinases, phosphatidylinositol 3-kinase (PI 3-kinase), and p70S6 kinase either as mediators or as modulators of TGF-β-dependent biological effects (de Caestecker MP *et al*., 2000). Importantly, several studies have demonstrated that MAPKs are involved in TGF-β-induced p21 expression on a variety of cell types (Hu pp *et al*., 1999; Kivinen L *et al*., 1999). In addition, both Smad- and Ras/MAPK mediated pathways target a proximal sequence of the p21 promoter that can recruit transcription factors of the Sp1 family together with Smads (Moustakas A *et al*., 2002). We examined the effect of U0126, which is a well known pharmacological inhibitor of MEK, on the induction of p21WAF1/Cip1 by TGF-β1. As shown in Figure. 16, pretreatment of human keratinocytes HaCaT cells with U0126 dramatically inhibited induction of $p21^{WAF1/Cip1}$ by TGF-β1. To investigate whether the other ERK family members, p38 and JNK, are involved in induction of $p21^{WAF1/Cip1}$ by TGF-β1, we next examined the effect of SB203580 and SP600125 which

are specific inhibitors of p38 and JNK, respectively. Pretreatment of cells with these inhibitors did not affect the induction of $p21^{WAF1/Cip1}$ by TGF-β1 (Fig. 16). These data indicate that induction of $p21^{WAF1/Cip1}$ by TGF-β1 may require the activation of ERK signaling pathway, but not of p38 or JNK signaling pathway. We determined if there were any effects of 4-O-methylhonokiol on the TGF-β1-induced activation of MAPK pathways. The increase in the phosphoryation of ERK following TGF-β1 treatment was apparent with maximum activity at 60 min, which was sustained longer than 120 min (Fig. 17A). However, TGF-β1 did not affect the phosphoryation of p38 and JNK (Fig.17B, C). TGF-β1-induced ERK activation was completely inhibited by pretreatment with 4-O-methylhonokiol (Fig. 17A). ERK signaling pathway was not only stimulated by H_2O_2 produced in response to TGF-β1, but also required in TGF-β1- induced p21 (Lafon C *et al.*, 1996; Herrera B *et al.*, 2001; Kim YK *et al.*, 2006). We examined the effect of 4-O-methylhonokiol on the production of ROS by TGF-β1. HaCaT cells were treated with TGF-β1 (10 ng/㎖) for 60 min after pretreatment with 4-O-methylhonokiol. 4-O-methylhonokiol significantly reduced the TGF-β1-induced ROS generation (Fig. 18B). In addition, we also evaluated whether 4-O-methylhonokiol could block production of superoxide by TGF-β1. Pretreatment of HaCaT cells with 4-O-methylhonokiol reduced the TGF-β1-induced ROS generation (included superoxide form). In addition, to examine whether 4-O-methylhonokiol could eliminate

TGF-β1-induced ROS, we measured the DPPH free radical scavenging activities, the SOD and CAT activities. As shown in Figure 19, 4-O-methylhonokiol did not affect the DPPH free radical scavenging activities, the SOD and CAT activities. Various mechanisms are involved in ROS production by TGF-β1. Mitochondrial ROS might increase by suppression of antioxidant genes (Franklin CC *et al*., 2003) and inducible NADPH oxidase-like system could account for extra-mitochondrial ROS production (Herrera B *et al*., 2004). In addition, it has been previously reported that NOX4 mediates TGF-β1-induced ROS production (Carmona-Cuenca I *et al*., 2008). We examined the effect of 4-O-methylhonokiol on TGF-β1-induced NOXs expression. As shown in Figure 20A, NOX4 mRNA expression increased at 30 min after TGF-β1 treatment. TGF-β1 did not affect the expression of NOX1 and NOX2. However, the expression of NOX4 was reduced by pretreatment of 4-O-methylhonokiol (Fig. 20B). These results suggest that 4-O-methylhonokiol could protect keratinocytes against TGF-β1-induced growth arrest by blocking ROS production through NADPH oxidase system.

In summary, we demonstrated that 4-O-Methylhonokiol might have the potential to promote hair growth via down regulation of TGF-β1 and TGF-β2, as well as the proliferation of dermal papilla and that 4-O-Methylhonokiol may prevent TGF-β1-induced keratinocyte growth arrest through both ERK and Smad pathways.

Ⅴ. Reference

Schneider MR, Schmidt-Ullrich R, Paus R. The hair follicle as a dynamic miniorgan. *Curr Biol*. 2009 10; 19:132-42.

Hardy MH. The secret life of the hair follicle. *Trends Genet*. 1992; 8:55-61.

Powell BC, Nesci A, Rogers GE. Regulation of keratin gene expression in hair follicle differentiation. *Ann N Y Acad Sci.* 1991; 642:1-20.

Paus R, Cotsarelis G. The biology of hair follicles. *N Engl J Med*. 1999; 341:491-7.

Oliver RF. Regeneration of dermal papillae in rat vibrissae. *J Invest Dermatol.* 1996; 47:496-7.

MacKinnon PJ, Powell BC, Rogers GE. Structure and expression of genes for a class of cysteine-rich proteins of the cuticle layers of differentiating wool and hair follicles. *J Cell Biol.* 1990; 111:2587-600.

Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell*. 1990; 61:1329-37.

Orwin DF. The cytology and cytochemistry of the wool follicle. *Int Rev Cytol*. 1979; 60:331-74.

Cotsarelis G, Sun TT, Lavker RM. Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. *Cell.* 1990; 61:1329-37.

Lajtha LG. Stem cell concepts. *Differentiation.* 1979; 14:23-34.

Lavker RM, Cotsarelis G, Wei ZG, Sun TT. Stem cells of pelage, vibrissae, and eyelash follicles: the hair cycle and tumor formation. *Ann N Y Acad Sci.* 1991; 642:214-24.

Yang JS, Lavker RM, Sun YY. Upper human hair follicle contains a subpopulation of keratinocytes with superior in vitro proliferative potential. *J Invest Dermatol.* 1993; 101:652-9.

Oliver RF, Jahoda CA. Dermal-epidermal interactions. *Clin Dermatol.* 1988; 6:74-82.

Randall VA, Hibberts NA, Thornton MJ, Merrick AE, Hamada K, Kato S, Jenner TJ, de Oliveira I, Messenger AG. Do androgens influence hair growth by altering the paracrine factors secreted by dermal papilla cells?. *Eur J Dermatol.* 2001; 11:315-20.

Elliott K, Stephenson TJ, Messenger AG. Differences in hair follicle dermal papilla volume are due to extracellular matrix volume and cell number: implications for the control of hair follicle size and androgen responses. *J Invest Dermatol.* 1999; 113:873-7.

Jahoda CA, Oliver RF, Reynolds AJ, Forrester JC, Horne KA. Human hair follicle regeneration following amputation and grafting into the nude mouse. *J Invest Dermatol.*

1996; 107:804-7.

Pisansarakit P, du Cros DL, Moore GP. Cultivation of mesenchymal cells derived from the skin and hair follicles of the sheep: the involvement of peptide factors in growth regulation. *Arch Dermatol Res*. 1991; 283:321-7.

Jahoda CA, Horne KA, Oliver RF. Induction of hair growth by implantation of cultured dermal papilla cells. *Nature.* 1984; 311:560-2.

Watson SA, Pisansarakit P, Moore GP. Sheep vibrissa dermal papillae induce hair follicle formation in heterotypic skin equivalents. *Br J Dermatol.* 1994; 131:827-35.

Stenn KS, Combates NJ, Eilertsen KJ, Gordon JS, Pardinas JR, Parimoo S, Prouty SM. Hair follicle growth controls. *Dermatol Clin.* 1996; 14:543-58.

Fusenig NE, Limat A, Stark HJ, Breitkreutz D.Modulation of the differentiated phenotype of keratinocytes of the hair follicle and from epidermis.J *Dermatol Sci.* 1994; 7:142-51.

Reynolds AJ, Jahoda CA. Hair matrix germinative epidermal cells confer follicle-inducing capabilities on dermal sheath and high passage papilla cells. *Development*. 1996; 122:3085-94.

Jave-Suarez LF, Winter H, Langbein L, Rogers MA, Schweizer J. HOXC13 is involved in the regulation of human hair keratin gene expression. *J Biol Chem*. 2002; 277:3718-26.

DasGupta R, Fuchs E. Multiple roles for activated LEF/TCF transcription complexes during

hair follicle development and differentiation. *Development.* 1999; 126:4557-68.

Kulessa H, Turk G, Hogan BL. Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle. *EMBO J.* 2000; 19:6664-74.

Paus R, Cotsarelis G. The biology of hair follicles. *N Engl J Med.* 1999; 341:491-7.

van Steensel MA, van Geel M, Steiljen PM. The molecular basis of hair growth. *Eur J Dermatol.* 2001; 11:348-5.

Widelitz RB, Jiang TX, Lu J, Chuong CM. beta-catenin in epithelial morphogenesis: conversion of part of avian foot scales into feather buds with a mutated beta-catenin. *Dev Biol.* 2000; 219:98-114.

Nusse R. WNT targets. Repression and activation. *Trends Genet.* 1999; 15:1-3.

van Genderen C, Okamura RM, Fariñas I, Quo RG, Parslow TG, Bruhn L, Grosschedl R. Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in LEF-1-deficient mice. *Genes Dev.* 1994; 8:2691-703.

Kratochwil K, Dull M, Farinas I, Galceran J, Grosschedl R. Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes Dev.* 1996; 10:1382-94.

Hardy MH. The secret life of the hair follicle. *Trends Genet.* 1992 ; 8:55-61.

Price VH. Treatment of hair loss. *N Engl J Med.* 1999; 341:964-73.

Hodak E, Gottlieb AB, Anzilotti M, Krueger JG. The insulin-like growth factor 1 receptor is expressed by epithelial cells with proliferative potential in human epidermis and skin appendages: correlation of increased expression with epidermal hyperplasia. J *Invest Dermatol.* 1996; 106:564-70.

Rudman SM, Philpott MP, Thomas GA, Kealey T. The role of IGF-I in human skin and its appendages: morphogen as well as mitogen?. *J Invest Dermatol*. 1997; 109:770-7.

Rosenquist TA, Martin GR. Fibroblast growth factor signalling in the hair growth cycle: expression of the fibroblast growth factor receptor and ligand genes in the murine hair follicle. *Dev Dyn*. ; 205:379-86.

Hébert JM, Rosenquist T, Götz J, Martin GR. FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell.* 1994; 78:1017-25.

Stenn KS, Combates NJ, Eilertsen KJ, Gordon JS, Pardinas JR, Parimoo S, Prouty SM. Hair follicle growth controls. *Dermatol Clin.* 1996; 14:543-58.

Randall VA, Thornton MJ, Hamada K, Messenger AG. Mechanism of androgen action in cultured dermal papilla cells derived from human hair follicles with varying responses to androgens in vivo. *J Invest Dermatol.* 1992; 98:86S-91S.

Sawaya ME, Price VH. Different levels of 5alpha-reductase type I and II, aromatase, and androgen receptor in hair follicles of women and men with androgenetic alopecia. *J*

Invest Dermatol. 1997; 109:296-300.

Itami S, Kurata S, Takayasu S. Androgen induction of follicular epithelial cell growth is mediated via insulin-like growth factor-I from dermal papilla cells. *Biochem Biophys Res Commun.* 1995; 212:988-94.

Hibberts NA, Messenger AG, Randall VA. Dermal papilla cells derived from beard hair follicles secrete more stem cell factor (SCF) in culture than scalp cells or dermal fibroblasts. *Biochem Biophys Res Commun.*. 1996; 222:401-5.

Lachgar S, Charveron M, Sarraute J, Gall Y, Bonafe JL. Anti-androgens and estrogens: modulators of VEGF expression in cultured hair dermal papilla cells. *Exp Dermatol*. 1999; 8:336-8.

Inui S, Fukuzato Y, Nakajima T, Yoshikawa K, Itami S. Androgen-inducible TGF-β1 from balding dermal papilla cells inhibits epithelial cell growth: a clue to understand paradoxical effects of androgen on human hair growth. *FASEB J*. 2002; 16:1967-9.

Yu DW, Yang T, Sonoda T, Gaffney K, Jensen PJ, Dooley T, Ledbetter S, Freedberg IM, Lavker R, Sun TT. Message of nexin 1, a serine protease inhibitor, is accumulated in the follicular papilla during anagen of the hair cycle. *J Cell Sci.* 1995; 108 :3867-74.

Sonoda T, Asada Y, Kurata S, Takayasu S. The mRNA for protease nexin-1 is expressed in human dermal papilla cells and its level is affected by androgen. *J Invest Dermatol.*

1999; 113:308-13.

Pratt WB, Toft DO. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr Rev.* 1997; 18:306-60.

Albert O. Brinkmann, ph,D, August 4, 2003. Chaptor 3-androgen physiology: receptor and metabolic disorders.

Andersson S, Berman DM, Jenkins EP, Russell DW. Deletion of steroid 5 alpha-reductase 2 gene in male pseudohermaphroditism. *Nature.* 1991; 354:159-61.

Kaufman KD. Androgen metabolism as it affects hair growth in androgenetic alopecia. *Dermatol Clin.* 1996 ; 14:697-711.

Ando Y, Yamaguchi Y, Hamada K, Yoshikawa K, Itami S. Expression of mRNA for androgen receptor, 5alpha-reductase and 17beta-hydroxysteroid dehydrogenase in human dermal papilla cells. *Br J Dermatol.* 1999 ; 141:840-5.

Kaufman KD, Olsen EA, Whiting D, Savin R, DeVillez R, Bergfeld W, Price VH, Van Neste D, Roberts JL, Hordinsky M, Shapiro J, Binkowitz B, Gormley GJ. Finasteride in the treatment of men with androgenetic alopecia. Finasteride Male Pattern Hair Loss Study Group. *J Am Acad Dermatol.* 1998; 39:578-89

Price VH. Treatment of hair loss. *N Engl J Med.* 1999; 341:964-73.

Li M, Marubayashi A, Nakaya Y, Fukui K, Arase S. Minoxidil-induced hair growth is

mediated by adenosine in cultured dermal papilla cells: possible involvement of sulfonylurea receptor 2B as a target of minoxidil. *J Invest Dermatol.* 2001; 117:1594-600.

Shorter K, Farjo NP, Picksley SM, Randall VA. Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil. *FASEB J.* 2008; 22:1725-36.

Han JH, Kwon OS, Chung JH, Cho KH, Eun HC, Kim KH. Effect of minoxodil on proliferation and apoptosis in dermal papilla cells of human hair follicle. *J Dermatol Sci* 2004; 34: 91-8

Buhl AE. Minoxidil's action in hair follicles. *J Invest Dermatol.* 1991; 96:73S-74S

Song WZ, Cui JF, Zhang GD. Studies on the medicinal plants of Magnoliaceae tu-hou-po of Manglietia. *Yao Xue Xue Bao.* 1989; 24:295-9.

Watanabe K, Watanabe HY, Goto Y, Yamamoto N, Yoshizaki M. Studies on the active principles of magnolia bark. Centrally acting muscle relaxant activity of magnolol and hōnokiol. *Jpn J Pharmacol.* 1975; 25:605-7.

Watanabe K, Watanabe H, Goto Y, Yamaguchi M, Yamamoto N, Hagino K. Pharmacological properties of magnolol and honokiol extracted from Magnolia officinalis: central depressant effects. Watanabe K, Watanabe H, Goto Y, Yamaguchi M,

Yamamoto N, Hagino K. *Planta Med.* 1983; 49:103-8.

Watanabe, K. Pharmacology of magnolia bark with special reference to gastrointestinal functions. *Gendai Toyo Igaku* 1986; 7:54-9.

Teng CM, Yu SM, Chen CC, Huang YL, Huang TF. EDRF-release and Ca+ (+)-channel blockade by magnolol, an antiplatelet agent isolated from Chinese herb Magnolia officinalis, in rat thoracic aorta. *Life Sci.* 1990; 47:1153-61.

Hamasaki Y, Kobayashi I, Zaitu M, Tsuji K, Kita M, Hayasaki R, Muro E, Yamamoto S, Matsuo M, Ichimaru T, Miyazaki S. Magnolol inhibits leukotriene synthesis in rat basophilic leukemia-2H3 cells. *Planta Med.* 1999; 65:222-6.

Bae EA, Han MJ, Kim NJ, Kim DH. Anti-Helicobacter pylori activity of herbal medicines. *Biol Pharm Bull.* 1998; 21:990-2.

Fukuyama Y, Otoshi Y, Miyoshi K, Nakamura K, Kodama M, Nagasawa M, Hasegawa T, Okazaki H, Sugawara M. Neurotrophic sesquiterpene-neolignans from Magnolia obovata: structure and neurotrophic activity. *Tetrahedron.* 1992; 48:377-92.

Oh JH, Kang LL, Ban JO, Kim YH, Kim KH, Han SB, Hong JT. Anti-inflammatory effect of 4-O-methylhonokiol, a novel compound isolated from Magnolia officinalis through inhibition of NF-kappaB. *Chem Biol Interact*. 2009; 180:506-14

Lee YK, Choi IS, Kim YH, Kim KH, Nam SY, Yun YW, Lee MS, Oh KW, Hong JT.

Neurite Outgrowth Effect of 4-O-methylhonokiol by Induction of Neurotrophic Factors Through ERK Activation. *Neurochem Res*. 2009 Jun 26.

Lee YK, Yuk DY, Kim TI, Kim YH, Kim KT, Kim KH, Lee BJ, Nam SY, Hong JT. Protective effect of the ethanol extract of Magnolia officinalis and 4-O-methylhonokiol on scopolamine-induced memory impairment and the inhibition of acetylcholinesterase activity. *J Nat Med*. 2009; 63:274-82.

Filsell W, Little JC, Stones AJ, Granger SP, Bayley SA. Transfection of rat dermal papilla cells with a gene encoding a temperature-sensitive polyomavirus large T antigen generates cell lines a differentiated phenotype. *J Cell Sci*.1994; 107: 1761-72.

Philpott MP, Kealey T. Cyclical Changes in Rat Vibrissa Follicles Maintained *In Vitro*. *J Invest Dermatol*. 2000; 115: 1152-5.

Buhl AE, Waldon DJ, Baker CA, Johnson GA. Minoxidil sulfate is the active metabolite that stimulates hair follcles. *J Invest Dermatol*. 1990; 95: 553-7.

Müller-Röver S, Handjiski B, van der Veen C, Maurer M, Eichmüller S, Ling G, Hofmann U, Foitzik K, Mecklenburg L, Handjiski B. A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol*. 2001; 117:3-15.

Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a

tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res*. 1987; 47: 936-42.

Krishan A. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* 1975; 66: 188–93.

Bradford LW, Problems of ethics and behavior in the forensic sciences. *J Forensic Sci*. 1976; 21:763-8.

Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.*1972; 247: 3170-5.

Carrillo MC, Kanai S, Nokubo M, Kitani K. Deprenyl induces activities of both superoxide dismutase and catalase but not of glutathione peroxidase in the striatum of young male rats. *Life Sci.* 1991; 48:517-21.

Hirosumi J, Nakayama O, Fagan T, Sawada K, Chida N, Inami M, Takahashi S, Notsu Y, Okuhara M. FK143, a Novel Nonsteroidal Inhibitor of Steroid 5α-Reductase: (1) In Vitro Effects on Human and Animal Prostatic Enzymes. *J Steroid Biochem Mole Biol* 1995; 52: 357-63.

Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett*. 2002; 82:85-91.

Massague J, Wotton D. Transcriptional control by TGF-beta/Smad signaling sustem. *EMBO*

J. 2000; 19:1745-54.

Datto MB, Yu Y, Wang XF. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/p21 promoter. *J Biol Chem.* 1995; 270: 28623-8.

Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A. Role of smad proeins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem.* 2000; 275: 29244-56.

Hu pp, Shen X, Huang D, Liu Y, Counter C, Wang XF. The MEK pathway is required for stimulation of p21 (WAF1/CIP1) by transforming growth factor-beta. *J Biol Chem*. 1999; 274: 35381-7.

Yoon YS, Lee JH, Hwang SC, Choi KS, Yoon G. TGF beta1 induces prolonged mitochondrial ROS generation through decreased complex Ⅳ activity with senescent arrest in Mv1Lu cells. *Oncogene*. 2005; 24:1895-903.

Jiang Z, Seo JY, Ha H, Lee EA, Kim YS, Han DC, Uh ST, Park CS, Lee HB. Reactive oxygen species mediate TGF-beta1-induced plasminogen activator inhibitor-1 upregulation in mesangial cells. *Biochem Biophys Res Commun.* 2003; 309:961-6.

Carmona-Cuenca I, Roncero C, Sancho P, Caja L, Fausto N, Fernandez M, Fabregat I.Upregulation of the NADPH oxdase NOX4 by TGF-beta in hepatocytes is required for its pro-apoptotic activity. *J Hepatol.* 2008; 49:965-76.

Philpott MP, Green MR, Kealy T. Human hair growth *in vitro. J Cell Sci.* 1990; 97: 463-71.

Philpott MP, Green MR, Kealy T. Rat hair follicle growth *in vitro*. *Br J Dermatol.* 1992; 127: 600-7.

Williams D, Siock P, Stenn KS. 13-cis-retinoic acid affects sheath-shaft interaction of equine hair follicles *in vitro*. *J Invest Dermatol.* 1996; 106: 356-61.

Buhl AE, Waldon DJ, Conrad SJ, Mulholland MJ, Shull KL, Kubicek MF, Johnson GA, Brunden MN, Stefanski KJ, et al. potassium channel conductance: a mechanism affecting hair growth both *in vitro* and *in vivo*. *J Invest Dermatol.* 1992; 98: 315-9.

Taylor M, Ashcroft AT, Messenger AG. Cyclosporin A prolongs human hair growth *in vitro*. *J Invest Dermatol.* 1993; 100: 237-9.

Buhl AE, Waldon DJ, Baker CA, Johnson GA. Minoxidil sulfate is the active metabolite that stimulates hair follcles. *J Invest Dermatol.* 1990; 95: 553-7.

Ibrahim L, Wright EA. The growth of rats and mice vibrissae under normal and some abnormal conditions. *J Embryol Exp Morphol.* 1975; 33:831-44.

Philpott MP, Kealey T. Cyclical Changes in Rat Vibrissa Follicles Maintained *In Vitro*. *J Invest Dermatol.* 2000; 115: 1152-5.

Lee J, Wu W, Kopan R. Murine vibrissae cultured in serum-free medium reinitiate anagen. *J Invest Dermatol.* 2008; 128:482-5.

Hoffmann R, Happle R. Current understanding of androgenetic alopecia. Part I: etiopathogenesis. *Eur J Dermatol.* 2000; 10:319-27.

Davies GC, Thornton MJ, Jenner TJ, Chen YJ, Hansen JB, Carr RD, Randall VA. Novel and established potassium channel openers stimulate hair growth *in vitro*: implications for their modes of action in hair follicles. *J Invest Dermatol.* 2005; 124:686-94.

Jahoda CAB, Horne KA, Oliver RF. Induction of hair growth by implantation of cultured the dermal papilla cells. *Nature.* 1984; 311: 560-2.

Elliott K, Stephenson TJ, Messenger AG. Differences in hair follicle dermal papilla volume are due to extracellular matrix volume and cell number: implications for the control of hair follicle size and androgen responses. *J Invest dermatol.* 1999; 113: 873-7.

Hall PA, Levison DA, Woods AL, Yu CC, Kellock DB, Watkkins JA, Barnes DM, Gillett CE, Camplejohn R, Dover R, Waseem NH, Lane DP. Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol.* 1990; 162: 285-94.

Danilenko DM, Ring BD, Pierce GF. Growth factors and cytokines in hair follicle development and cycling: recent insights from animal models and the potentials for clinical therapy. *Mol Med Today.* 1996; 2: 460-7.

Ozeki M, Tabata Y. In vivo promoted growth of mice hair follicles by the controlled release

of growth factors. *Biomaterials*. 2003; 24: 2387-94.

Jindo T, Tsuboi R, Imai R, et al. Hepatocyte growth factor/scatter factor stimulates hair growth of mouse vibrissae in organ culture. *J Invest Dermatol*. 1994; 103: 306-9.

Paus R, Foitzik K, Welker P, et al. Transforming growth factortype I and type II expression during murine hair follcle development and cycling. *J Invest Dermatol.* 1997; 109: 518-26.

Foitzik K, Paus R, Doetschman T, et al. The TGF-2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. *Dev Biol.* 1999; 212: 278-89.

Soma T, Tsuji Y, Hibino T. Involvement of transforming growth factor-2 in catagen induction during the human hair cycle. *J Invest Dermatol.* 2002; 118: 993-7.

Soma T, Dohrmann CE, Hibino T, et al. Profile of transforming growth factor- responses during the murine hair cycle. *J Invest Dermatol*. 2003; 121: 969-75.

Soma T, Ogo M, Suzuki J, et al. Analysis of Apoptosis Cell Death in Human Follicles In Vivo and In Vitro. *J Invest Dermatol.* 1998; 111:948-54.

Hibino T, Nishiyama T. Role of TGF-beta2 in the human hair cycle. *J Dermatol Sci.* 2004; 35: 9-18.

Foitzik K, Lindner G, Mueller-Roever S, Maurer M, Botchkareva N, Botchkarev V, Handjiski B, Metz M, Hibino T, Soma T, Dotto GP, Paus R. Control of murine hair

follicle regression (catagen)by TGF-β1 in vivo. *FASEB J*. 2000; 14:752–760.

Paus R, Foitzik K, Bulfoned-Paus S, Eichmuller S. Transforming growth factor-beta receptor type I and type II expression during murine hair follicle development and cycling. *J Invest Dermatol.* 1997; 109:518–526.

Seiberg M, Marthinuss J, Stenn KS. Changes in expressionof apoptosis-associated genes in skin mark early catagen. *J Invest Dermatol.*1995; 104:78–82.

Welker P, Foitzik K, Bulfone-Paus S, Henz BM, Paus R. Hair cycle-dependent changes in the gene expression and protein content of transforming factor beta 1 and beta 3 in murine skin. *Arch Dermatol Res.* 1997; 289:554–7.

Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett*. 2002; 82:85-91.

Peter M, Herskowitz I. Joining the complex: cyclin-dependent kinase inhinitory proteins and the cell cycle. *Cell. 1*994; 79:181-4.

Pardali K, A Kurisaki, A Moren, Pt Dijke, Kardassis D, Moustakas A. Role of Smad proteins and transcription factor Sp1 in p21WAF/Cip regulation by transforming growth factor-beta. *J Biol Chem.* 2000; 275:29244–56.

Massague J, Wotton D. Transcriptional control by the TGF-beta/Smad signaling system. *EMBO J.* 2000; 19:1745-54.

Datta PK, Blake MC. Regulation of plasminogen activator inhibitor-1 expression by transforming growth factor-beta-induced physical and functional interactions between Smads and Sp1. *J Biol Chem.* 2000; 275:40014-9.

Pardali K, Kurisaki A, Moren A, ten Dijke P, Kardassis D, Moustakas A. Role of smad proeins and transcription factor Sp1 in p21(Waf1/Cip1) regulation by transforming growth factor-beta. *J Biol Chem.* 2000; 275: 29244-56.

de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor-β signaling in cancer. *J Natl Cancer Inst.* 2000; 92:1388-402.

Hu pp, Shen X, Huang D, Liu Y, Counter C, Wang XF. The MEK pathway is required for stimulation of p21 (WAF1/CIP1) by transforming growth factor-beta. *J Biol Chem.* 1999; 274: 35381-7.

Kivinen L, Laiho M. Ras- and mitogen-activated protein kinasse-dependent and – independent pathways in p21Cip1/Waf1 induction by fibroblast growth factor-2, platelet-derived growth factor, and transforming growth factor-beta1. *Cell Growth Differ.* 1999; 10: 621-8.

Moustakas A, Pardali K, Gaal A, Heldin CH. Mechanisms of TGF-beta signaling in regulation of cell growth and differentiation. *Immunol Lett.* 2002; 82: 85-91.

Kim YK, Bae GU, Kang JK, Park JW, Lee EK, Lee HY, Choi WS, Lee HW, Han JW.

Cooperation of H_2O_2-mediated ERK activation with Smad pathway in TGF-beta1 induction of $p21^{WAF1/Cip1}$. *Cell Signal.* 2006; 18:236-43.

Lafon C, Mathieu C, Guerrin M, Pierre O, Vidal S, Valette A. Transforming growth factor beta 1-induced apoptosis in human ovarian carcinoma cell: protection by the antioxidant N-acetylcyteine and bcl-2. *Cell Growth Differ*. 1996; 7:1095-104.

Herrera B, Alvarez AM, Sanchez A, Fernandez M, Roncero C, Benito M, Fabregat I. Reactive oxygen species (ROS) mediates the mitochondrial-dependent apoptosis induced by transformin growth factor (beta) in fetal hepatocytes. *FASEB J.* 2001; 15:741-51

Franklin CC, Rosenfeld-Franklin ME, White C, Kavanagh TJ, Fausto N. TGF beta1-induced suppression of glutathione antioxidant defenses in hepatocytes:caspase-dependent post-translational and caspase-independent transcriptional regulatory mechanisms. *FASEB J.* 2003; 17:1535-7.

Ⅵ. Abstract in Korean

탈모인구는 해마다 계속 증가하고 있으며, 최근에는 중년 남성뿐만 아니라 젊은 층과 여성층에서도 탈모가 보편적으로 나타나고 있다. 특히 유전적인 요인, 호르몬의 과다, 정신적 스트레스, 자가면역질환 및 화학요법 등이 탈모에 관여하는 것으로 보고되고 있다. 그런 까닭에 모발 소실 방지 및 모발 성장 증진을 위한 새로운 치료물질을 개발하는 것은 무엇보다 중요하다. 새로운 탈모치료제를 찾기 위한 일환으로 천연물에서 유래하는 물질을 이용하여 육모효능을 검색 한 결과 후박 추출물이 탁월한 육모 효능을 확인 할 수 있었다. 후박은 중국 및 일본에서 전통적으로 위장질환, 불안장애 및 천식을 포함한 알레르기 질환 치료에 이용 되고 있다. 후박추출물 및 그 성분은 anti-inflammatory, antiallergic, antibacterial 및 neurite spouting activity등 많은 약리활성이 보고 되고 있다.

본 연구에서 후박추출물 및 후박추출물의 성분인 4-O-metylhonokiol의 육모 효능에 대해 조사하였다. Rat vibrissa 모낭에 후박 추출물을 처리하였을 때, vibrissa 모낭의 hair fiber 길이는 유의성 있게 증가 하였다. 특히 후박추출물의 성분인 4-O-methtylhonokiol 역시 vibrissa 모낭의 hair fiber 길이를 증가 시켰다. 게다가 C57BL/6 마우스에 4-O-methtylhonokiol을 도포한 경우, 성장기 모낭을 유도하였다.

그리고 4-O-methtylhonokiol은 bulb region에서 PCNA 발현과 immortalized vibrissa dermal papilla 세포 증식을 증가 시켰다. 4-O-methtylhonokiol의 육모 효능 작용기전을 알아보기 위해, keratinocyte apoptosis 유도에 의한 anagen에서 catagen 전이시 중요한 역할을 하는 TGF-β1과 TGF-β2의 발현을 조사하였다. 성장기 모낭에 4-O-methtylhonokiol를 처리하여 7일간 배양 후 TGF-β1과 TGF-β2의 발현을 조사 한 결과, TGF-β1은 대조군 보다 outer root sheat (ORS)와 epithelial strand에 약하게 발현되는 것을 관찰되었다. 또한 4-O-methylhonokiol을 처리한 7일째 모낭의 bulb matrix region에서는 TGF-β2 발현이 감소하였다. 이와 같은 결과는 TGF-β1과 TGF-β2의 down-regulation과 dermal papilla 세포 증식을 통하여 4-O-methtylhonokiol이 모낭 성장을 촉진한다고 생각됩니다.

다음은 TGF-β1에 의해 유도된 keratinocyte growth arrest을 4-O-methtylhonokiol의해 어떻게 억제 할 수 있는지 조사 하였습니다. HaCaT cell에 4-O-methtylhonokiol을 전처리 하였을 때, TGF-β1에 의해 유도되는 cell cycle arrest가 감소되는 것을 확인하였다. Cell cycle arrest와 밀접한 관계가 있는 p21 발현에 4-O-methylhonokiol의 효과를 알아본 결과, 4-O-methylhonokiol처리에 의해 TGF-β1에 의해서 유도된 p21 발현은 감소하였다. TGF-β1에 의한 p21 발현은 Smad pathway와 non-Smad pathway를 통하여 일어난다. TGF-β1에 의해서 유도되는 Smad2/3, Smad4의 nuclear translocation와 Sp1 활성에 4-O-methylhonokiol의 효과를

알아본 결과, 4-O-methylhonokiol은 TGF-β1에 의해서 유도된 Smad2/3, Smad4의 nuclear translocation와 Sp1 활성을 억제하는 것을 확인하였다. 또한 TGF-β1에 의해 유도되는 ERK 활성에 4-O-methylhonokiol이 효과를 알아본 결과, 4-O-methylhonokiol를 처리하였을 때 ERK 활성이 감소되는 것을 확인 할 수 있었다. 한편, TGF-β는 다양한 세포에서 ROS 생성을 증가시키고, 증가된 ROS는 MAPKs를 활성화시켜 growth arrest 및 apoptosis에 관여한다고 알려져 있습니다. TGF-β1에 의해 유도되는 ROS 증가 및 NOX4 mRNA 발현을 4-O-methylhonokiol 처리에 의해 감소하는 것을 확인 할 수 있었다. 따라서 4-O-methylhonokiol은 Smad2/3, Smad4 및 NOX4의 down regulation함으로써 TGF-β1에 의해 유도되는 cell cycle arrest를 막을 수 있을 것으로 사료됩니다.

주요어: 후박, 육모, 4-O-methylhonokiol, 모유두세포, TGF-β1, TGF-β2, PCNA, p21, Sp1, Smad2/3, Smad4, NOX4

감사의 글

오늘도 유난히 처음 박사과정을 선택할 때와 같이 날씨가 변덕스럽습니다. 기대와 아쉬움, 절망과 희망, 쉽지 않은 4년, 어느덧 시간이 흘러 논문을 완성하였으나 무언가를 해 내었다는 뿌듯함보다는, 미완의 아쉬움과 아직 많이 부족하다는 자책이 더 앞서 갑니다. 부족하지만 제가 논문을 마무리하기까지 많은 도움과 격려를 주신분들께 이 지면을 빌어 감사의 마음을 전하려 합니다.

먼저, 석사과정부터 지금까지 항상 부족한 저를 믿어주시고 지속적인 관심과 가르침을 주신 저의 지도교수님이신 **강희경 교수님**께 감사 드립니다. 교수님의 믿음만큼 학위과정 중 저의 열정이 따라가지 못해 죄송합니다. 하지만 제자에 대한 믿음과 연구에 대한 열정을 버리시지 않는 교수님의 모습은 저에게 학위과정 동안 큰 힘이 되었습니다. 교수님의 그 열정 언제나 가슴 깊이 간직하겠습니다. “교수님 정말 감사합니다. 늘 건강하세요”. 그리고 바쁘신 와중에도 저의 미흡한 논문을 정성껏 다듬어주신 **유은숙 교수님**, **박덕배 교수님**, **현진원 교수님**, **이영재 교수님께** 깊은 감사의 말씀을 드립니다. 아울러 대학원 과정 동안 늘 관심과 조언으로 학문의 길을 이끌어주신 **이영기 교수님, 강현욱 교수님, 정영배 교수님, 고영상 교수님, 이근화 교수님, 김수영 교수님, 은수용 교수님**을 비롯하여 의대 모든 교수님들께도 감사의 마음을 전합니다.

대학원 생활 내내 몸과 마음은 고단 했지만, 국립 암센터에서 열심히 포닥을 하는 **혜자**, 늘 말 벗이 되어 줘서 고마워, 지금은 아기 때문에 정신 없는 **원종**이, 우리 실험실 재정을 담당하는 **재희**, 언제나 말이 없는 **경진**, 실험이 점점 재미있어 하는 **정일**, TEPS을 열심히 공부하는 막내 **혜진**, 그리고 학과 후배이자 진정한 내편 **민경,** 도움이 필요할 때 아낌없이 도와준 생화학교실 **영미**누나와 **희경**누나에게 고마움을 전하며, 말없는 눈웃음으로 인사하는 **진영**, 이제는 연구교수인 **경아**, 가끔 마주 치는 **정은**, 항상 웃는

얼굴로 인사하는 **지강**, **연희**, **은희,** 사랑스런 아내와 같이 생활하는 수의독성화학교실 대장 **희경**, 살림꾼 **현주**, 똑순이 **원영**, 어릴 때만 과학신동인 **동건**에게도 고마운 마음을 전합니다.

학위과정 중 몸과 마음이 너무 힘들 때 늘 옆에서 힘을 준 나의 사랑스런 **아내**.....고마워요.....사랑해요.....중간에 포기하지 않게 마칠 수 있도록 영혼의 힘을 준 나의 아들 **복덩이**에게 무한한 사랑을 느끼면 감사의 마음을 표합니다. 항상 부족한 아들을 믿어주시고 지켜 봐주신 **어머니**, 누구보다도 동생을 믿어준 우리 **형**, **매형, 누나**, 사랑스런 조카 **아령**, **아연**, 그리고 아무것도 없는 나에게 소중한 딸을 믿고 보내주신 부산에 계신 **장인어른**, **장모님**, 나의 든든한 지원군 **처남**에게도 진심 어린 고마움을 전합니다.....오래오래 건강하세요.....사랑합니다!!!

지난 일들을 되돌아보면 그토록 힘들고 어려웠던 순간에 저 혼자만 있었던 것은 아닌 것 같습니다. 많은 분들의 도움과 그들의 염려하는 마음이 항상 함께하였기에 지금의 내가 있는 것 같습니다. 그리고 앞으로도 이 모든 것들을 언제나 감사하고 기억하며 살아가겠습니다.

저의 소박한 노력의 결과가 이제 조그마한 매듭을 맺고 또 다른 한발을 내딛고자 합니다. 정말 고맙습니다.