การศึกษาการเปลี่ยนแปลงของการหลั่งสารไซโตไคน์ และโกรทแฟคเตอร์ ของเซลล์เดอร์มอลพาพิลาจาก
หนังศีรษะของผู้ชายที่มีภาวะผมบางจากพันธุกรรม

นางรัชต์ธร ปัญจประทีป

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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THE STUDY OF THE ALTERING OF CYTOKINES AND GROWTH FACTORS SECRETED BY CULTURED BALDING DERMAL PAPILLA CELLS

Mrs. Ratchathorn Panchaprateep

A Dissertation Submitted in Partial Fulfillment of the Requirements

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รัชต์ธร ปัญจประทีป : การศึกษาการเปลี่ยนแปลงของการหลั่งสารไซโตไคน์และโกรทแฟคเตอร์ ของเซลล์เดอร์มอลพาพิลาจากหนังศีรษะของผู้ชายที่มีภาวะผมบางจากพันธุกรรม (THE STUDY OF THE ALTERING OF CYTOKINES AND GROWTH FACTORS SECRETED BY CULTURED BALDING DERMAL PAPILLA CELLS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ประวิตร อัศวานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ.นพ.ถนอม บรรณประเสริฐ, 107 หน้า.

ที่มา จากผลการศึกษาที่ตีพิมพ์ในปัจจุบัน พบว่าการเปลี่ยนแปลงของการหลั่งสาร cytokines/ growth factors ของเซลล์เดอร์มอลพาพิลาที่ได้รับอิทธิพลจากฮอร์โมนแอนโดรเจน เป็นสิ่งสำคัญในกลไกการเกิดภาวะผมร่วงจากพันธุกรรม การศึกษานี้ได้ทำการตรวจหาความแตกต่างของการหลั่งสาร cytokines/ growth factors เหล่านี้จากเซลล์เดอร์มอลพาพิลา บริเวณหนังศีรษะด้านหน้า เทียบกับบริเวณด้านหลังของหนังศีรษะในผู้ชายที่มีภาวะผมบางจากพันธุกรรม ซึ่งพบว่าสาร neurotrophins (NTs) ได้แก่ brain-derived nerve factor (BDNF), neurotrophin (NT)-3 และ beta-nerve growth factor (NGF) หลั่งจากเซลล์ เดอร์มอล พาพิลาบริเวณด้านหน้ามากกว่าด้านหลังอย่างมีนัยสำคัญทางสถิติ

วิธีการศึกษา ได้ทำการศึกษาเป็น 2 ระยะ โดยระยะที่ 1 ดูผลของสาร NTs ทั้ง 4 คือ BDNF, NGF, NT-3 และ NT-4 ที่ความเข้มข้นต่างๆ ต่อการเจริญเติบโตของเซลล์เดอร์มอลพาพิลาบริเวณหนังศีรษะด้านหน้า เพาะเลี้ยงในหลอดทดลอง ด้วยวิธี MTT assay และการเจริญเติบโตของเส้นผมในระยะ anagen โดยการวัดความยาวในหลอดทดลอง ส่วนการศึกษาในระยะที่ 2 ดูผลของแอนติบอดี้ต่อสาร NTs ในการหักล้างผลของ NTs

ผลการศึกษา ระยะที่ 1 เซลล์เดอร์มอลพาพิลาที่ 2 วันหลังเพาะเลี้ยง ในกลุ่มที่ได้รับสาร NGF, NT-3 และ NT-4 ที่ความเข้มข้น 10ng/mL และ NT-4 0.1ng/ mL มีจำนวนน้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ และที่ 4 วัน กลุ่มที่ได้รับสาร NGF และ NT-4 ที่ความเข้มข้น 0.1, 1 และ 10 ng/ mL มีจำนวนเซลล์น้อยกว่ากลุ่มควบคุมอย่างมีนัยสำคัญตามความเข้มข้นของสารที่มากขึ้น ในการทดสอบผลต่อการเติบโตของเส้นผมในหลอดทดลอง พบว่าความยาวของเส้นผมในกลุ่มที่ได้รับสาร BDNF และ NGF ที่ความเข้มข้น 1ng/ mL สั้นกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ โดยมีค่า *P*-value เท่ากับ 0.003 และ0.05 ตามลำดับ ส่วนผลการศึกษาระยะที่ 2 แอนติบอดี้ต่อสาร NTs ทั้ง 4 ตัว แสดงแนวโน้มที่จะออกฤทธิ์หักล้างผลของ NTs ที่มีฤทธิ์ยับยั้งการเจริญเติบโตของเซลล์เดอร์มอลพาพิลา และเส้นผม อย่างไรก็ตามการเปลี่ยนแปลงไม่พบมีนัยสำคัญทางสถิติ

สรุปผลการศึกษา: สาร NTs ที่หลั่งจากเซลล์เดอร์มอลพาพิลาที่ได้รับอิทธิพลจากฮอร์โมนแอนโดรเจน สามารถยับยังการเจริญเติบโตของเซลล์เดอร์มอลพาพิลาเพาะเลี้ยง และเส้นผมในหลอดทดลอง ซึ่งแสดงให้เห็นว่าสาร NTs นี้เป็นสาระสำคัญตัวหนึ่งในการก่อให้เกิด ภาวะผมบางจากพันธุกรรม

ภาควิชา	อายุรศาสตร์	ลายมือชื่อนิสิต	
สาขาวิชา	อายุรศาสตร์	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	
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RATCHATHORN PANCHAPRATEEP: THE STUDY OF THE ALTERING OF CYTOKINES AND GROWTH FACTORS SECRETED BY CULTURED BALDING DERMAL PAPILLA CELLS. ADVISOR: ASSOC.PROF. PRAVIT ASWANONDA, M.D., CO-ADVISOR: TANOM BUNPRASERT, M.D. 107 pp.

BACKGROUND: Recent studies established that androgen-driven alteration to signals produced by dermal papilla (DP) cells may play an important role in androgenetic alopecia. Here we screened the difference in the protein expression of growth factors/ cytokines in balding vs nonbalding DP cells from the same individuals affected with AGA and found neurotrophins (NTs), namely brain-derived nerve factor (BDNF), neurotrophin (NT)-3 and beta-nerve growth factor (NGF), were the most upregulated protein.

OBJECTIVES: To further investigate the effects of four recombinant human (rh) NTs: BDNF, NGF, NT-3 and NT-4 on the proliferation of cultured balding DP cells and hair follicle (HF) growth in vitro. We also assessed whether the neutralizing NTs antibody can reverse the negative effects of NTs.

MATERIALS AND METHODS: We evaluated the effects the effects of various concentrations of rhNTs on the proliferation of cultured balding DP cells by MTT assay at day 2 and 4. We also measured elongation of anagen hair follicles in organ culture. Furthermore, the reversible effects of NTs were investigated when the neutralizing NTs antibody was added.

RESULTS: NGF, NT-3 and NT-4 at concentration of 10ng/mL and NT-4 at 0.1ng/ mL significantly inhibited proliferation of DP cells at day 2. The more inhibitory effects were observed on day 4, NGF and NT-4 at concentration of 0.1, 1 and 10 ng/ mL showed significantly inhibit DP cells proliferation in dose dependent manner. Moreover, after 10-day of HF organ culture, 1ng/ mL BDNF and NGF significantly inhibited hair shaft growth by 610.2 μm,-44.3% (P=0.003) and 395.7μm, -28.7% (P=0.05), respectively compared with control. Lastly, all NTs neutralizing antibodies failed to reverse NTs-induced cultured DP cells and hair follicles growth inhibition. However, we could observe reversible trend from our results.

CONCLUSIONS: NTs produced by balding DP cells inhibit the proliferation of DP cells and human hair follicle growth in vitro. This data, together with the higher expression of NTs in balding compared with nonbalding DP cells suggests that NTs may be one of the key factors involved in pathogenesis of AGA.

Department: Medicine Student's Signature .....

Field of Study: Medicine Advisor's Signature .....

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Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

AGA	Androgenetic alopecia
Ab	Antibody
DHT	Dihydrotestosterone
DP	Dermal papilla
DPC	Dermal papilla cell
BDNF	Brain-derived nerve factor
bFGF	Basic fibroblast growth factor
CTS	Connective tissue sheath
FGF	Fibroblast growth factor
GF	Growth factor
HF	Hair follicle
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IRS	inner root sheath
KC	Keratinocyte
KGF	Keratinocyte growth factor
NGF	Nerve growth factor
NT	Neurotrophin
OPG	Osteoprogerin
ORS	Outer root sheath
rh	Recombinant human
T	Testosterone
TGF	Transforming growth factor
VEGF	Vascular endothelial growth factor

CHAPTER I
INTRODUCTION

1.1 Background and rationale

Androgenetic alopecia (AGA) or male pattern baldness is the most common type of hair loss and at least half of the male population is affected at some point in their life. AGA is invariably typified by recession of the bitemporal hairline and in some cases coupled by thinning of the vertex hair and later two areas are joined together like a horseshoe-shape resulting in "patterned" hair loss. To date, the exact molecular mechanism of AGA is not fully understood. This is known to be due to genetic (polygene involvement) and hormone modification resulting in higher expression of 5-α reductase in the balding area and higher expression of aromatase in the occipital hair follicles (1).

Androgens modulate hair growth by their actions on several targets, whereby one of the most important "players" in AGA is the mesenchyme-derived dermal papilla (DP) cell. DP cell reside at the base of the epithelial portion of hair follicle and has a crucial role in epidermal-mesenchymal interaction. These cells are thought to function as "inducer" sending signals to regulate hair growth, control hair cycle and renewal of neighboring tissues, while epithelial cells works as "responder". Itami and colleagues reported that androgens stimulate proliferation of outer root sheath keratinocytes when cocultured with beard DP cells but not nonbalding DP cells (2).

Besides hormonal regulations, hair follicles are also under control of neural and immunologic stimulations as well as autocrine/ paracrine factors and even inflammatory mediators. Several cytokines/growth factors, such as insulin-like growth factor (IGF-1), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and transforming growth factor (TGF)-βsecreted by DP cells are known to be involved in hair cycling. These have been elegantly studied by several groups (3, 4), however, most studies have been performed in mice (5-7), and very few in men. Moreover, most were done during morphogenesis and "normal" hair cycling. Whether the same paradigm occurs in

diseased human hair with AGA, under the effects of androgens as occurs in pattern baldness is not known. The exact molecular mechanisms underlying androgen related actions in AGA remain largely elusive, let alone the complete "big picture" of the entire cascade. This may explain the paucity of available treatment modalities, limited only to 5-α reductase inhibitors and topical minoxidil that US-FDA approved. Recent publications have shown that mediators secreted from dermal papillae in AGA patients include insulin-like growth factor (IGF)-1, which acts as a mitogenic factor for hair growth (8) and transforming growth factor (TGF)-β and DKK-1as negative paracrine factors (9). Apart from the structural changes of hair follicles and hair shafts, advanced AGA is characterized by shortening of the hair cycles. Numerous cytokines and growth factors have been shown to accelerate catagen formation. Some of these growth factors are produced by DP cells.

In this study we sought to determine the difference in the expressions of these growth factors/cytokines in balding vs. nonbalding scalp specimens from the same individuals with AGA using Quantibody Growth Factor Array. The specific effect of these novel and interesting mitogenic and inhibitory factors on the DP cell proliferation and hair follicle elongation in hair organ culture system are also further investigated.

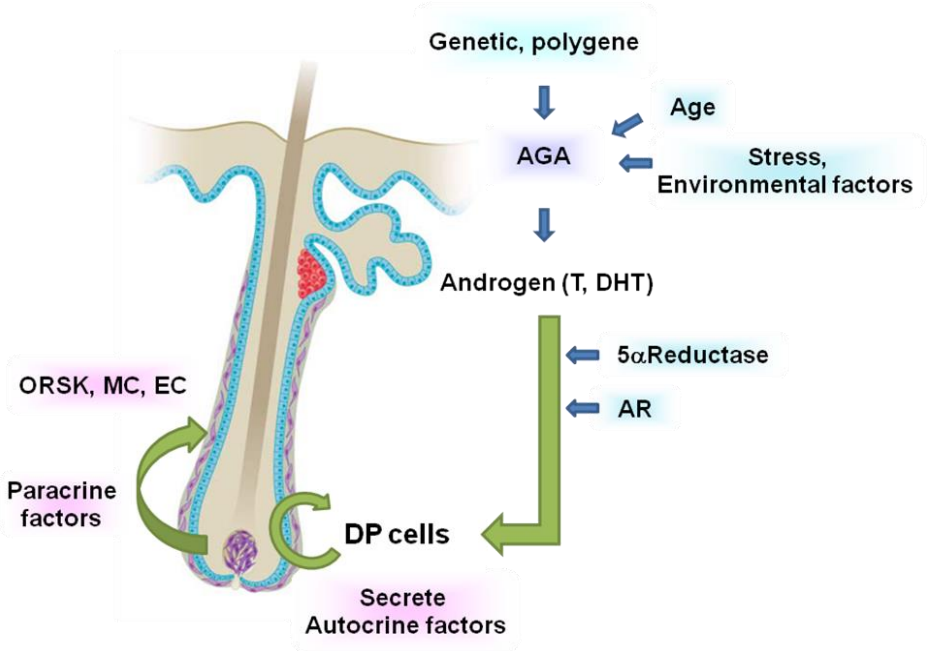
1.2 Hypothesis of the studies

1. The level of growth factors secreted by balding and nonbalding DP cells from AGA patients are different.
2. These mitogenic and inhibitory factors have effects on cultured balding DP cell proliferation in vitro.
3. These mitogenic and inhibitory factors have effects on hair follicle elongation in hair organ culture system.
4. The neutralizing antibodies can reverse the negative effects of the altering inhibitory factors on cultured balding DP cell proliferation in vitro.
5. The neutralizing antibodies can reverse the negative effects of the altering inhibitory factors on hair follicle elongation in hair organ culture system

1.3 Objectives

1. To determine the difference in the expressions of growth factors/ cytokines in balding versus nonbalding scalp specimens from the same individuals affected with AGA.
2. To assess whether these altering factors have effects on cultured balding DP cell proliferation in vitro
3. To assess whether these altering factors have effects on hair follicle elongation in hair organ culture system.
4. To assess whether these neutralizing antibodies can reverse the negative effects of the altering inhibitory factors on cultured balding DP cell proliferation in vitro.
5. To assess whether these neutralizing antibodies can reverse the negative effects of the altering inhibitory factors on hair follicle elongation in hair organ culture system.

1.4 Conceptual frameworks



1.5 Assumption

The level of growth factors secreted from balding and nonbalding DP cells, the characteristics of hair follicle and dermal papilla cells among AGA affected patients are the same.

CHAPTER II
LITERATURE REVIEW

2.1 Hair follicle (HF)

Hair severs many functions, its primary roles are insulation and protection from elements in mammals, however, in humans, it is extremely important to one's social and psychological equilibrium.

2.1.1 Structural of hair follicle and pilosebaceous unit

HFs are different varied to sites, age, racial and individual variation as well as season. All hair follicles follow a common architecture. Together with the sebaceous gland (*SG*) and the arrector pili muscle (*M*), the hair follicle is part of the so-called pilosebaceous unit.

At its most basic, HF anatomy can be made between the permanent, superficial structure and the transient cycling component of the hair follicle. The permanent portion of the hair follicle, the part that extends from the skin surface down to lower end of the bulge region, does not undergo significant cyclic changes (10). Furthermore, HF can be divided according to its anatomy into 3 parts: infundibulum, isthmus and inferior parts (figure 1).

1. Infundibulum: superficial part starts from follicular the opening to opening of SG. The superficial part of the hair follicle infundibulum, the acro-infundibulum, is lined by intact epidermis including a well-developed stratum corneum and a stratum granulosum.
2. Isthmus: between the opening to opening of SG and insertion of arrector pili (AP) muscle. The insertion site of the arrector pili muscle is called "bulge region", a specialized compartment of the outer root sheath (ORS) which forms a niche for hair follicle stem cells.
3. Inferior: the lowest part of HF which extends from the bulge to the base of the hair follicle "bulb".

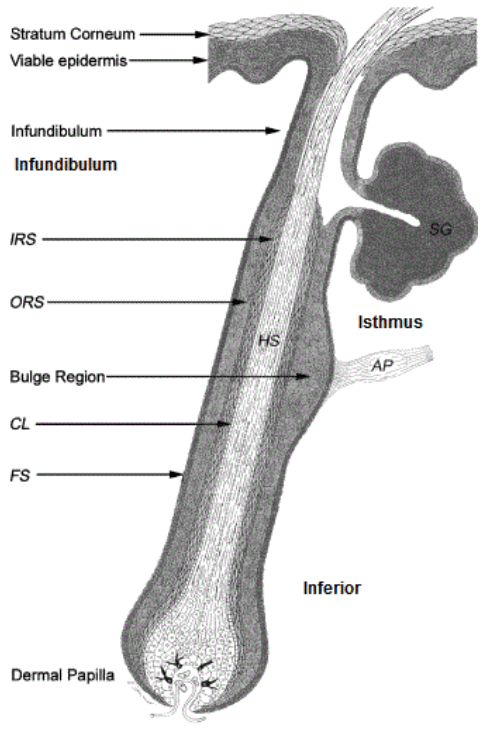


Figure 1 Longitudinal section of anagen HF. The fibrous sheath (*FS*) and the outer and inner root sheaths (*ORS, IRS*) form concentric layers, which ensheath the hair shaft (*HS*).

Microstructure

Microstructure of HF composes of 3 parts: epithelial part, hair bulb and hair shaft (11).

1. **Epithelial part:** this part continues from epidermis. It composed of keratinocytes which differentiate to, from outer most to innermost: ORS, IRS and companion layer. Each cell is characterized by distinct expression of the hair follicle-specific keratins, keratin associated protein, trichohyalin, adhesion and matrix molecule (12).
    - Outer root sheaths: The ORS is continuous with the basal layer of epidermis at the infundibulum and down to the bulb. ORS keratinocytes form the bulge at the base of isthmus in 3-4 layers. These cells are generally cuboidal shape, possess a higher nuclear to cytoplasmic ratio. Moving downward, ORS becomes much larger and contains abundant glycogen. In the bulb, ORS consisted of only 1-2, flattened cell layers.

- Inner root sheath: IRS extends from the base of HF to the level of opening of SG of the isthmus portion. It composed of 3 parts from outermost to innermost: Henle's, Huxley's and IRS cuticle. Henle's layer is single-layer thick and the first to keratinize and develop keratohyaline granules. Huxley's layer composes of 2-4 layers or KCs and keratinizes above Henle's layer at the region called Adamson's fringe.
- Companion layer: it attaches to Henle's layer and provide a slippage plane between the stationary ORS and upward moving IRS.

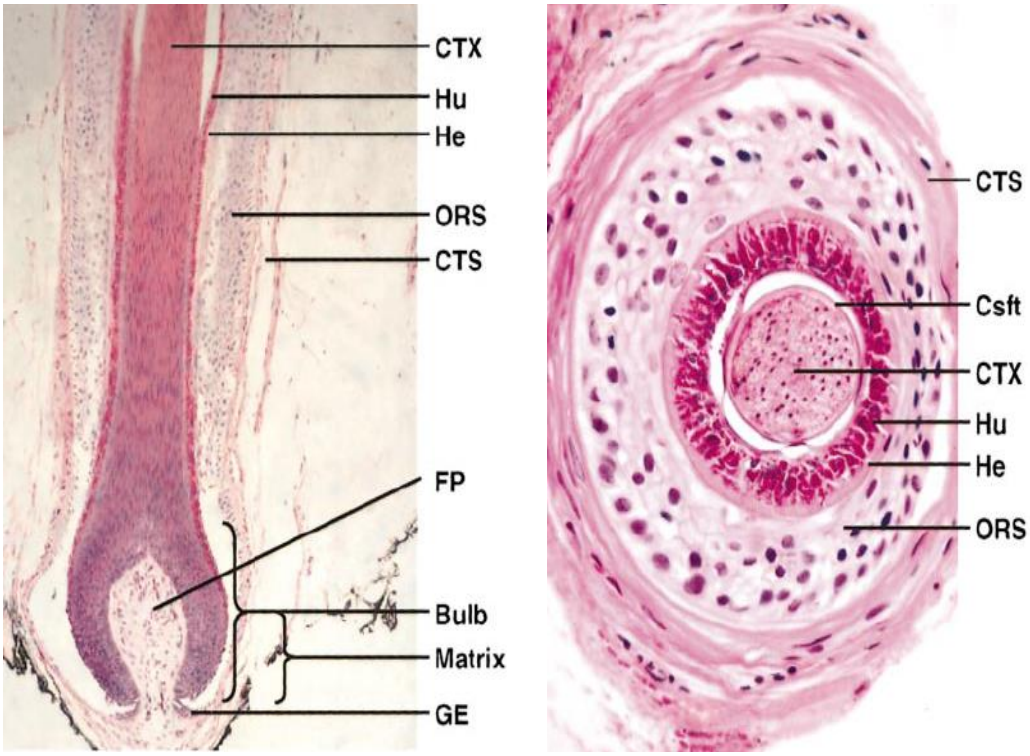


Figure 2 Hair anatomy in longitudinal section (left) and cross-sectional section (right). (CTX= cortex, Hu = Hunle's layer, He = Huxley's layer, FP = follicle papilla, GE = germinal epithelium, CTS = connective tissue sheath)(13)

2. **Hair bulb:** located at the base of hair follicle. It composed of matrix cells, dermal papilla (DP) และ dermal sheath (DS).

- Matrix keratinocytes are rapid dividing cells which give rise to the hair shaft and the inner root sheath. They are localized in the bulb where they sit on the dermal papilla.
- Dermal Papilla is the tear-shaped, condensate of specialized mesenchymal cells in the sub-bulbar region. DP cells are embedded in extracellular matrix (ECM) and contain their own blood supply. DP cells produce and secrete several growth factors to hair matrix cells and stimulate their proliferation and differentiation (14, 15). Most researchers think that DP cells play essential roles in the induction of new hair follicles and maintenance of hair growth. From previous study, surgical removal of the dermal papilla and the lower dermal sheath prevents hair growth (16, 17).
- Dermal sheath or connective tissue sheath. It composed of 3 layers of woven collagen fibers and fibroblasts in the thick middle collagen layer. Follicle DS cells act as specialized progenitor fibroblast populations that are reservoirs for DP cells (18), induce new hair follicle formation (19) and activated in response to wounding (20).

3. **Hair shaft**: arises from rapidly proliferating matrix keratinocytes. It composed of 3 layers of cuticle, cortex and medulla
    - Medulla: sits at the center of large or terminal hairs, not in vellus or fine hairs
    - Cortex: arrange in large, cable-like shape structures called macro fibrils.
    - Cuticle: composes of 6-10 layers of overlapping cuticle cells. It provides integrity of hair shaft.

2.1.2 Hair cycle

The average number of hairs in the adult scalp is 100,000, of which 85-90% are in anagen, 1% in catagen and 10% in telogen (21). The average duration of anagen is 2-6 years, of catagen is 2-3 weeks, of telogen is 3 months, and of exogen is weeks to months.

Hair cycle is the morphological evidence of rhythmically re-occurring growth, regression and tissue re-modeling events in this complex neuroectodermal-mesodermal interaction system. Each hair follicle undergoes cycle 10-20 times in a lifetime. The hair cycle is composed of 4 phase including anagen (active growth), catagen (regression/ involution), telogen (resting) and exogen phases (figure 3).

Anagen to catagen

The anagen bulb produces the hair shaft and the inner and outer root sheaths. The inner root sheath molds the hair shaft in the lower follicle and is shed in the isthmus region of the follicle. Several years of hair growth, HF receive signal to enter catagen phase. The matrix cells stop proliferating and the production of melanin. The DP contracts and is released from follicle. The middle portion of follicle constricts and the lower portion expands to become bulb. The lower portion retracts up to the level of the bulge, leaving behind a streamer of undifferentiated epithelial cells or fibrous tract. The mechanism of retraction is due to apoptosis or control cell death (22). Although the exact signals for catagen induction are not known, there are 7 important morphogenetic molecular families implicated, in animal models, including fibroblast growth factor (FGF), transforming growth factor (TGF)-beta, sonic hedgehog (Shh), Wingless or Wnt pathway (Wnt), BMP, Neurotrophins and homeobox (hox) gene family(23, 24) (figure 4)

Catagen to telogen

The catagen phase is generally less than 4 weeks. It is difficult to distinguish it from early telogen. There is no specific molecular signaling characterized at this time.

Telogen to anagen

During telogen, the epithelial streamer moves upward and forms a short projection underneath the club hair called the secondary hair germ. The dermal papilla shrinks to its smallest size during telogen. At some point, anagen phase is initiated by complex signals between DP, secondary hair germ and hair follicle stem cells in bulge area. Although exact

molecular controls of hair follicle cycling are still not fully understood (23, 24), most evidences support “Bulge activation hypothesis” that entry into anagen occurs when stem cells in the bulge region are activated by signals from the DP. Then transient-amplifying (TA) cells in matrix area differentiate to cell products (4). With a new anagen cycle, the follicle follows the papilla down the fibrous tract, and the papilla generally enlarges once again (4, 13).

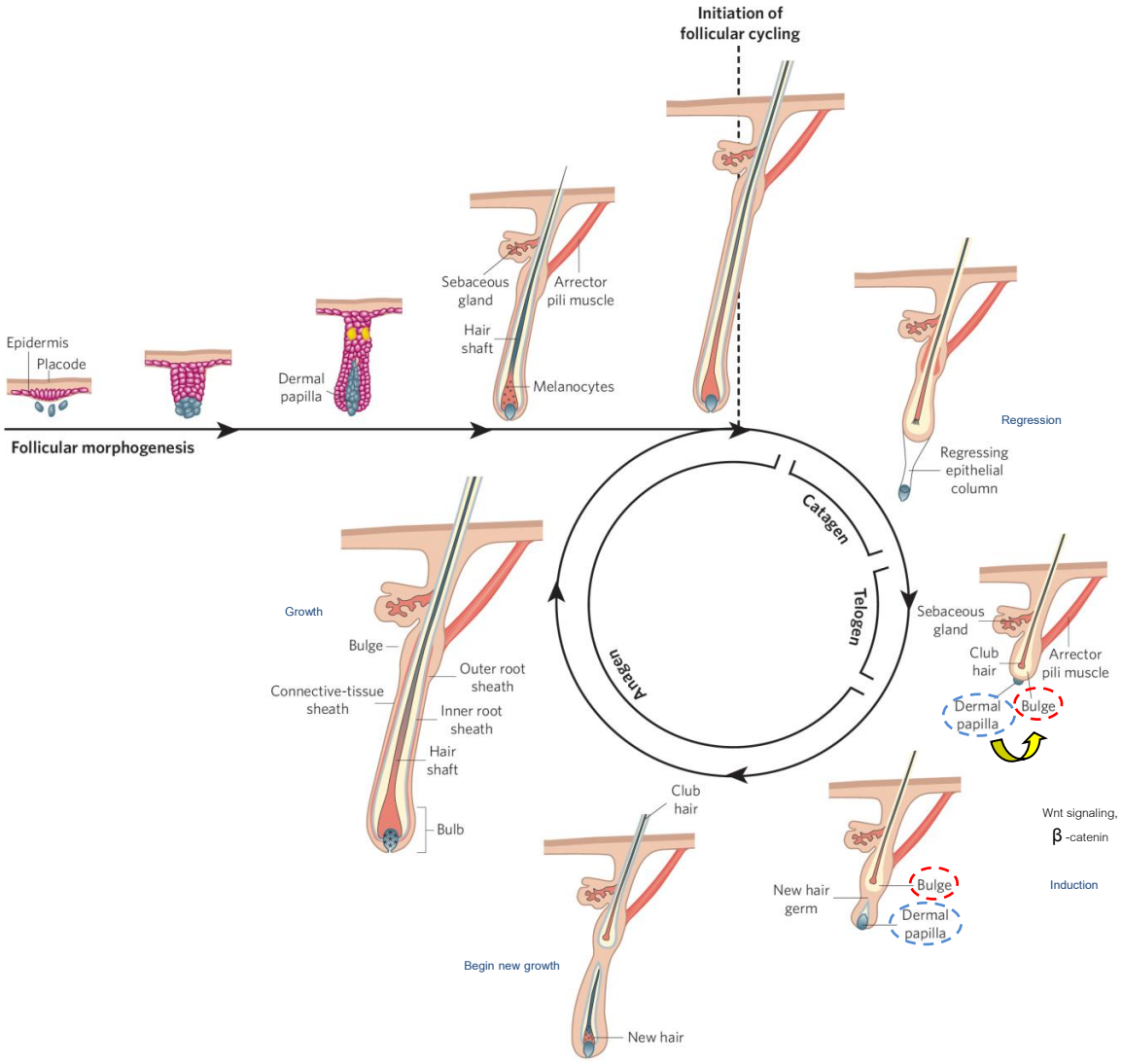


Figure 3 Hair follicle development and cycling. There is no clear distinguish between the end of follicular morphogenesis and the beginning of the first follicle growth cycle (25). Many

studies suggest that the first true cycle begins after morphogenesis with the first catagen (26) or the first full anagen (27).

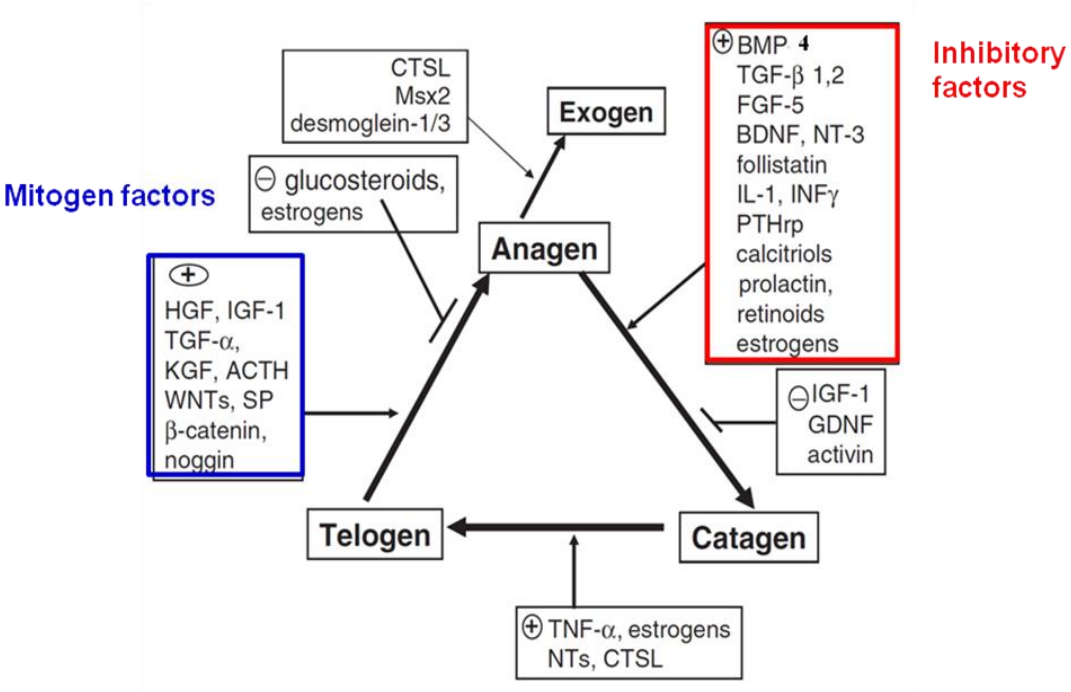


Figure 4 Hair cycle-modulatory effects of endogenous cytokines, growth factors, hormones, neuropeptides and enzymes, recognized as in mice (4, 23, 24, 28)

Exogen

The exogen phase begins with the complete formation of club hair and ends with its release.

2.1.3 Types of hair

Hair can be categorized according to its size into 2 groups:

1. Terminal hair: are larger hairs with a diameter exceeding 0.06 mm and a length longer than 1 cm. They are often pigmented and medullated
2. Vellus hairs: are small, soft, non-pigmented, non-medullated hairs with diameter less than 0.03 mm and a length shorter than 1 cm. The term vellus hair is often include true vellus hairs at certain area of body such as face and miniaturized hair by AGA.

2.2 Androgenetic alopecia

Androgenetic alopecia (AGA), or pattern hair loss, is the most common disorder in both men and women all around the world. The name has evolved from its presumed dependence on androgens and genetic factors. It is characterized by a progressive hair miniaturization whereby large, pigmented terminal hairs are replaced by fine, less pigmented vellus hairs and shortening anagen phase of hair follicle (13).

Medically, alopecia is viewed as a relatively mild dermatological condition. However, people suffering from the condition feel that alopecia is a serious condition with major distress on social life (29).

2.2.1 The prevalence of AGA

The prevalence of AGA varies widely. Several authors have reported an incidence greater than 96% in Caucasian men (30-32). The prevalence of male pattern baldness increases with age: 53% of men 40 to 49 years of age are affected with AGA, in contrast to 16% of men 18 to 29 years of age (33). While in Asia, reported incidences are lower at 73% among general population (34). The reported prevalence of cosmetically significant MPB (Norwood III-VII) in Thailand is 38.52% and steadily increases with age, approaching that of Caucasians (35).

Female pattern hair loss (FPHL) is less common than male balding but the prevalence still increase with age. 2-5% of women by the age of 30 years and nearly 40% at the age of 70 show some thinning of hair. FPHL begins in women at the late 20s and reach its peak after 50 years of age (36).

2.2.2 Clinical features

Male pattern hair loss (MPHL)

Male pattern alopecia often presents in the first decade after puberty and is characterized by deep bitemporal recession and loss of hair on the vertex. Hairs become

shorter and thinner, ultimately complete loss except the lateral and posterior scalp (figure 5). Small portion of men show diffuse hair loss that called diffuse, unpatterned alopecia (DUPA). Hamilton (30) produced the first grading scale for Caucasian men and women, ranging from type I to VIII. Norwood (37) modified Hamilton's classification which is widely used until now.

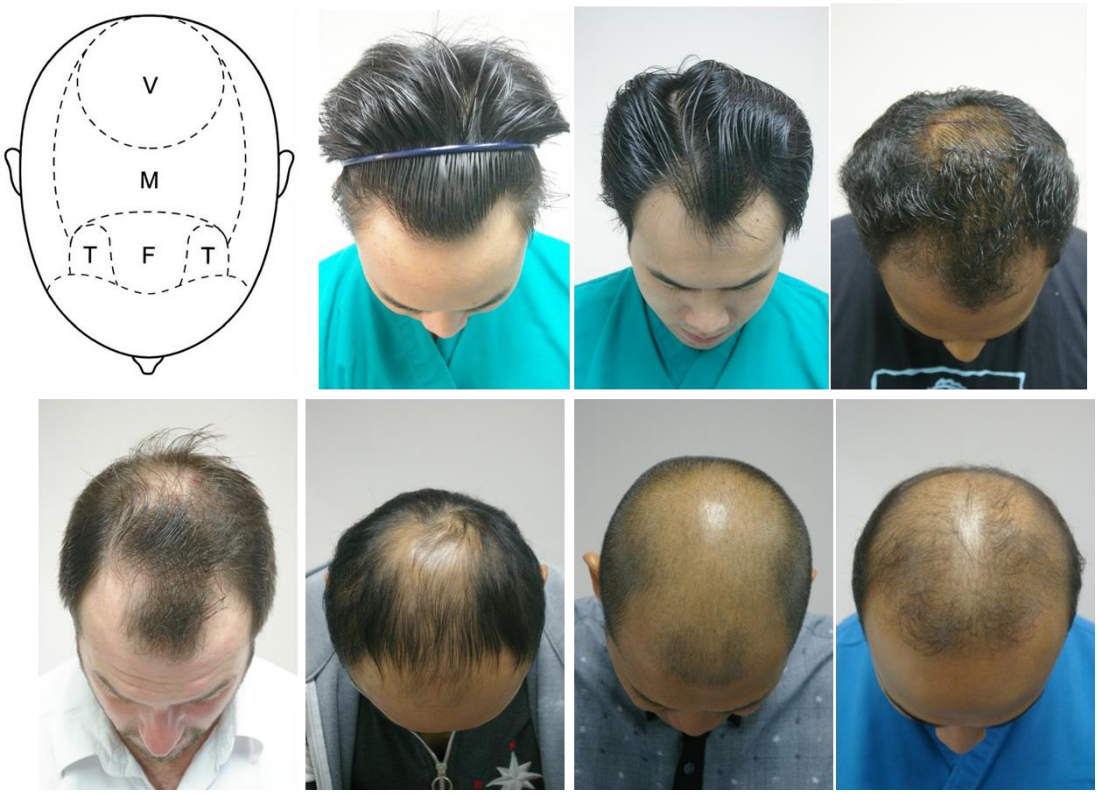


Figure 5 Clinical characteristic of male pattern baldness

Female pattern hair loss (FPHL)

FPHL often presents a decade later than male pattern alopecia and is characterized by either an intact frontal hairline and a diffuse thinning over the crown (Ludwig pattern)(38) or a frontal accentuation of hair loss (Christmas tree pattern) (39). Hair thinning is more extensive on the top than on the back and sides of the head. Marked bitemporal recession is not usually a feature of female pattern hair loss, and often there is no temporal recession (figure6).

Furthermore, actual balding such as that seen in men is rare, and scalp hair thinning is far more common in women who are not severely virilized. Those women with a Ludwig pattern of hair loss show no obvious virilization.

Figure 6 Clinical characteristic of female pattern hair loss classified by Ludwig pattern

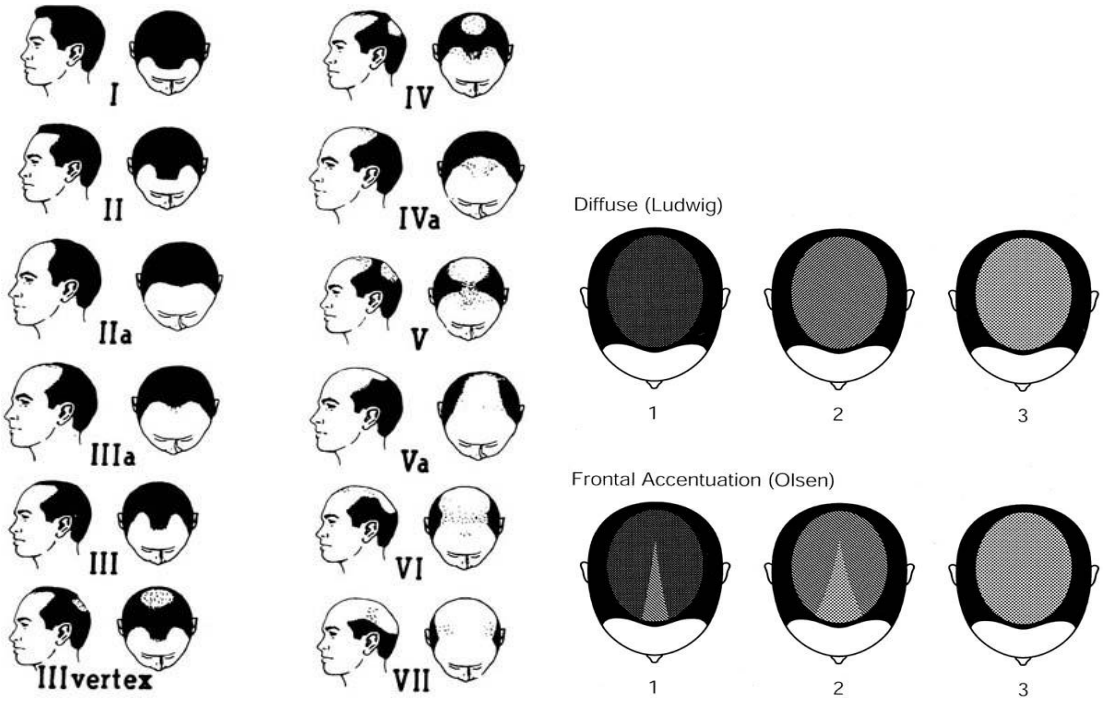


Figure 7 Severity classification of both male and female pattern alopecia. Left is Hamilton-Norwood pattern and right are diffuse (ludwid) and frontal accentuation (olsen) patterns

2.2.3 Diagnosis of AGA

The diagnosis of AGA in men is usually not difficult. The hair loss in AGA is nonscarring which the follicular openings are preserved. It is characterized by its special pattern; by a variation in hair shaft diameter; and by the occurrence of miniaturized, vellus-like hairs that sometimes can be seen only with a magnifier or a dermatoscope.

2.2.4 Pathogenesis of AGA

There are 3 main etiologies of AGA: genetics, androgens and micro inflammation.

2.2.4.1 Genetic involvement

Polygene is most likely to explain mode of inheritance. Multiple genes are involved as follows:

- Androgen-receptor genes (AR): is located on X-chromosome and men inherit it form their mother. It is considered as the most important because higher expression of AR has been found in balding compared to nonbalding scalp (40) .
- 5α-receptor genes (SRD5A1, SRD5A2): regulated 5α-reductase (5αR) enzyme. 5α-reductase type 1 is located on chromosome 5 and type 2 on chromosome 2 (41)
- Aromatase gene (Cyp19)

2.2.4.2 Androgens

The role of androgens in MPHL has been considered since an observation of Hamilton. Eunuchoids and castrated men did not experience baldness. After administration of testosterone, baldness developed in predisposed individuals (30).

Testosterone (T) is the major circulating androgen in men, however dihydrotestosterone (DHT) is the principle causative agent. The conversion of T to DHT is catalyzed by the 5α-reductase enzyme. There are 2 5αR isozymes, type I and II, encoded by different genes. Even though both isozymes converse T to DHT, they differ in their pH

optima, substrate affinities and tissue distribution. Type I 5αR is widely distributed in the skin as well as liver, adrenal glands and kidneys (42). Type II 5αR has been found in DP, inner layer of ORS, SG and proximal IRS of scalp HFs. Furthermore, it is also found in prostate, testis and liver. The important of 5αR in AGA is supported by the absence of baldness in subjects with type II 5αR deficiency(43).

The skin is also an endocrine target tissue for androgen hormones (32). The circulating androgens dihydroepiandrosterone sulfate (DHEA-S) and androstenedione are produced predominantly in the adrenal glands, and T and DHT are synthesized mainly in the gonads (44). DHEA-S and androstenedione have a relatively weak androgen potential. They can be metabolized to more potent androgens, such as T and DHT. The androgen metabolic pathways are shown in figure 8 with six enzymes involved in the each step.

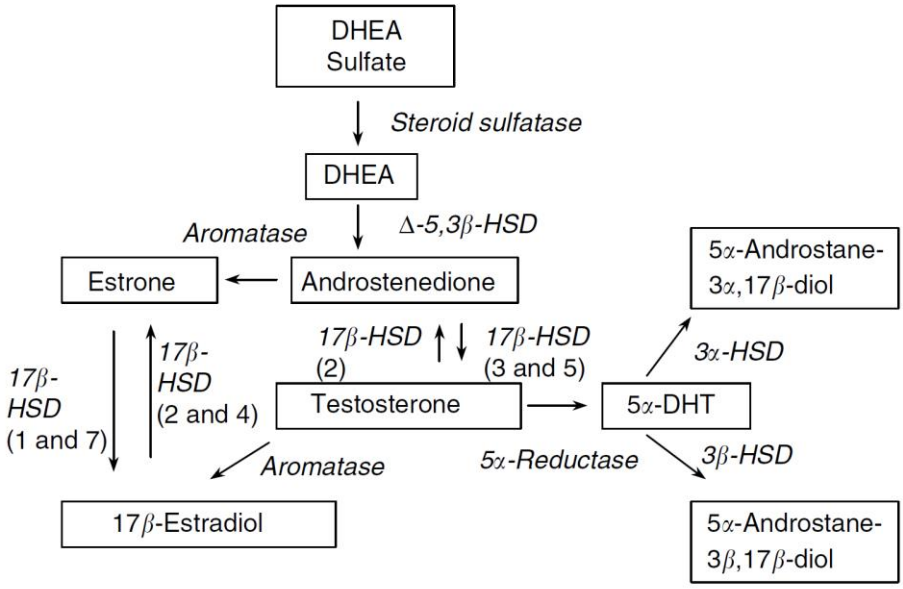


Figure 8 Metabolism of androgens in human skin

Androgens and hair growth

HF is considered as a paradoxical androgen target organ. After puberty, androgens promote transformation of vellus HFs in many areas, e.g. the beard, pubic and axilla, to terminal ones. While on the scalp, it is opposite, androgens lead to the replacement of

terminal hairs by vellus ones in genetic predisposed people. However, they have no apparent effect on the eyelashes and occipital scalp (45).

Molecular mechanism of androgenetic alopecia

AGA is characterized by progressive shortening of the duration of anagen, leading to decreased numbers of hair in anagen at any given time, and progressive follicular miniaturization (13). The telogen phase is also prolonged in AGA, which leads to increase in shedding of short-lived telogen hairs (telogen effluvium). Moreover, AGA involves a process of premature catagen induction.

In association with the changes in hair cycle dynamics, the entire follicular apparatus is progressively miniaturized in AGA, making hair significantly finer and more susceptible to falling out. The follicular miniaturization occurs between anagen cycles and is global, affecting the papilla and the matrix, as well as the hair shaft (39). Therefore, it is important to dissever the molecular controls of the anagen–catagen transformation of the hair cycle (26) and of follicular miniaturization.

DP is a target HF structure of androgens (46). Circulating testosterone enters HF via DP's blood supply (capillaries) and catalyze by 5α-reductase to DHT. When DHT binds to AR in DP cells, the changes in gene transcription and protein expression are result (47). Therefore many researches are now concentrated on identifying androgen-regulated factors deriving from dermal papilla cells.

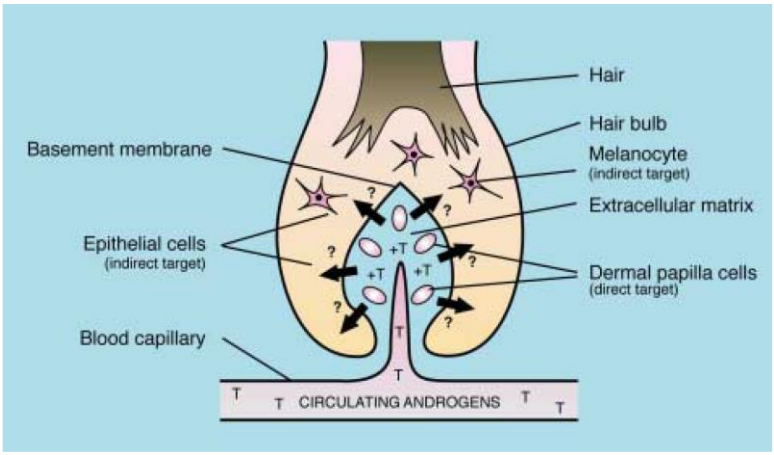


Figure 9 Effect of androgen on hair follicle via DP.

Cytokines/ growth factors and hair growth

Of all several cytokines/ growth factors that have been suggested to play a role in HF growth, only 5 factors have been reported as altered in vitro by androgens. (table1)

1. Insulin-like growth factor (IGF-1)(8)

Itami et al. reported that androgen significantly stimulated the proliferation of ORS cells that are cocultured with beard DP cells without cell contact. However ORS cells do not show significant increase in number when they are cocultured with non-balding DP cells. Furthermore, the expression of IGF-I mRNA in beard DP is stimulated by androgen and antagonized by cyproterone acetate, while ORS cells do not express mRNA for IGF-I either in the presence or absence of androgen. Neutralizing antibody against IGF-I antagonizes the stimulatory effect of androgen on the growth of ORS cells cocultured with beard DP cells. Lastly, IGF-1 receptor knockout mice showed abnormal hair follicular pattern and abnormal hair differentiation.

2. Glia-derived nexin-1 (48)

Protease nexin-1, is a potent inhibitor of serine protease (thrombin, urokinase, plasmin). Its function is regulating cellular growth, differentiation and death of cells by modulating proteolytic activity in many tissues. Protease nexin-1 mRNA is expressed in all dermal papilla cells examined in a hair cycle-dependent fashion and its levels are well correlated with the

ability of dermal papilla cells to support hair growth. Furthermore, it is also identified in the lower part of the connective tissue sheath. The expression of protease nexin-1 mRNA is inhibited by dihydrotestosterone in cultured dermal papilla cells obtained from balding scalp. Tagayasu et al. also suggest that the inhibition of DP cells might cause the decrease in ECM production, e.g. collagen type IV and ultimately the alteration in size of DP and HF.

3. Transforming growth factor (TGF)-β1 and TGF-β2 (49-51)

Inui et al. explored the roles of androgens in regulation of hair growth using an in vitro, human balding DPCs and KCs coculture system. Androgen showed no significant effect on the growth of KCs when they are cocultured with DPCs from AGA because the expressions of mRNA of androgen receptor (AR) decrease during subcultivation of DPCs in vitro. Then they transiently transfected the AR expression vector into the DPCs and cocultured them with KCs, androgen showed significant suppression of the growth of KCs by approximately 50% and the level of total and active TGF-β1 increase in the conditioned medium. The expression of TGF-β1 mRNA is also upregulated in the cocultured DPCs. Moreover, the neutralizing anti-TGF-β1 antibody reverses the androgen-elicited growth inhibition of KCs in a dose-dependent manner. These findings suggest that androgen-inducible TGF-β1 derived from DPCs of AGA is involved in epithelial cell growth suppression in coculture system.

Inoue K et al. compared gene signatures of human DP cells at different passages and human dermal fibroblasts, and found TGF-β2 is highly expressed in cultured DP cells. They also screened the factors secreted from keratinocytes and found that the vitamin D3 analogue promotes TGF-β2 expression and ALP activity of DP cells. In animal models using rat epidermis and cultured human DP cells, inhibition of TGF-β2 signaling strongly impaired hair folliculogenesis and maturation. Their results suggest an important role for TGF-β2 in hair follicle morphogenesis and possibility of future cell therapies for hair regrowth by transplanting expanded DP cells.

In addition, the study by Hibino T et al endorses the role of TGF-β2 in the human hair cycle. DHT stimulates cultured DP cells to secrete TGF-β2 which inhibits the epithelial cells

proliferation through apoptotic cell death. TGF-β antagonists are effective in preventing catagen-like morphological changes and in promoting elongation of hair follicles in vivo and in vitro.

4. SCF/C-kit (52)

Since stem cell factor (SCF) have important roles in maintain hair pigmentation and migration into regenerating hair bulb. Randall VA, et al. investigated SCF/c-Kit signaling in human hair follicles to determine whether this is altered in AGA. Cultured beard DPC secreted more SCF than non-balding DPCs, while balding DPC secreted less SCF than normal cells, measured by ELISA. Androgens appear to reduce alopecia hair colour by inhibiting dermal papilla SCF production, impeding bulbar melanocyte pigmentation. These results may facilitate new treatments for hair colour changes in hirsutism, alopecia or greying.

5. Dickkoft-1 (53)

Kwack MH et al. screened DHT-regulated genes in balding DP cells using cDNA gene microarray and found that dickkopf 1 (DKK-1) is one of the most upregulated genes. DKK-1 is a potent endogenous secreted Wnt antagonist, binding to low-density lipoprotein receptor-related protein co-receptor of Wnt signaling and then inhibiting. They further investigated the effect of DKK-1 and found that DKK-1 messenger RNA is upregulated in 3-6 hours after 50-100 nM DHT treatment and ELISA showed that DKK-1 is secreted from DP cells in response to DHT. A co-culture system using ORS keratinocytes and DP cells showed that DHT inhibits the growth of ORS cells, and neutralizing antibody against DKK-1 significantly reversed the growth inhibition of ORS cells. In addition, DHT-induced epithelial cell death in cultured hair follicles was reversed by neutralizing DKK-1 antibody. Moreover, immunoblotting showed that the DKK-1 level is up in the bald scalp compared with the haired scalp of patients with androgenetic alopecia. From data, researchers suggest that DHT-inducible DKK-1 is involved in DHT-driven balding.

Table1 Summary of growth modulators from androgen-sensitive DP

Hair length & enlargement	
IGF-I	Cultured beard DPC secrete IGF-I in response to androgen
Hair miniaturization	
TGF-β1, TGF-β2	TGF-β1 and 2 secreted from cultured balding DP cells cause KC proliferation inhibition, hair follicle regression, premature catagen induction in response to androgen
Dickkopf-1 (DKK-1)	Cultured balding DPC upregulated DHT-inducible DKK-1 from cDNA microarray. Exogenous DKK-1 induces apoptosis of follicular keratinocytes in co-cultured system
Nexin-1	Androgen inhibit expression of protease nexin-1 from balding DPC → change in production of ECM component of DP → decrease in size
Hair pigmentation	
SCF	Cultured balding DPC secrete less SCF (measured by ELISA) SCF is the ligand for c-kit on melanocytes

2.2.4.3 Hair follicle microinflammation

From histopathology, microscopic follicular inflammation is found at AGA affected hair follicle. It is slow, silent, and painless process in AGA, different to classic inflammation. The implication of various activators of inflammation in the etiology of AGA has progressively and recently emerged from several independent studies (54).

The micro-inflammation process can divided into 3 stages.

1. Inflammatory stage: an inflammatory infiltrate of lymphocytes (activated T cells), macrophages, and langerhans cells are found at infundibulum. It is speculated that several inhabitants of the scalp, such as the ‘‘triad’’ (*Propionibacterium* sp.; *Staphylococcus* sp.; *Malassezia ovalis*) or other members of the transient flora, could be triggers of this complex inflammatory process (55). These pathogens are able to induce the production of pro-inflammatory cytokines by keratinocytes, which are known to

respond to chemical stress, pollutants, UV irradiation or even mechanical stress (56). Not only are radical oxygen species (57), nitric oxide (58) and prostaglandins produced, but also IL-1α, TNFα, TGFβ, IL-8, monocyte chemoattractant protein(MCP)-1, MCP-3 are released (56, 59, 60). By itself, these pro-inflammatory cytokines are able to inhibit the growth of isolated hair follicles in culture *in vitro* (61, 62).

2. Perifollicular fibrosis: T lymphocytes together with mononuclear cells, located in the upper perifollicular adventitial dermal sheath, perpetuate a local inflammatory stage (63). Eventually, a fibroplasia of the dermal sheath and collagen bundle surrounds the hair follicle, especially at active progressive alopecia.
3. Permanent alopecia: if inflammation sustains, it will lead to a terminal process resulting in the miniaturization and then permanent alopecia.

2.2.5 Treatment of AGA

The aim of AGA treatment is.

1. To increase good quality of hairs enough to cover the scalp by restore or regeneration of healthy, normal cycle hair
2. To retard or decrease progression of thinning hairs

Currenly, there are now only two US-FDA approved medications, Topical 2% and 5% minoxidil (Hair growth stimulator) and finasteride at a dose of 1 mg per day (androgen modulator)(64). Finasteride is a competitive inhibitor of type 2 5a-reductase and inhibits the conversion of testosterone to DHT. Because of risk of malformation of the external genitalia of male fetuses, finasteride is contraindicated in women who are or may become pregnant. Minoxidil is a potassium-channel opener and vasodilatator. The exact mechanism in helping hair loss is still unknown. It has been reported to stimulate the production of VEGF in cultured dermal papilla cells (65). There is also a evidence that this effect is mediated by adenosine and sulfonylurea receptors, which are well-known target receptors for adenosine-triphosphate

(ATP)-sensitive potassium channel openers (66). Topical solutions of 2 and 5 % minoxidil are recommended for treatment of AGA in men and women, respectively. Regrettably, the efficacy of minoxidil is variable and temporary, making it difficult to predict the success of treatment on an individual basis.

AGA treatment strategies according to pathologic mechanism (figure 10) (67)

1. Gene therapy (currently not available)
2. Hormone modifier of androgen and its metabolism (available for men)
    a. Finasteride, Lanoprost
    b. Curcuma Aeruginosa extract
    c. Othern Herbal extract
3. Antimicrobial shampoo (controversy)
4. Anti-androgens (available for women)
    a. Androgen receptor protein inhibitors (cyproterone acetate, spironolactone)
    b. Anti-androgen (spironolactone)
    c. Topical estrogen
5. Hair growth promotors (available for both men and women)
    a. Topical Minoxidil 2-5%
6. Anti-inflammatory agents (controversy)
7. Apoptosis modulating agents (currently not available)
8. Hair transplantation, implantation of dermal papilla cells or cells of follicle dermal-sheath (impending)

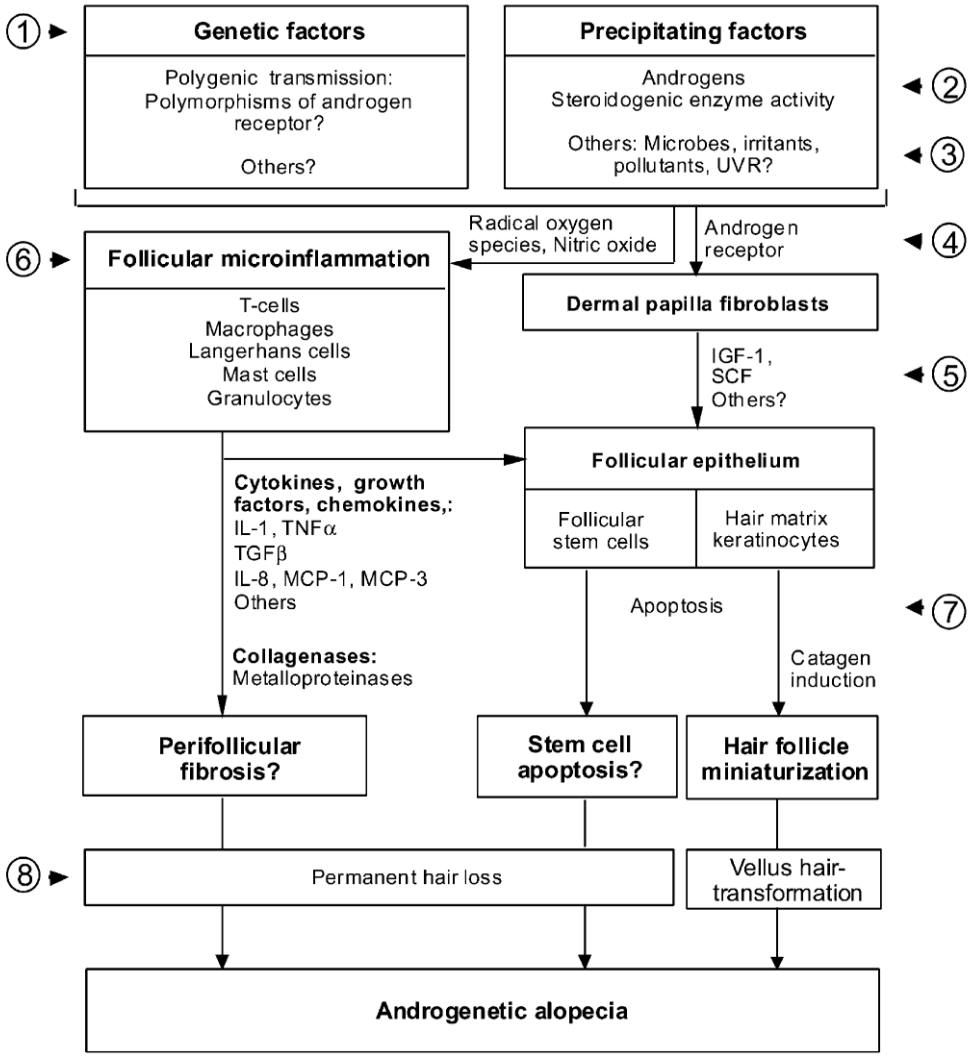


Figure 10 Mechanisms and therapeutic strategies of androgenetic alopecia

2.3 Neurotrophins and hair follicle

Neurotrophins (NTs) belong to a family of growth factors, which control the development, maintenance, and apoptotic death of neurons and also fulfill multiple regulatory functions outside the nervous system particularly the hair follicle and the skin because they share common ectodermal in origin.

2.3.1 NTs and their receptors

The NT family consists of four structurally and functionally related polypeptides: nerve growth factor (NGF), brain derived nerve factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). Mature NT proteins are approximately 13 kDa in size, share about 50% of amino-acid sequence homology, and exert their biological effects as dimers interacting with specific receptors (68). Summary of NTs and their receptors is shown in table 2.

High affinity receptors for NTs belong to the tyrosine kinase family: TrkA, TrkB and TrkC, which are the high affinity receptors for BDNF and NT4, NGF and NT-3, respectively. All NT members bind with the low-affinity p75 kDa NT receptor (p75NTR), a transmembrane glycoprotein which belongs to a family of tumor necrosis factor receptors containing the cytoplasmic ‘‘death’’ domain. It mediates responses independently or in association with Trk receptors (69, 70).

p75NTR performs diverse functions depending on whether it is coexpressed with Trk receptors or whether it is expressed alone. According to current information, the coexpression of p75NTR with Trk receptors leads to signaling through Trk receptors and to enhancement of cell survival by NT stimulation, while expression of p75NTR alone results in stimulation of apoptosis, activation of the nuclear transcription factor kB and modulation of growth factor production by target cells (71, 72).

Table 2 Summary of NTs and their receptors

NTs	High affinity receptors	Low affinity receptors
BDNF	TrkB	p75NTR
NGF	TrkA	p75NTR
NT-3	TrkC (NT-3 may also bind with low affinity to TrkA and TrkB receptors)	p75NTR
NT-4	TrkB	p75NTR

2.3.2 NTs and hair morphogenesis

During progressing HF development, BDNF, NT-3, TrkA, TrkB, TrkC and p75NTR spatiotemporally expression in defined HF compartments, for example, early during HF morphogenesis, TrkA-C are expressed in epithelial hair placode and p75NTR in underlying mesenchymal condensation that develops into follicular dermal papilla. Furthermore, data obtained from genetically engineered mice with overexpression or deletion of NGF, BDNF, and/or NT-3/ TrkC revealed that NGF and NT-3 significantly accelerate HF morphogenesis, whereas BDNF do not show any significant effects (6, 73). On the other hand, p75NTR KO mice accelerates HF development (74).

2.3.3 NTs and hair cycle control

The levels of all NTs expressively fluctuate during hair cycle. NGF increases dramatically in ealry anagen while NT-3, BDNF and NT-4 protein is significantly upregulated prior to and during catagen as determined by semi-quantitative RT-PCR on RNA extracted from full thickness back skin of mice (6).

Botchkarev et al. extensively studied about NTs and hair cycle. The IR expression of NTs and their receptors show spatiotemporal patterns in hair follicle epithelium and mesenchyme. NGF prominently upregulated in follicular epithelial cells during early anagen and BDNF, NT-3 and NT-4 expression are increased during late angagen-catagen. p75NTR show coexpression with TUNEL in the regressing ORS. Therefore, the roles of NTs secreted from DPs and p75NTR in catagen control are hypothesized.

Furthermore, BDNF, NT-3, NT-4 and p75NTR KO mice show catagen retardation, while BDNF, NGF and NT-3 overexpression mice show premature catagen development (73-76).

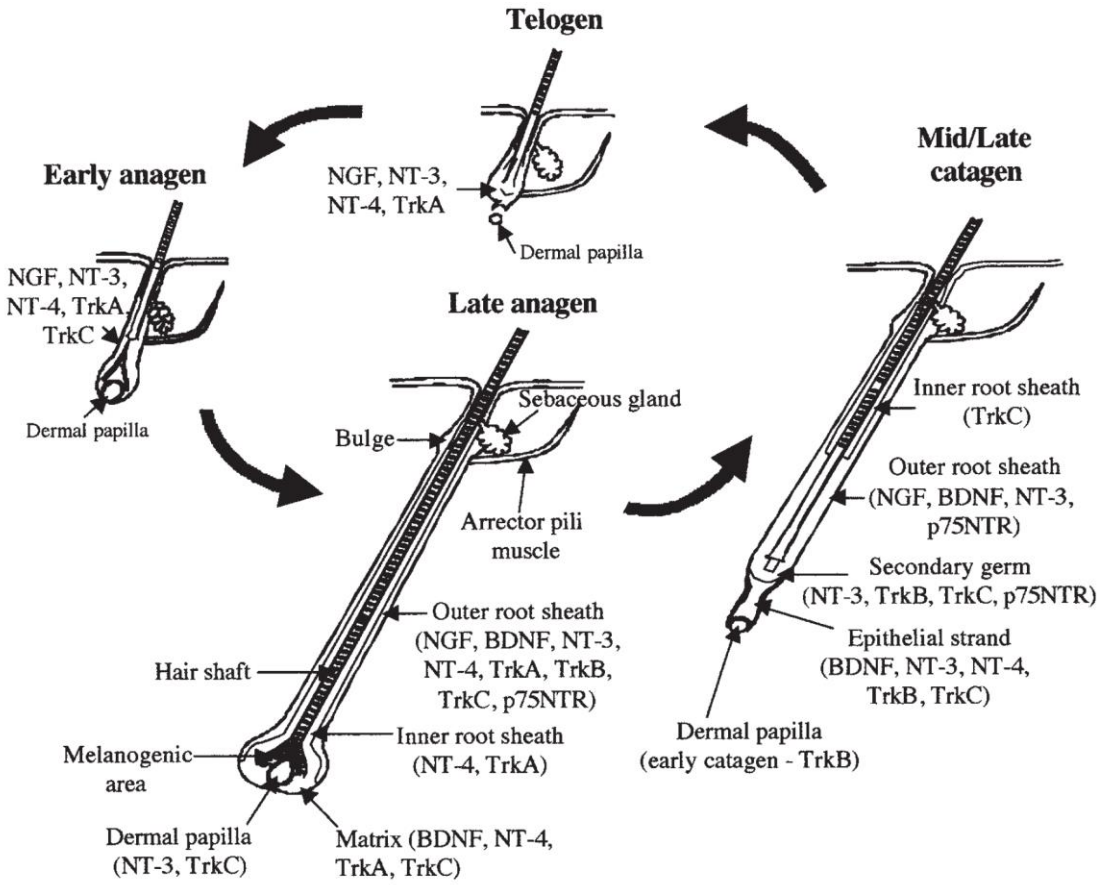


Figure 11 Summary of expression pattern of NTs and their receptors during hair cycle (73)

2.3.4 NTs and human hair growth

There are some studies evaluating the effects of BDNF and NGF on human hair growth. Similar to the result in mouse, NGF and BDNF decrease hair shaft elongation in human anagen hair organ culture system. Furthermore, they also inhibit KC proliferation, induce premature development of catagen and increase number of apoptotic cells in hair bulb. In immunohistochemistry study, NGF, BDNF, TrkA, TrkB are also expressed in human hair follicle epithelium and DP as in mice (73, 77).

CHAPTER III
METHODOLOGY

3.1 Study design:

This is an experimental study. We divided our study into 3 phases; the first phase was a cross sectional experimental study to determine the difference in the expressions of growth factors/cytokines in balding vs. nonbalding scalp hair follicle specimens from the same individuals with AGA using Quantibody Growth Factor Array. The second phase was designed to evaluate the effect of these factors on the proliferation of cultured DP cells (autocrine activities) and hair follicle elongation in hair organ culture system. The third phase was to further investigate whether these effects could be reversed when neutralizing antibodies were added.

3.2 Target Population

Androgenetic alopecia affected patients

3.3 Study population

Hair follicles from volunteer AGA affected patients during 2009-2010 in King Chulalongkorn Memorial Hospital and DHT clinic, Bangkok, Thailand.

3.4 Inclusion Criteria

Subjects eligible for enrollment in the study must meet all of the following criteria:

1. Male patients, 40 to 55 years-old
2. AGA classified as type III - V anterior utilizing Norwood-Hamilton Classification
3. Willing to comply with study requirements

3.5 Exclusion criteria

1. Diagnosis of diffuse unpattern alopecia (DUPA)
2. History of hair transplantation within 6 months prior to screening

3. History of hair loss other than AGA
4. Use of any cosmetic product aimed at improving hair loss within 2 weeks prior
5. Use of light or laser treatment of scalp within 3 months prior
6. Use of dutasteride within 18 months prior
7. Use of finasteride within 12 months prior
8. Use of minoxidil (oral/topical) within 6 months prior
9. Use of drugs that will affect hair growth within 6 months prior

3.6 Sample-size/statistical power considerations

Due to assumption above, the sample size calculation is unnecessary.

3.7 Definition

The diagnosis of AGA was based on the history, family history and clinical characteristics. The staging of AGA is based on Hamilton-Norwood patterns.

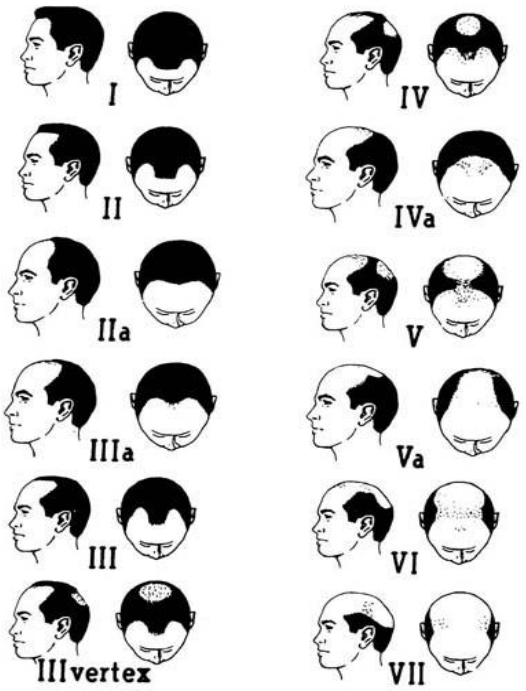


Figure 12 Staging of AGA based on Hamilton-Norwood classification

3.8 Materials and methods

Materials

10 pieces of both balding (frontotemporal) and nonbalding (occipital) scalp hair follicle specimens were collected from men with AGA (Hamilton–Norwood classifications III–V anterior) who consented to the study in outpatient department King Chulalongkorn Memorial Hospital. The protocol followed the Declaration of Helsinki and was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University.

Methods

1. An explanation about the study including the risks, benefits and potential complications was provided to the patients.
2. After the informed consent was signed, subjects will be screened for eligibility. Each subject was interviewed and examined to determine that all selection criteria were met.
3. The medical, family and hair loss history were completed
4. The biopsy area were selected by using 2.5 times optical loupes as follows:
    a. Balding scalp (frontotemporal area): the intermediate hair follicles or the hairs which started to miniaturize were selected.
    b. Non-balding scalp (occipital area): normal healthy or terminal hair follicles were biopsied
5. The scalp specimens were taken by 1.5 mm minipunch technique under local anesthesia (1% xylocaine with adrenaline).

Isolation of human hair follicle dermal papilla cells

1. The specimens were transported in Williams' E serum free media (Gibco BRL, Paisley, Scotland) with 2% antibiotic-antimycotic (Gibco BRL) at 4^{o}C not more than 48 hours.
2. After transferred to the laboratory, the specimens were repeated washed with phosphate-buffered saline (PBS) 3 times.

3. To isolate DP cells, simple micro-dissection with one-step enzymatic digestion method was performed.

Surgical micro-dissection and one-step enzymatic digestion method

1. Briefly, the DP was released from each hair follicle by transection at the level of papilla, opened inversely to expose the inner side and cut at the stalk by using with ophthalmic corneal blade under a stereomicroscope. (Figure 13, 14)
2. Then the isolated DPs were transfered to the Petri dish by forceps and half of them were anchored to the plastic plate by scratch through center using fined needle.
3. After the DPs were isolated by simple micro-dissection method as above (Figure 15), the isolated DPs were then digested with Liberase DH (dispase high) research grade (Roche, Basel, Switzerland) at 37 ºC for 2 hours or until dermal sheath and stalk of DP were digested and the papilla just began to be digested.
4. After the enzymatic dissociation, the DPs were cultured in a 6-well plate.

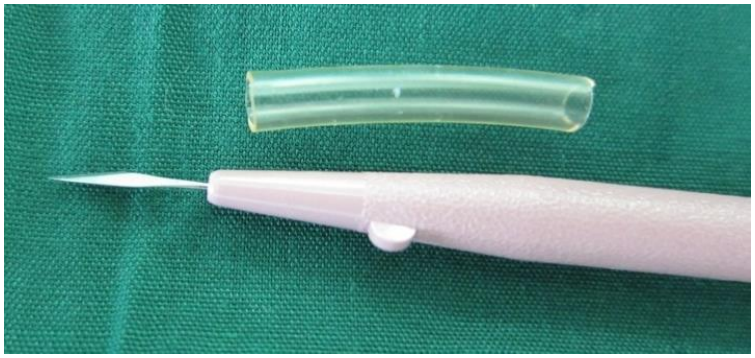


Figure 13 Ophthalmic corneal blade that used for dissect dermal papilla in isolation process

Figure 14 Instruments used for isolation process namely stereomicroscope and class II laminar flow

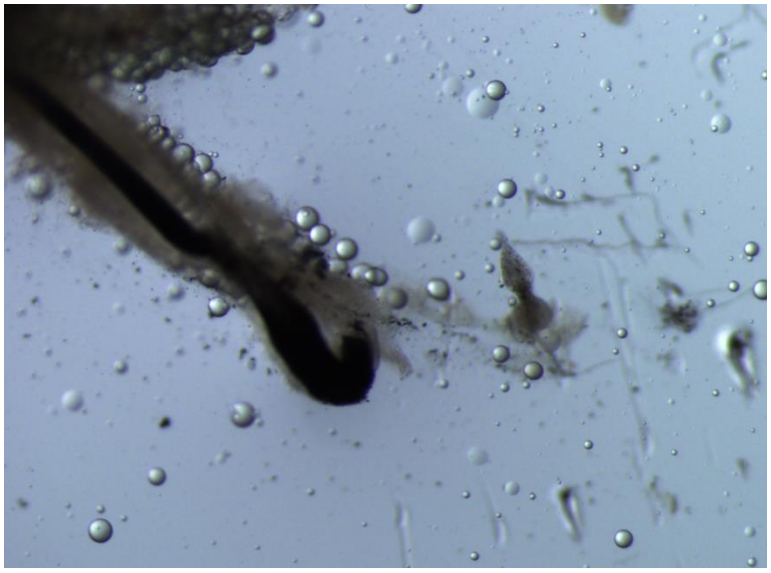


Figure 15 Isolated dermal papilla (tear drop shape) after dissection with blade under 40xstereomicroscope

Cultivation and Proliferation of human hair follicle dermal papilla cells

1. DP from all methods were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma Co) supplemented with nutrient Ham F12 (3 parts of DMEM, 1 part of Ham's

F12), 10% fetal bovine serum (FBS), 200 mmol/L L-glutamine and 1% antibiotic-antimycotic at 37ºC under a humidified atmosphere of 95% air and 5% CO_2.

2. Primary cultures were left untouched for 3-5 days or until the DP cells were attached and the first cell migration became apparent.
3. Thereafter the medium was changed every 2 days. After the cell outgrowth become confluent, the cells were subcultured in a 1:3 spilt using 0.25% trypsin/EDTA solution.
4. The cultured DP cells in the 2^{nd} or 3^{rd} passages were used for every experimental study.

Phase 1: Quantitative human Growth factor array

For growth factors secretion study, cultured DP cells in the 2^{nd} or 3^{rd} passages from 3 patients were seeded at concentration of 4×10^4 cells per well in a six-well plate and grown for 7 days or until 50-70% confluence. To minimize serum effects and promote cell quiescence, the DP cells were cultured in serum-free DMEM with 200 mmol/L L-glutamine and 1% antibiotic-antimycotic. The supernatants were collected at 48 h after incubation and spun at 2000 r.p.m. in a centrifuge at 4°C for10 min. Then the culture media were frozen at 20 C until assay. All experiments were conducted in triplicate.

Quantifying the levels of growth factors and cytokines expressed by cultured balding and non-balding DP cells were performed using Quantibody Growth Factor Array-1 (RayBiotech, Inc., Norcross, GA), which is a multiplexed sandwich enzyme-linked immunosorbent assay-based technology and has the capability to determine the concentration of 40 human growth factors simultaneously. This multiplex ELISA-based array has high detection sensitivity (pg/ml) and using only small volume of sample (50-100 µL per array).

The growth factors and cytokines of interest were as follows: androgen receptor (AR), brain-derived nerve factor (BDNF), bFGF, bone morphogenetic protein (BMP)-4, BMP-5, BMP-7, b-nerve growth factor (NGF), epidermal growth factor (EGF), EGF receptor, endocrine gland-derived VEGF (EG-VEGF), fibroblast growth factor (FGF)-4, FGF-7 or keratinocyte

growth factor, glial cell-derived neurotrophic factor, growth differentiation factor-15 (GDF-15, TGF-bPL or macrophage inhibitory cytokine-1), growth hormone, heparin-binding EGF, hepatocyte growth factor, insulin, IGF-1, IGF binding protein (IGFBP)-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-6, macrophage colony stimulating factor receptor, NGF receptor, neurotrophin (NT)-3, NT-4, osteoprotegerin (OPG), placenta growth factor (PLGF), platelet-derived growth factor (PDGF)-AA, stem cell factor (SCF), SCF receptor, TGF-a, TGF-b1, TGF-b3, VEGF, VEGF receptor (VEGFR)-2, VEGFR-3 and VEGF-D.

The arrays were performed according to the manufacturer's instructions (Figure 16). Briefly, one standard glass slide was spotted with 16 wells of identical cytokine antibody arrays. Each antibody, together with the positive controls, was arrayed in quadruplicate. The slide comes with a 16-well removable gasket which allows for the processing of 16 samples in one slide. Four slide chips can be nested into a tray, which matches a standard microplate and allows for automated robotic high through put processing of 64 arrays simultaneously. For cytokine quantification, the array-specific cytokine standards, whose concentration has been predetermined, are provided to generate an eight-point standard curve for each cytokine. Before addition of standard cytokines and samples, the glass chip was blocked by incubation with 100 µL sample diluents for 30 min. The standard cytokines, balding and nonbalding DP cell supernatants (100 µL) were added and incubated at room temperature for 1 h. After a washing step, 80 µL of detection antibody was added (1 h), followed by a further washing step before addition of 80 µL Cy3 equivalent dye conjugated streptavidin for 1 hour. Fluorescent signals were detected by laser scanner (Axon GenePix; Molecular Devices, Sunnyvale, CA, U.S.A.) set at 555 nm excitation, 565 nm emission and 10 µm resolution. Data were extracted and analysed with RayBio Q-Analyzer software at RayBiotech, Inc., Norcross, GA, USA (Figure 17). The results from our experiments were shown in Figure 18 and 19.

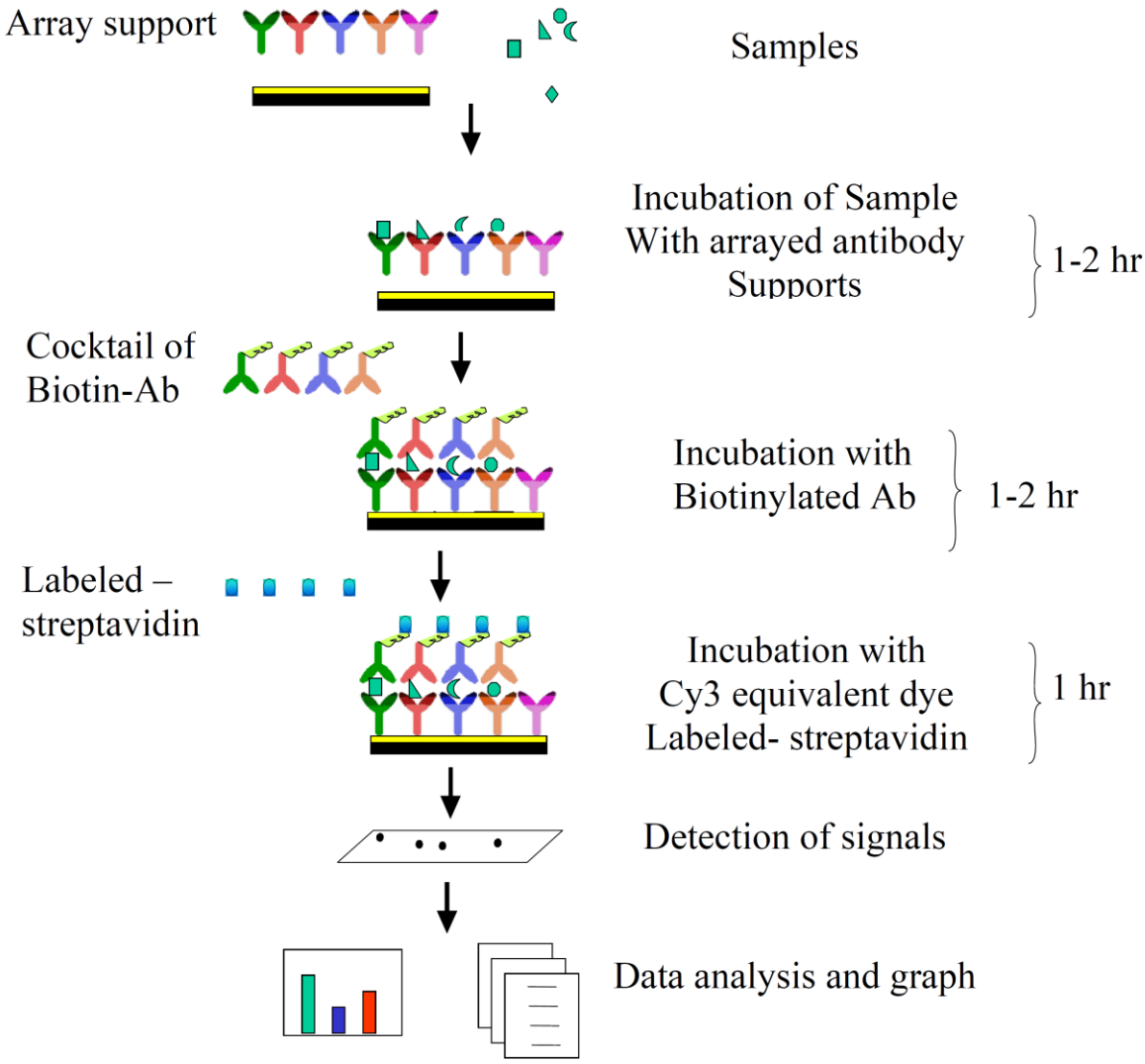


Figure 16 Steps in performing quantitative multiplex ELISA-based human growth factor array

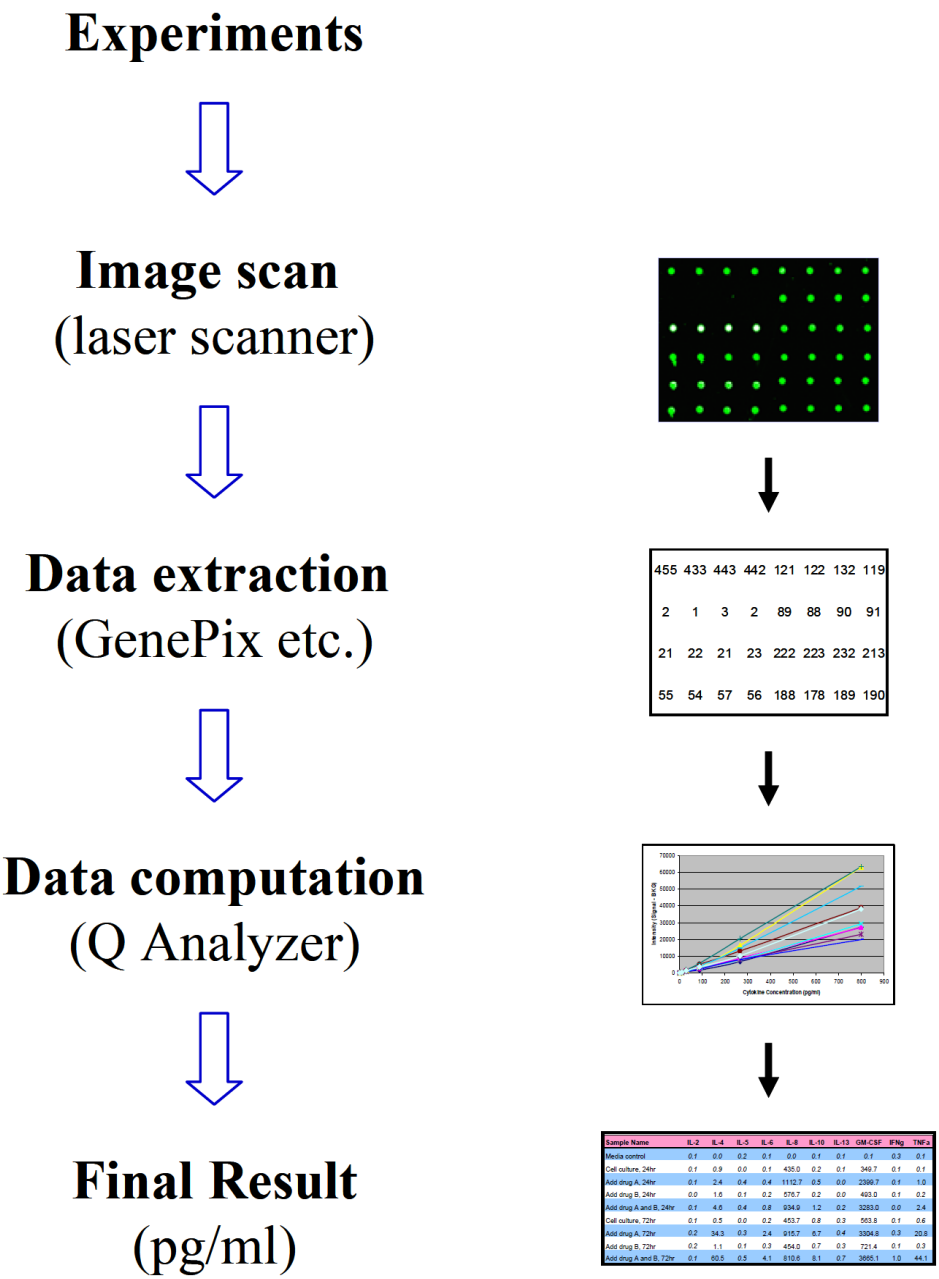
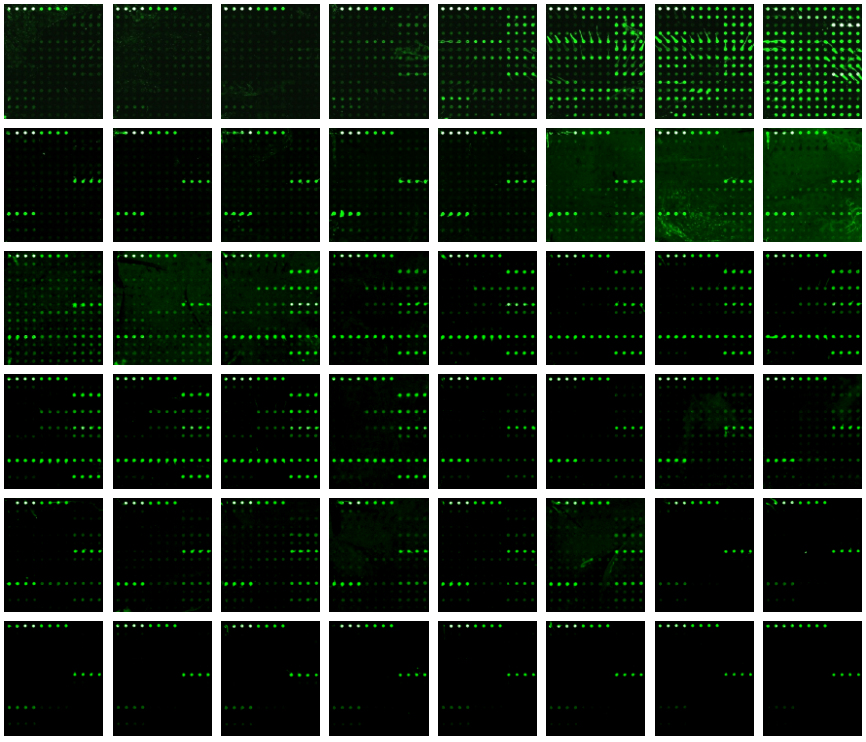


Figure 17 Steps in data analysis of quantitative growth factor array



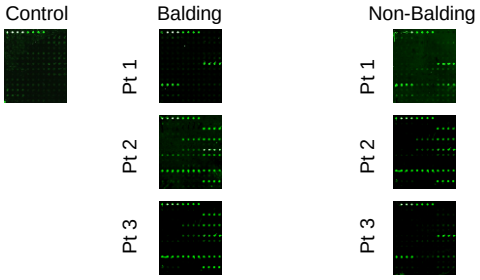
Array map

POS1	POS2	AR
BDNF	bFGF	BMP-4
BMP-5	BMP-7	b-NGF
EGF	EGF R	EG-VEGF
FGF-4	FGF-7	GDF-15
GDNF	GH	HB-EGF
HGF	IGFBP-1	IGFBP-2
IGFBP-3	IGFBP-4	IGFBP-6
IGF-I	Insulin	MCF R
NGF R	NT-3	NT-4
OPG	PDGF-AA	PIGF
SCF	SCF R	TGFa
TGFb	TGFb3	VEGF
VEGF R2	VEGF R3	VEGF-D

Sample ID

Cntrl	Std7	Std6	Std5	Std4	Std3	Std2	Std1
SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8
SA9	SA10	SA11	SA12	SA13	SA14	SA15	SA16
SA17	SA18	SA19	SA20	SA21	SA22	SA23	SA24
SA25	SA26	SA27	SA28	SA29	SA30	SA31	SA32
SA33	SA34	SA35	SA36	SA37	SA38	SA39	SA40

Figure 18 Quantibody array microplate and array map of 40 cytokines/ growth factors



Cntrl	Std7	Std6	Std5	Std4	Std3	Std2	Std1
SA1	SA2	SA3	SA4	SA5	SA6	SA7	SA8
SA9	SA10	SA11	SA12	SA13	SA14	SA15	SA16
SA17	SA18	SA19	SA20	SA21	SA22	SA23	SA24
SA25	SA26	SA27	SA28	SA29	SA30	SA31	SA32
SA33	SA34	SA35	SA36	SA37	SA38	SA39	SA40

Figure19 Array microplates with fluorescent signals detection by laser scanner of our patients

Phase 2: Specific cytokines/ growth factors study

To identify factors responsible for balding, we screened cytokines and growth factors secreted by cultured human balding DP cells versus nonbalding by Quantibody growth factor array. In this study, we found that neurotrophins (NT) including BDNF, NGF and NT-3 were the most upregulated, while osteoprotegerin (OPG) was recognized as the most downregulated cytokines secreted by balding DP cells. Thus we examined the effects of BDNF, NGF, NT-3, NT-4 and OPG with varying doses on the proliferation of DP cells and hair shaft elongation in hair organ culture system.

2.1 DP cell proliferation using MTT assay

The cultured balding DP cells in the 2^{nd} or 3^{rd} passages from 4 patients were seeded at concentration of 3×10^4 cells per well in a 48-well plate and grown for 1 day in DMEM (Sigma Co) supplemented 10% fetal bovine serum (FBS), 200 mmol/LL -glutamine and 1% antibiotic-antimycotic. Then the DP cells were changed to culture in a quiescent environment of DMEM with 1%FBS, 200 mmol/LL -glutamine and 1% antibiotic-antimycotic for another 24 hours before test. Prior to study, the 48-well plates were microscopically verified to guarantee that the cells were adherent and to assure that there was no confluence nor contamination

DP cells were cultured DMEM with 1%FBS, 200 mmol/L L-glutamine and 1% antibiotic-antimycotic with and without following NTs in variable concentration (table 3). A number of viable cells were quantified using MTT assay (R&D Systems Inc., Minneapolis, MN, USA) at 2 and 4 days after the incubation. For 48-well plate, culturing of cells longer than 4 days may affect the proliferation of cells due to the limitation in space for expanding. Absorbance was measured at 570nm using an ELISA reader. All experiments were conducted in triplicate. All NTs were purchased from R&D Systems Inc., Minneapolis, MN, USA. The below concentrations were chosen according to manufacturer's protocol.

Table 3 Varying doses of four NTs and OPG in MTT assay

Factors	Recommend dose*	I	II	III
BDNF	1-6 ng/ml	0.1 ng/ml	1 ng/ml	10 ng/ml
NGF	0.2-2 ng/ml	0.1 ng/ml	1 ng/ml	10 ng/ml
NT-3	1-10 ng/ml	0.1 ng/ml	1 ng/ml	10 ng/ml
NT-4	0.3-3 ng/ml	0.1 ng/ml	1 ng/ml	10 ng/ml
OPG	8-24 ng/ml	5 ng/ml	10 ng/ml	50 ng/ml

*According to manufacturer's recommendation (the ED_{50} activity of rhBDNF, NT-3 and NT-4 were measured in a cell proliferation assay using the TrkB-transfected BaF3 mouse pro-B cell line, BaF- Trk-B-BD and rH-NGF were measured using TF-1 human erythroleukemic cells)

2.2 Hair shaft elongation study

Isolation and cultured of hair follicle

Normal hair follicles from occipital scalp were cut individually into single hair follicles under 10x stereomicroscope. Each follicular sample was then cut at the level of dermo-subcutneous junction using a surgical number 10 blade. Only hairs in the anagen stage were selected for hair length elongation study.

Each follicle was cultured using submerged system in individual wells of 48-well plates in 5%CO_2 incubator at 37°C for 10 days. One hair follicles per well were cultured in 300µl of Williams E medium (Gibco BRL, Paisley, Scotland) supplement with 10 µg of insulin, 10 ng/ml of hydrocortisone, 200 mmol/L L-glutamine and 1% antibiotic-antimycotic. In the experiment, test groups received various concentrations of studied factors and control (table 4). 12-14 hair follicles were used in each study group and 25 hair follicles were used in control. The specimens were collected from 4 different subjects. All NTs were purchased from R&D Systems Inc., Minneapolis, MN, USA. The below concentrations were chosen according to manufacturer's protocol.

Hair shaft lengths were measured under stereomicroscope x16, immediately and at 10-day culture, using Olympus DP2-BSW picture software (version 2.1).

Table 4 Doses of four NTs and OPG in hair shaft elongation study

Factors	Recommend dose*	Study dose
BDNF	1-6 ng/ml	1 ng/ml
NGF	0.2-2 ng/ml	1 ng/ml
NT-3	1-10 ng/ml	1 ng/ml
NT-4	0.3-3 ng/ml	1 ng/ml
OPG	8-24 ng/ml	10 ng/ml

*According to manufacturer's recommendation (the ED_{50} activity of rhBDNF, NT-3 and NT-4 were measured in a cell proliferation assay using the TrkB-transfected BaF3 mouse pro-B cell line, BaF- Trk-B-BD and rH-NGF were measured using TF-1 human erythroleukemic cells)

Phase 3: Neutralizing antibody assay

3.1 DP cells proliferation study

The cultured balding DP cells in the 2^{nd} or 3^{rd} passages from 4 patients were seeded at concentration of 2.5×10^4 cells per well in a 48-well plate and the same method was done as previous described. Various concentrations of testing factors alone or in combination with neutralizing antibody is added in culture medium (table 5).In each factor, the study was divided into 4 groups: control, factor alone, antibody alone and both factor and antibody. Cell proliferation was evaluated using MTT assay kit (R&D Systems Inc., MN, USA) at 2 and 4 days after the incubation. All NTs and anti-NTs antibody were purchased from R&D Systems Inc., Minneapolis, MN, USA. The below concentrations were chosen according to manufacturer's protocol. All experiments were conducted in triplicate.

Table 5 Doses of 4 groups (I-IV) of 4 NTs and anti-NTs antibody in MTT assay

BDNF	I	II	III	IV	Unit
BDNF	0	1	0	1	ng/ml
Anti-BDNF	0	0	0.5	0.5	µg/ml

NGF	I	II	III	IV	Unit
NGF	0	1	0	1	ng/ml
Anti-NGF	0	0	0.1	0.1	µg/ml

NT-3	I	II	III	IV	Unit
NT-3	0	10	0	10	ng/ml
Anti- NT-3	0	0	0.1	0.1	µg/ml

NT-4	I	II	III	IV	Unit
NT-4	0	10	0	10	ng/ml
Anti- NT-4	0	0	1	1	µg/ml

*According to manufacturer's recommendation (the ND_{50} activity of rhBDNF = 0.5-1 µg/m for 5 ng/mL of rhBDNF, NT-3 = 0.1-0.5 µg/ml for 100 ng/mL of rhNT-3 and NT-4= 1-3 µg/ml for 40 ng/mL of rhNT-4 were determined by using TrkB-transfected cell line and the ND_{50} activity of rhNGF = 0.1-0.4 µg/m for 5 ng/mL of rhNGF were determined by using TF-1 cell line assay.)

2.2 Hair shaft elongation study

Isolation and cultured of hair follicle

In this experiment, test groups were received various concentrations of studied factors alone or in combination with neutralizing antibodies (table 6). 18-22 hair follicles were used in each study group and 21 hair follicles were used as control. All NTs and anti-NTs antibody were purchased from R&D Systems Inc., Minneapolis, MN, USA. The below concentrations were chosen according to manufacturer's protocol. The specimens were collected from 4 different subjects. Hair shaft lengths were measured under stereomicroscope x16, immediately and 10 days after culture using Olympus DP2-BSW picture software (version 2.1).

Table 6 Doses of 4 groups (I-IV) of 4 NTs and anti-NTs antibody in hair shaft elongation study

BDNF	I	II	III	IV	Unit
BDNF	0	1	0	1	ng/ml
Anti-BDNF	0	0	0.5	0.5	µg/ml

NGF	I	II	III	IV	Unit
NGF	0	1	0	1	ng/ml
Anti-NGF	0	0	0.1	0.1	µg/ml

NT-3	I	II	III	IV	Unit
NT-3	0	1	0	1	ng/ml
Anti- NT-3	0	0	0.1	0.1	µg/ml

NT-4	I	II	III	IV	Unit
NT-4	0	1	0	1	ng/ml
Anti- NT-4	0	0	1	1	µg/ml

*According to manufacturer's recommendation (the ND_{50} activity of rhBDNF = 0.5-1 µg/m for 5 ng/mL of rhBDNF, NT-3 = 0.1-0.5 µg/ml for 100 ng/mL of rhNT-3 and NT-4= 1-3 µg/ml for 40 ng/mL of rhNT-4 were determined by using TrkB-transfected cell line and the ND_{50} activity of rhNGF = 0.1-0.4 µg/m for 5 ng/mL of rhNGF were determined by using TF-1 cell line assay.)

3.9 Statistical Analyses

Summary of Data

Continuous outcomes were presented as mean ± standard deviation (SD) and median and categorical data were presented as numbers (percentage).

Hypothesis testing

Differences between two related groups were tested by paired t-tests for continuous variables and normal distribution or Wilcoxon signed rank for skew distribution

Comparison of more than two, unrelated groups were done by analysis of variance (one way ANOVA).

Phase 1: Median levels of protein expression of growth factors or cytokines (pg/ml.) detected from the balding scalps were compared with those of the corresponding nonbalding counterparts using Wilconxon signed rank test.

Phase 2 and 3: Number of cells (cells) and hair length (micron) were continuous data which were presented as mean ± standard deviation (SD) (table 7).

In both phases of study, the SPSS statistical software package (SPSS version 16.0; SPSS, Chicago, IL, U.S.A.) was used for analysis. A value of $P < 0.05$ was considered statistically significant.

Table 7 Statistical analyses of data

Type of data	Data (Unit)	Data shown	Statistical Analyses
Numerical Data (Continuous)	Growth factor level concentration (pg/ml)	Mean (SD) (normal distribution)	Paired T-test
		Median (skew distribution)	Wilcoxon sign rank test
	Number of cells	Mean (SD) (normal distribution)	one way ANOVA
		Median (skew distribution)	Kruskal Wallis
	Hair length (micron)	Mean (SD) (normal distribution)	one way ANOVA
		Median (skew distribution)	Kruskal Wallis

CHAPTER IV
RESULTS

4.1 Isolation of cultured human DP cells

Up to now, isolation of human DP cells is hard to perform simply and effectively. We reposted a more efficient method by using simple micro-dissection with one-step enzyme digestion to isolate the DP cells of hair follicles. We observed that our isolated DP cells grew well on the plate without the feeder layer in Dulbecco's modified Eagle's medium (DMEM) supplemented with Nutrient Ham's F12, 10% Fetal bovine serum, L-glutamine (200 mmol/L) and 1% antibiotic-antimycotic.

4.1.1 Characteristics of cultured DP cells isolated by simple micro-dissection with one-step enzymatic digestion

After simple microdissection (Figure 20), we digested the DP with Liberase DH (dispase high) research grade (Roche, Basel, Switzerland)at 37 ºC for 2 hours to digest directly the capsule sheath of follicular dermis to expose the DP cells.

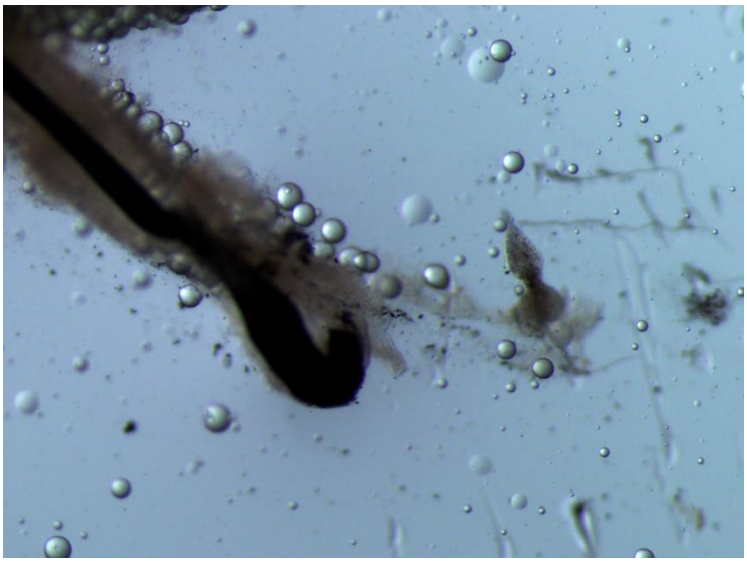


Figure 20 DP after isolated with simple micro-dissection technique before digest with Liberase DH (dispase high) research grade

The DPs after digestion are in spherical shape (Figure 21). Most of DPs attached quickly within one day on the plastic plate with higher attached rate 95% (N = 95/100). One day after DP is attached to the plate, the cells initially outgrowth from the DP explants and spread out like sunflowers (outgrowth rate for the 5 days is 92% (N= 92/100)). (ดังรูปที่ 22, 23) In this method, the culture plates are kept untouched for 3 days, allowing all DPs to adhere.

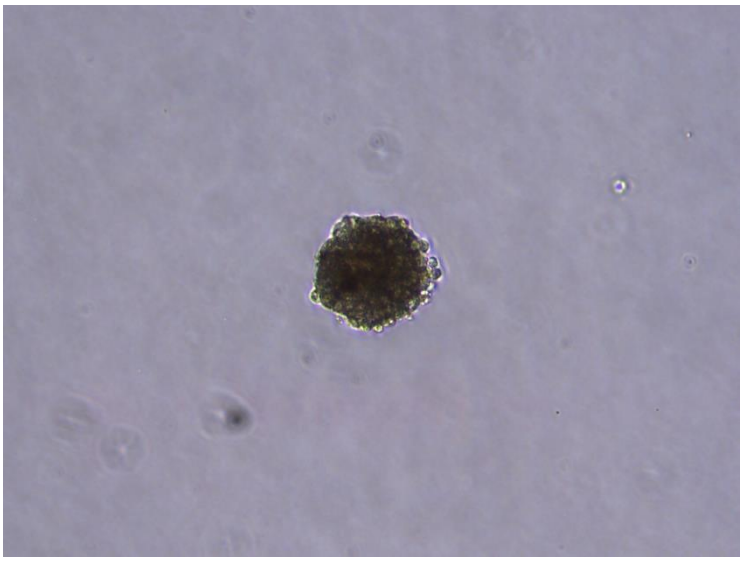


Figure 21 DP immediatly after dissection and enzymatic digestion with Liberase DH

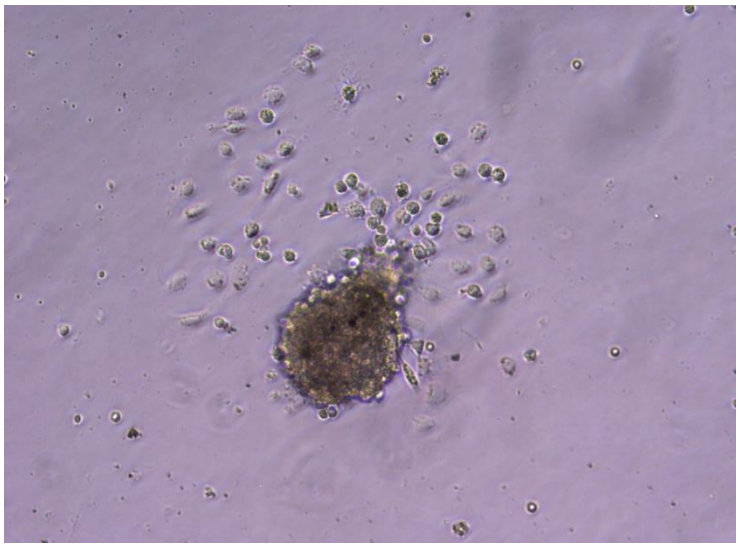


Figure 22 DP cells start to outgrow from the DP explant a few days after isolation

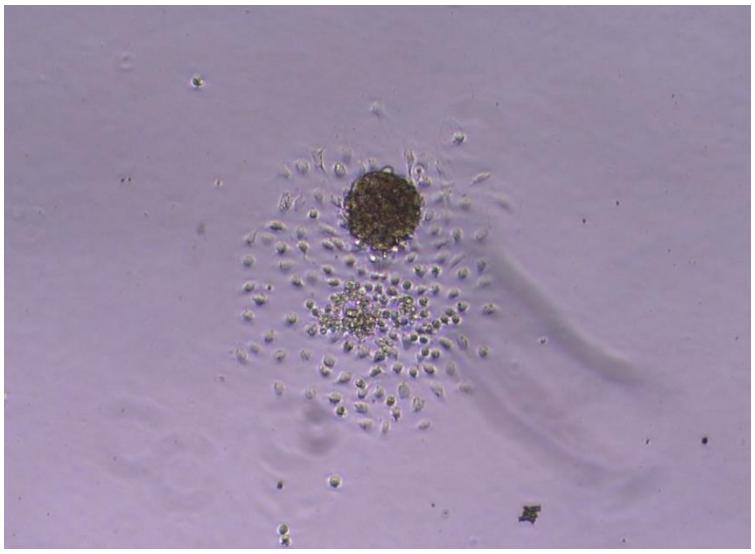


Figure 23 DP cells proliferate radiating from the DP explant like a sunflower

These cells show polygonal morphology with many various length processes and containing pigments in the large cytoplasm (Figure 24). The DP cells reach confluence and can be subcultured within 2 weeks after primary culture. Difference from the dermal fibroblasts and dermal sheath cells, DP cells generally spindle shaped at low density and form multilayer aggregation and clump at preconfluent density. (Figure 25, 26)

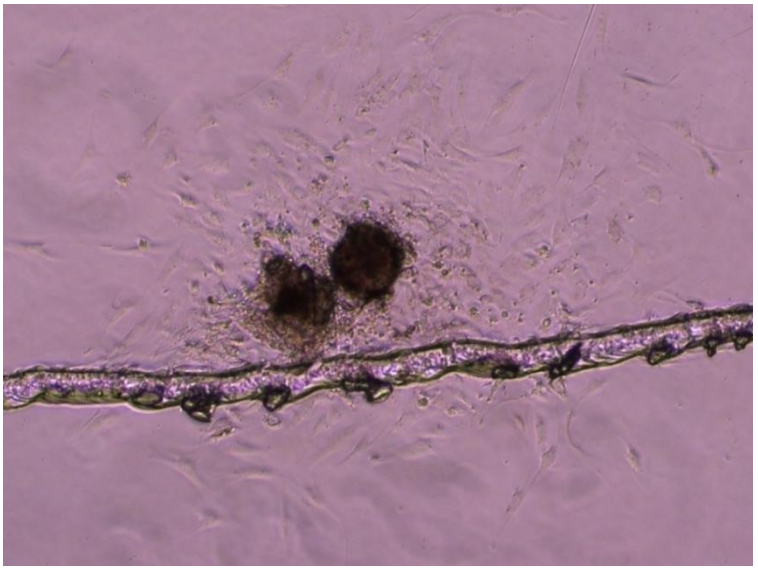


Figure 24 Cultured DP cells at 3 days after isolation

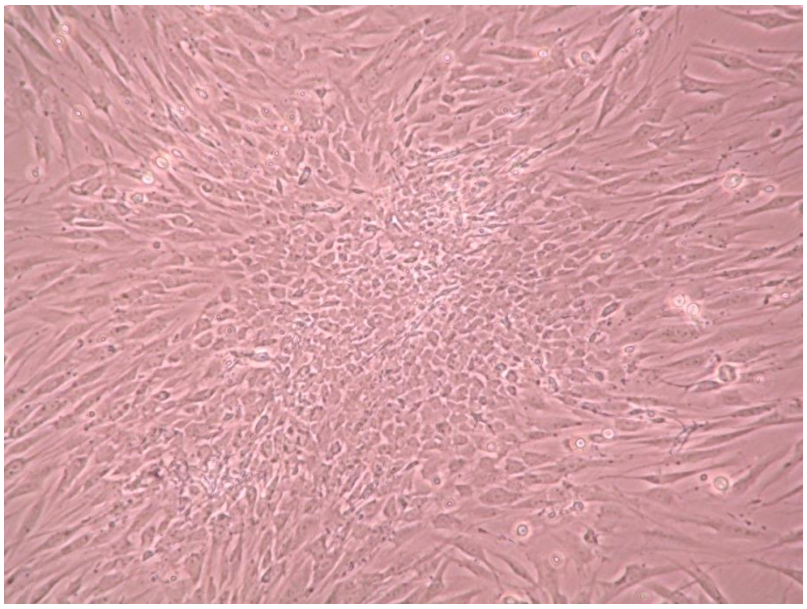


Figure 25 Characteristic multilayer aggregation and clump of DP cells

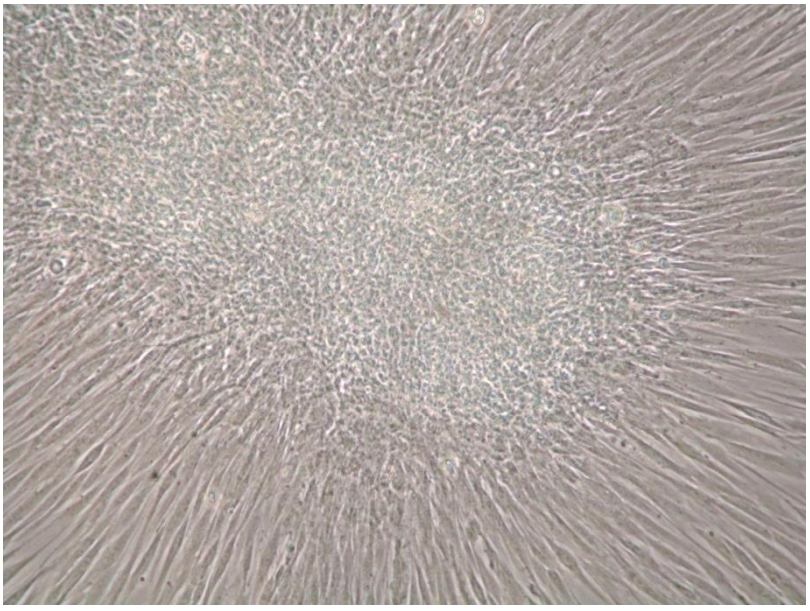


Figure 26 Cultured DP cells at 14 days after isolation, the characteristic of clumped DP cells are seen

Similar to the bulge outer root sheath cells, DP cells also show high proliferative capacity. In early passages, DP cells are smaller in size and fast growing. (Figure27) In the

later passages DP cells become larger, flat, irregular shape and grow less aggressively. (Figure 28) The aggregation of cells into multi-layer clumps, which is characteristic of cultured DP cells, is less seen in multiple passages (over 8 passages).

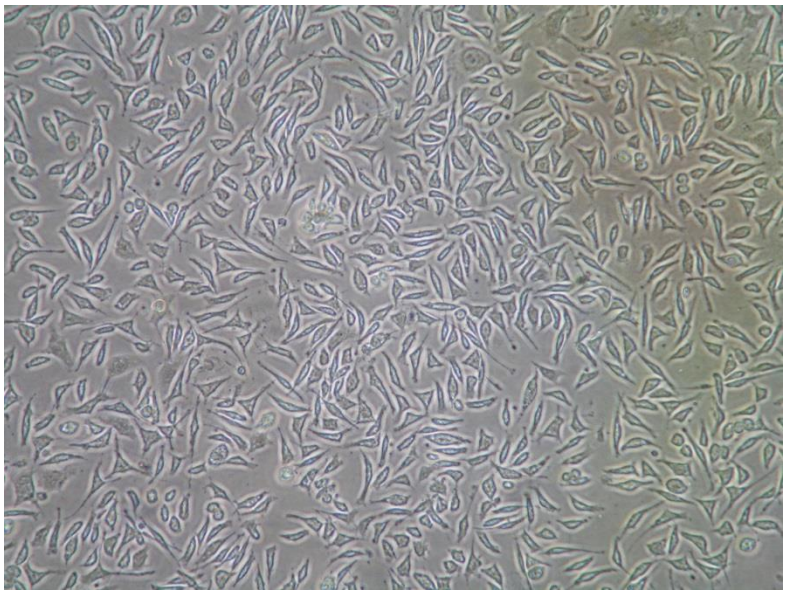


Figure 27 Cultured DP cells in 2nd passage; cells are small in size and fast growing

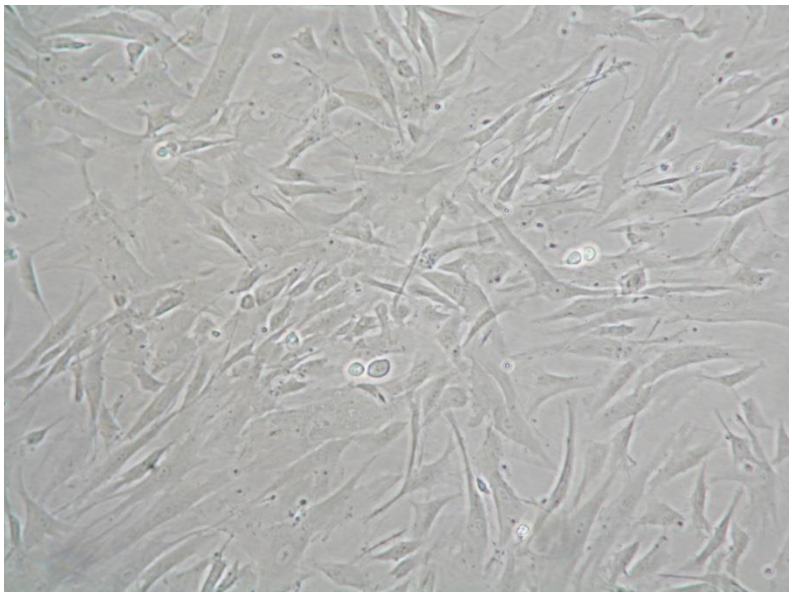


Figure 28 Cultured DP cells in 6th passage; cells are larger in size, slow growing and less active

4.1.2 Immunocytochemistry of human cultured DP cells

The DP cells in the third passages were cultured on sterile chamber slide (Nunc®, Lab-tex II™) and used for immunocytochemical staining (α-smooth muscle actin, AB-PAS, 0.1% toluidine blue O and vimentin) to clarify and characterize cell type. The cells at nearly confluence were fixed with cold acetone 10 minutes and rinsed with 0.01 M PBS for three times. The following steps of these cytochemical and immunocytochemical staining procedures were performed as usual.

The cultured DP cells are identified by positive immunocytochemistry (α-smooth muscle actin, AB-PAS, 0.1% toluidine blue O and vimentin), which are similar to the staining results of in situ hair follicle and consistent with published reports. When stained with alcian blue followed by periodic acid Schiff (AB-PAS), the DP cells show red cytoplasm. (Figure 29, 0) When stained with toluidine blue O, both nucleus and cytoplasm of DP cells stain purple. (Figure 31) For alpha-smooth muscle actin, DP cells cytoplasm show positive brown color in multi-layer aggregation and clumps (Figure 32, 33). DP cells, DS cells and dermal fibroblasts are all positive for vimentin (Figure 34). DS cells show positive with AB and AB-PAS staining except toluidine blue O. Dermal fibroblasts are all negatively stained with AB, AB-PAS and toluidine blue O. The results showed as in Table 8.

Table 8 Immunocytochemical staining characteristics of DP cells, DS cells and dermal fibroblasts

Type of cells	AB	AB-PAS	Toluidine blue	Vimentin	SMA
DP cells	+	++	+	+	+
DS cells	+	+/-	-	+	+
Fibroblasts	-	--	-	+	-

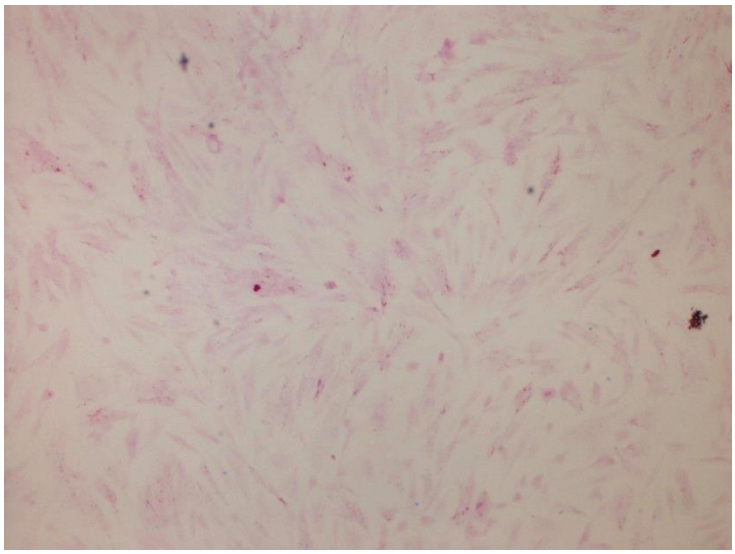


Figure 29 DP cells show positive red cytoplasm when stained with AB-PAS (x100)

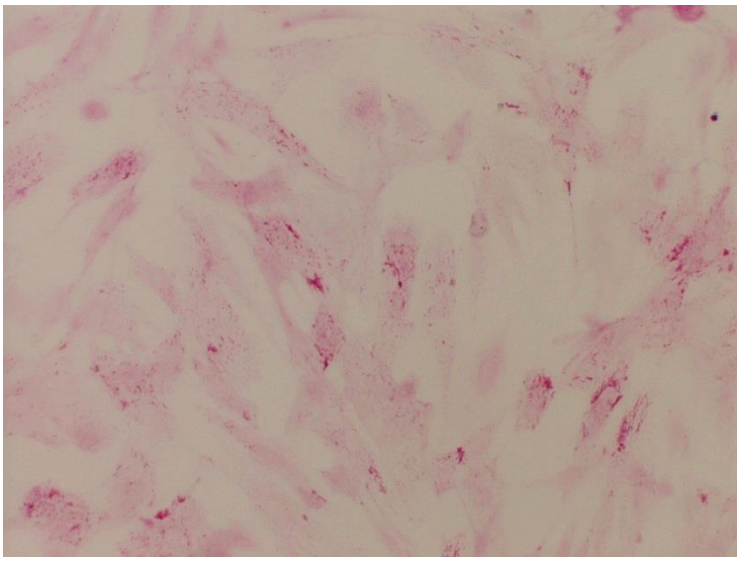


Figure 30 Higher magnification of DP cells with positive AB-PAS (red cytoplasm) (x200)

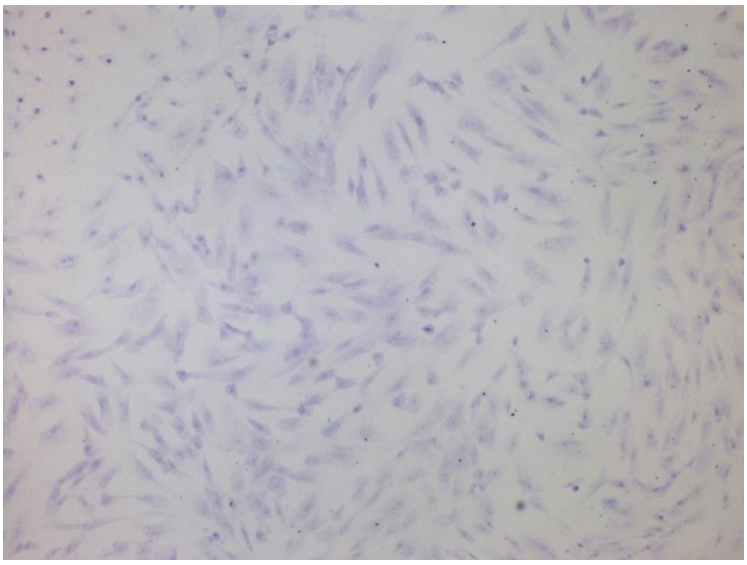


Figure 31 DP cells show positive purple color of toluidine blue O in both nucleus and cytoplasm (x200)

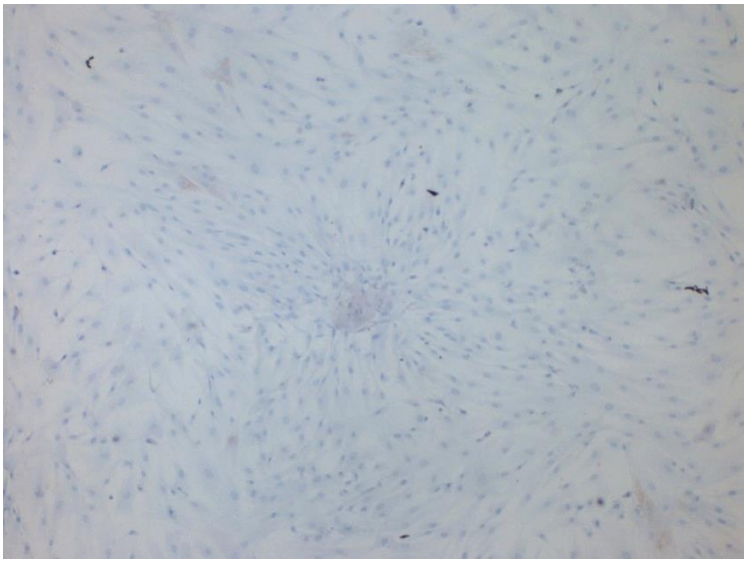


Figure 32 DP cells show positive brown color of alpha-smooth muscle actin at cell clumps(x100)

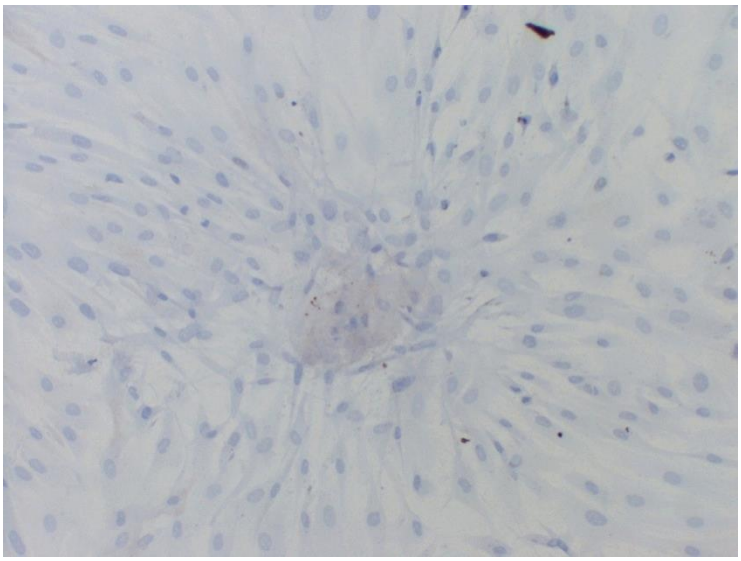


Figure 33 Higher magnification of DP cells clump with positive alpha-smooth muscle actin (brown color) (x200)

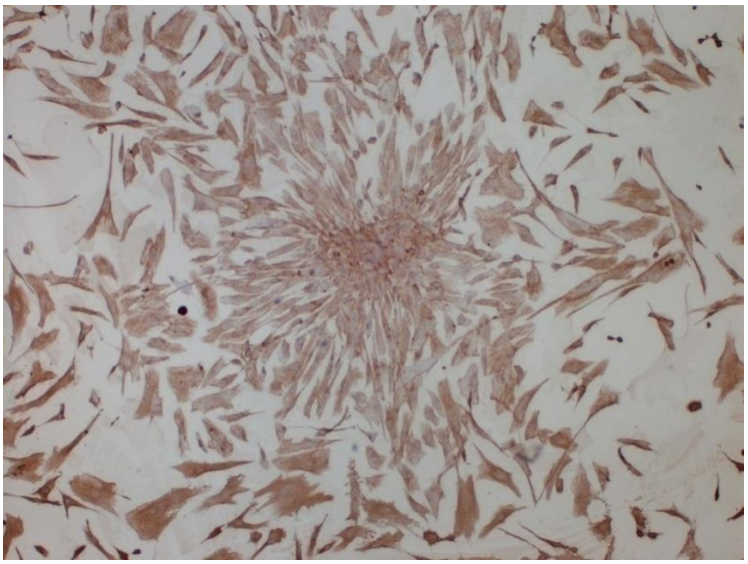


Figure34 DP cells show positive vimentin (x100)

4.2 Phase 1 study

Balding (frontotemporal) and nonbalding, occipital scalp specimens were collected by 1.5 mm minipunch technique from 3 men aged between 42 and 51 years with AGA (Hamilton–Norwood classifications III-Va) who consented to the study.

4.2.1 Upregulated growth factors by balding dermal papilla cells

After the production of the DP cells from balding and nonbalding scalp specimens, 40 cytokines/ growth factors were measured in the supernatants at 48 h. Among the 40 cytokines /growth factors examined, protein expression of 22 in the balding DP cell supernatants was different from that in the nonbalding cells. DP cells from balding scalp follicles secreted approximately12-fold more BDNF at 135 pg mL^{-1} and 3-fold more NT-3 at 594 pg mL^{-1} from those of the nonbalding counterpart at 11.2 and 203 pg mL^{-1}, respectively. These difference are statistically significant at P = 0.007 and 0.048, respectively. Furthermore, we found that expression of another neurotrophins, b-NGF, showed almost three fold increases; however, this did not reach statistically significant levels (Table 9).

HB-EGF was another factor that significantly upregulated by balding DP cells ($P < 0.05$). Two other growth factors, FGF-4 and GDF-15, also showed tendencies to be upregulated in the balding scalp supernatants. Specifically, FGF-4 was not detectable in the supernatant obtained from the nonbalding DP cell cultures, while it expression was as high as 30.4pg mL^{-1}in the balding DP cell supernatants.

Table 9 Upregulated growth factors/ cytokines (n =9)

Growth factors	Balding DP cells (pg mL^{-1})		Nonbalding DP cells (pg mL^{-1})		Conc. Ratio[a]	P value
	mean (SD)	median	mean (SD)	median		
BDNF	135.03 (18.5)	166.7	11.17 (16.7)	0	12.11	0.007
NT-3	594.23 (445.6)	889.4	202.50 (303.7)	0	2.93	0.048
β-NGF	5.50 (9.9)	0	1.98 (3.5)	0	2.76	0.083
FGF-4	30.40 (45.6)	0	0	0	NA	0.083
FGF-7	5.91 (8.9)	0	0	0	NA	0.083

GDF-15	17.03 (25.5)	0	6.87 (10.3)	0	2.48	0.083
HB-EGF	56.67 (42.5)	81.7	26.33 (39.5)	0	2.15	0.024
PDGF-AA	190.97 (214.1)	52.1	96.63 (67.7)	76.9	1.98	0.369
VEGF	909.73 (1147.4)	205.6	430.33 (291.6)	565.7	2.11	0.369

[a]balding/nonbalding supernatants; NA, not applicable

4.2.2 Downregulated growth factors by balding dermal papilla cells

As expected, DP cells from balding scalp follicles secreted significantly less IGF-1 and its respective binding proteins (IGFBP-2, IGFBP-4) than those from nonbalding scalp. Additionally, VEGF-D, EG-VEGF, OPG, NT-4 and TGF-α were also significantly downregulated (Table 10).

Table 10 Downregulated growth factors/ cytokines (mean (SD); n = 9)

Growth factors	Balding DP cells (pg mL^{-1})		Nonbalding DP cells (pg mL^{-1})		Conc. Ratio[a]	P value
	mean (SD)	median	mean (SD)	median		
IGF-1	113.20 (84.9)	165.1	700.83 (530.8)	965.5	6.19	0.024
IGFBP-2	5120.70 (2901.4)	3555.4	6101.17 (3951.1)	4750.1	1.19	0.048
IGFBP-4	2331.30 (1923.4)	4422.6	14722.07 (11840)	1714.8	6.31	0.024
IGFBP-6	637.23 (955.8)	0	1541.47 (1416.2)	1367.6	2.42	0.335
EGF	0	0	0.3 (0.4)	0	NA	0.083
EGF-R	12.33 (18.5)	0	21.37 (32)	0	1.73	0.335
EG-VEGF	3.53 (5.3)	0	13.27 (10.8)	14.9	3.77	0.024
VEGF-D	41.07 (32.4)	32.5	193.23 (157.2)	157.2	4.71	0.024
NT-4	94.80 (102.0)	57.6	473.10 (359.5)	642.8	4.99	0.024
OPG	14032.40 (12931.8)	11084	52265.57 (39208.1)	71481	3.72	0.048
PLGF	83.57 (102.9)	31	173.93 (136.3)	214.9	2.08	0.335
TGF-α	13.97 (20.9)	0	67.93 (53.2)	83.9	4.87	0.024
TGF-β3	259.37 ± 71.8	295.6	505.87 (351.1)	337.2	1.95	0.369

[a]nonbalding/balding supernatants; NA, not applicable

4.3 Phase 2 study

The various concentrations of neurotrophic factors and OPG were examined as previously described.

4.3.1 DP cells proliferation in vitro

Recombinant human neurotrophins inhibit DP cells proliferation in vitro

We examined the effects of NTs on the proliferation of cultured balding DP cells. Using MTT assay, we found the number of expanded human DP cells was smaller in NTs-supplemented media than in the control media at both 2- and 4-day culture. On day 2, NGF, NT-3 and NT-4 at concentration of 10 ng/mL and NT-4 at 0.1 ng/ mL significantly inhibited proliferation of DP cells. On day 4, the more inhibitory effect was observed, NGF and NT-4 at concentration of 0.1, 1 and 10 ng/ mL showed significant inhibition of DP cells proliferation in dose dependent manner. Of all NTs, NT-4 had the largest inhibitory effect on DP cells proliferation at 4-day culture; -42.7% at 10ng/ mL, -29.7% at 1ng/ mL and -24.6% at 0.1ng/ mL respectively. NGF also had significant inhibitory effect; -53.4% at 10ng/ mL, -42% at 1ng/ mL and -40.6% at 0.1ng/ mL respectively. However, BDNF10, 1 and 0.1ng/mL showed minimal effect on the DP cells growth inhibition. (Table 11-12, Figure 35-36)

Table 11 Number of DP cells after treatment with rhNTs determined by MTT assay. BDNF, NGF, NT-3 and NT-4 10, 1, 0.1ng/mL and OPG 50, 10, 5ng/mL inhibit the proliferation of cultured balding DP cells (n=12). (* $P < 0.05$; ** $P < 0.001$compared with control)

Growth factor	Day 2			Day 4		
	Mean DPCs	SD	*P* value	Mean DPCs	SD	*P* value
Control	4.479	1.81	-	4.216	2.80	-
BDNF 10ng/ mL	4.186	1.82	0.648	4.126	1.70	0.843
BDNF 1ng/ mL	3.849	2.00	0.326	4.111	1.30	0.818
BDNF 0.1ng/ mL	3.931	1.71	0.393	4.000	1.24	0.635
NGF 10ng/ mL	2.851	8.36	0.012*	2.416	0.82	0.000**

Growth factor	Day 2			Day 4		
	Mean DPCs	SD	*P* value	Mean DPCs	SD	*P* value
NGF 1ng/ mL	4.044	1.30	0.497	2.963	0.60	0.006*
NGF 0.1ng/ mL	3.984	1.22	0.440	3.181	0.88	0.024*
NT-3 10ng/ mL	3.143	1.63	0.038*	3.698	1.83	0.256
NT-3 1ng/ mL	3.646	1.77	0.194	3.964	1.44	0.580
NT-3 0.1ng/ mL	3.346	1.82	0.078	4.024	1.54	0.673
NT-4 10ng/ mL	2.648	5.38	0.005**	1.966	0.88	0.000**
NT-4 1ng/ mL	3.346	1.33	0.078	2.446	0.7	0.000**
NT-4 0.1ng/ mL	2.686	1.40	0.006**	2.506	1.03	0.000**
OPG 50ng/ mL	4.626	1.37	0.818	4.051	0.57	0.717
OPG 10ng/ mL	4.305	1.46	0.787	5.131	0.76	0.046*
OPG 5ng/ mL	4.001	2.17	0.456	5.011	0.81	0.082

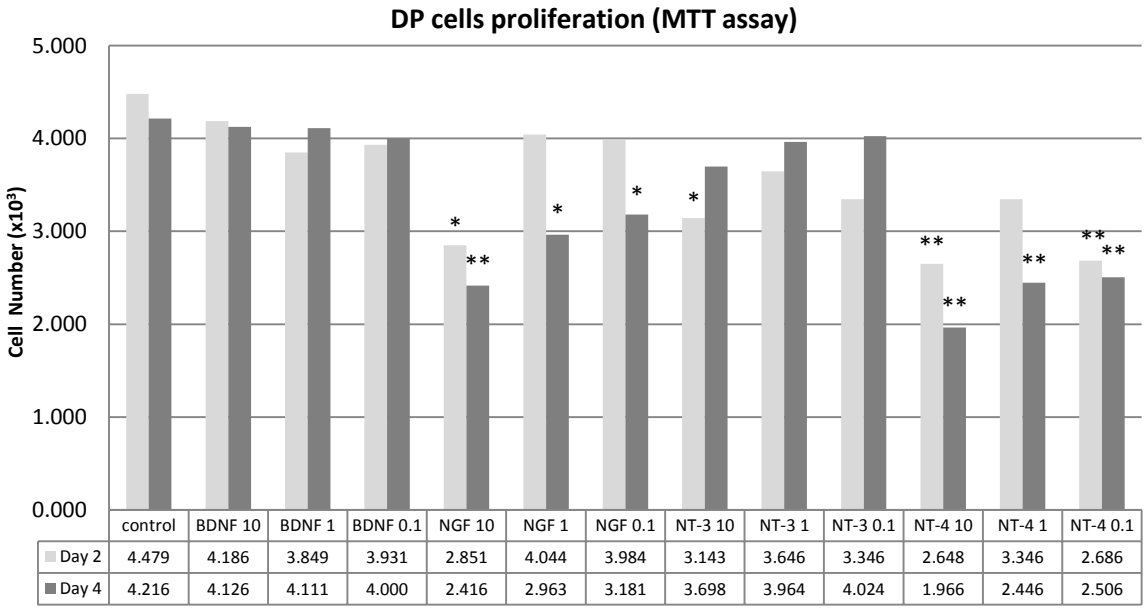
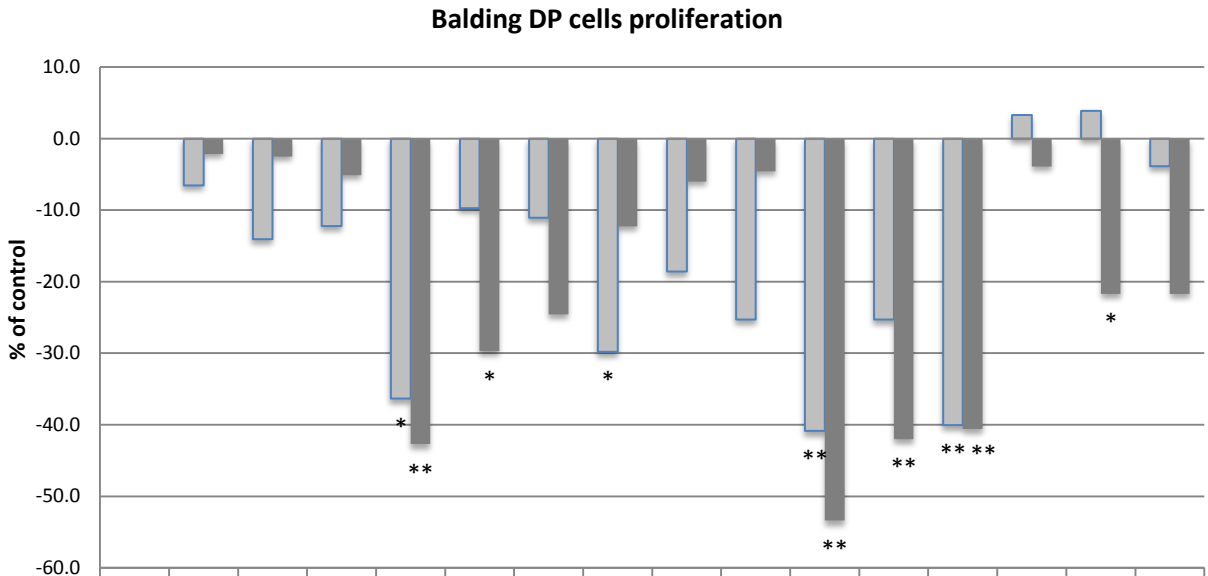


Figure 35 Number of DP cells after treatment with rhNTs determined by MTT assay (n=12). (* *P*< 0.05; ** *P* < 0.001compared with control)

Table12 Percentage of number of DP cells compared to control when treated with NTs and OPG determined by MTT assay. (* $P < 0.05$; ** $P < 0.001$)

Growth factor	Percent of (mean of DP cells) control	
	Day 2	Day 4
Control	0.0%	0.0%
BDNF 10	-6.5%	-2.1%
BDNF 1	-14.1%	-2.5%
BDNF 0.1	-12.2%	-5.1%
NGF 10	-36.3%*	-42.7%**
NGF 1	-9.7%	-29.7%*
NGF 0.1	-11.1%	-24.6%*
NT-3 10	-29.8%*	-12.3%
NT-3 1	-18.6%	-6.0%
NT-3 0.1	-25.3%	-4.6%
NT-4 10	-40.9%**	-53.4%**
NT-4 1	-25.3%	-42.0%**
NT-4 0.1	-40.0%**	-40.6%**
OPG 50	3.3%	-3.9%
OPG 10	3.9%	-21.7%*
OPG 5	-3.9%	-21.7%



	control	BDNF 10	BDNF 1	BDNF 0.1	NGF 10	NGF 1	NGF 0.1	NT-3 10	NT-3 1	NT-3 0.1	NT-4 10	NT-4 1	NT-4 0.1	OPG 50	OPG 10	OPG 5
Day 2	0.0	-6.5	-14.1	-12.2	-36.3	-9.7	-11.1	-29.8	-18.6	-25.3	-40.9	-25.3	-40.0	3.3	3.9	-3.9
Day 4	0.0	-2.1	-2.5	-5.1	-42.7	-29.7	-24.6	-12.3	-6.0	-4.6	-53.4	-42.0	-40.6	-3.9	-21.7	-21.7

Figure 36 Percentage of number of DP cells compared to control when treated with NTs and OPG determined by MTT assay. (* $P < 0.05$; ** $P < 0.001$compared with control)

Recombinant human osteoprogerin (OPG) stimulates DP cells proliferation in vitro

The effect of OPG on the proliferation of cultured DP cells was also investigated using MTT assay. We found that recombinant human OPG at concentration of10 ng/mL significantly stimulated proliferation of DP cells compared to control (P=0.046) at 10-day culture. (Figure 37)

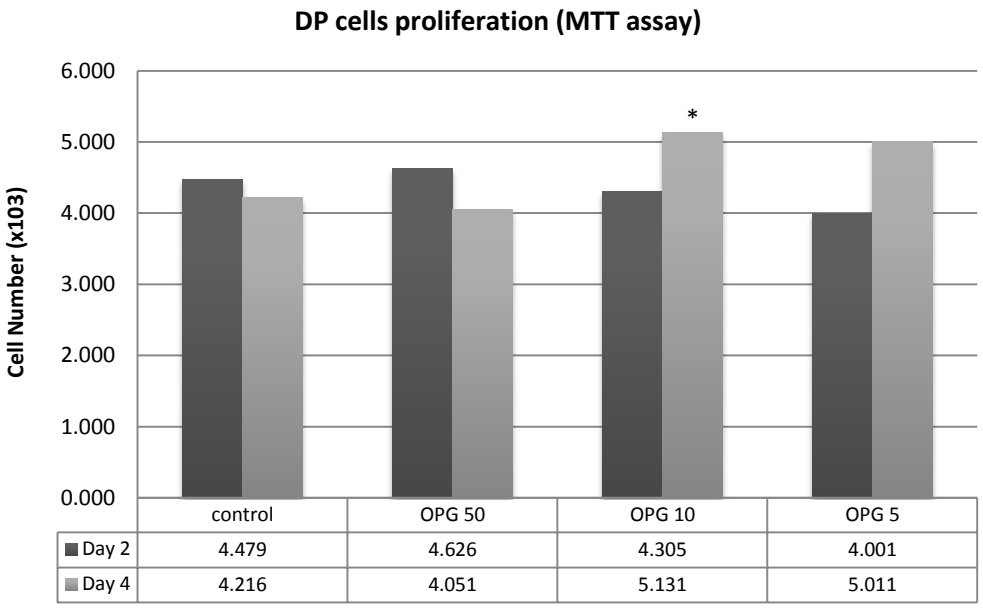


Figure 37 Number of DP cells after treatment with OPG determined by MTT assay. OPG at concentration of 50, 10, 5 ng/mL stimulates proliferation of cultured DP cells. Values are mean (n=4). (* $P< 0.05$ compared with control)

4.3.2 Hair shaft elongation study in hair organ culture system

Recombinant human neurotrophins inhibit human hair growth in vitro

We next treated cultured human hair follicles with the recommended dose of recombinant human NTs. After 10-day culture, BDNF and NGF at concentration of 1 ng/ mL significantly inhibited hair shaft growth by 610.2 μm, -44.3% (P=0.003) and 395.7μm, -28.7% (P=0.05) respectively, compared with control. NT-3 (146.8 μm, -10.7%) and NT-4 (259 μm, -18.8%) also showed inhibitory effect at but the number did not show reach statistically significance. (Table 13, Figure 38)

Recombinant human osteoprogerin (OPG) promotes human hair growth in vitro

We found that the hair follicle growth following treatment with 10 ng/ mL OPG for 10 days was 110.7μm (8%) higher than control but no statistical significance.

Table 13 Mean length of hair shafts after treatment with NTs and OPG in hair follicle organ culture. Isolated hair follicles were cultured for 10 days. (* *P*< 0.05 compared with control)

Growth factor	Mean length of hair growth (micron)	SD	Number of HFs per experiment	*P* value
Control	1377.52	818	25	-
BDNF	767.32	549.3	13	0.003*
NGF	981.84	377.7	13	0.050*
NT-3	1230.72	403.9	12	0.475
NT-4	1118.49	544	12	0.209
OPG	1488.24	376.3	14	0.057

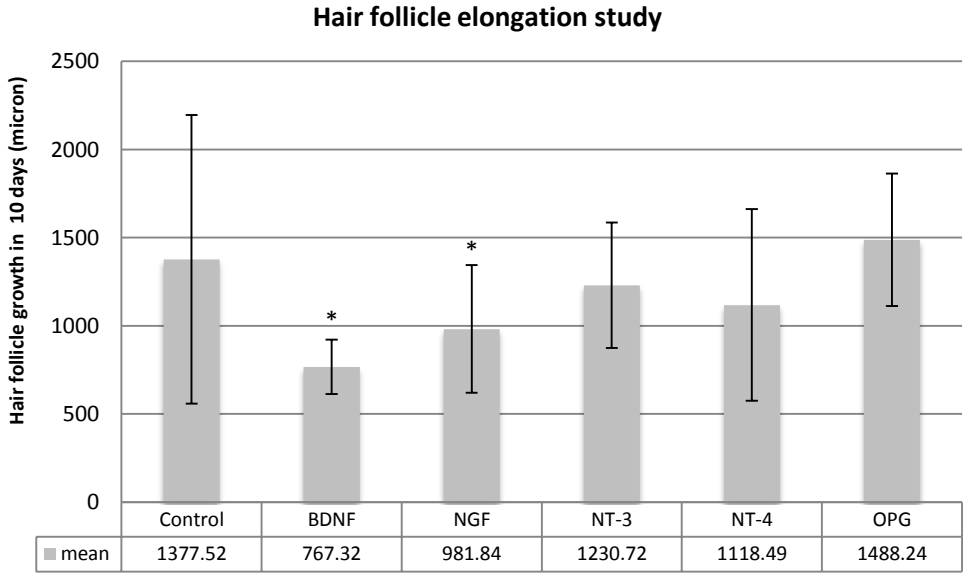


Figure 38 Mean length of hair shafts after treatment with NTs and OPG in hair follicle organ culture. Isolated hair follicles were cultured in Williams E medium supplement with 10 µg of insulin, 10 ng/ml of hydrocortisone, 200 mmol/L L-glutamine, 1% antibiotic-antimycotic and 5 different factors (BDNF 1ng/ mL, NGF 1ng/ mL, NT3 1ng/ mL, NT-4 1ng/ mL and OPG 10ng/ mL) for 10 days. Values are mean ± SD of hair shaft growth from baseline (micron). (* *P*< 0.05 compared with control)

4.4 Phase 3 study

Each anti-human NTs antibody was added to culture medium to determine whether it can reverse the NT inhibitory effect on the DP cells proliferation and hair follicle growth. In this phase, the study was divided into 4 test groups: control, factor alone, antibody alone and both factor and antibody.

4.4.1 DP cells proliferation

The influence of the NTs-neutralizing antibody against NTs-induced growth inhibition of cultured balding DP cells

Using MTT assay to measure the DP cell activity and viability, we found that during 4-day culture, the number of expanded human DP cells was lower in all NTs-supplemented groups than those in the control but these numbers were not statistically significant. Of four NTs, NT-4 showed the highest inhibitory effect on DP cells culture at 20.2% decrease compared to control. In the presence of human anti-NTs, we found that these neutralizing antibodies had trend to reverse the NTs- induce growth inhibition effect of cultured balding DP cells. (table 14-15, figure 39)

Table14 Number of DP cells after treatment with neutralizing antibodies against NTs determined by MTT assay. The balding DP cells were cultured for 4 days in the absence (control) or presence of BDNF, NGF, NT-3, NT-4 and their neutralizing antibodies. (control group; n=48, test group; n=12). (* $P < 0.05$; ** $P < 0.001$)

NTs and antibody	Day 2		Day 4	
	Mean DPCs	SD	Mean DPCs	SD
Control	2368.56	729.7	3389.993	1365.3
BDNF 1 ng/mL	2644.96	614.1	2775.74	1107.6
anti-BDNF 0.5 µg/mL	2415.64	566.6	2911.387	525.7
BDNF + anti-BDNF	2550.68	877.3	2832.311	1006.081
NGF 1 ng/mL	2670.71	585.1	3060.816	834.3

NTs and antibody	Day 2		Day 4	
	Mean DPCs	SD	Mean DPCs	SD
Anti-NGF 0.1 µg/mL	2588.19	599.8	3615.964	1486.6
BDNF + Anti-BDNF	2303.11	706.2	3128.334	818.3
NT-3 1 ng/mL	2063.05	759.5	2740.731	1363.9
anti-NT-3 0.1 µg/mL	2333.12	769.1	3523.306	1520.5
NT-3 + anti-NT-3	2430.65	908.4	3449.297	2351.7
NT-4 1 ng/mL	1980.53	629.6	3038.31	1463.5
anti-NT-4 1 µg/mL	2580.69	652.9	3525.94	1887.9
NT-4 + anti-NT-4	2243.10	681.8	2873.266	1078.6

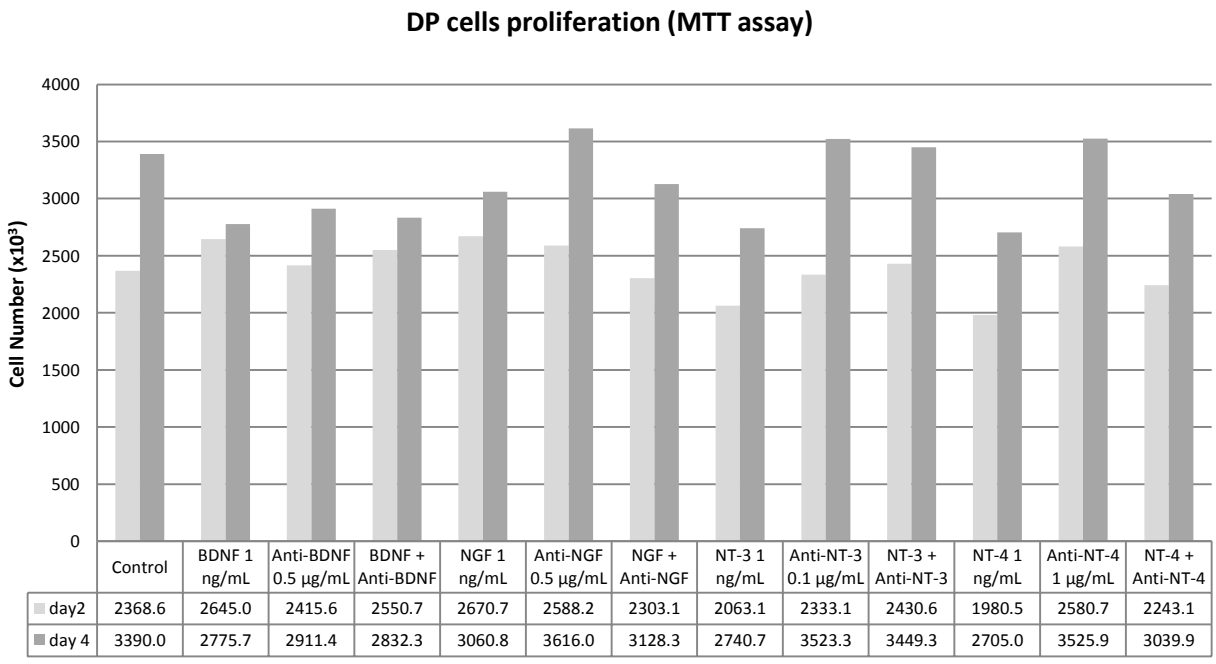


Figure 39 Number of DP cells after treatment with anti-BDNF, NGF, NT-3 and NT-4 neutralizing antibodies determined by MTT assay. Values are mean of DP cells number.

Table 15 Percentage of number of DP cells after treatment with anti-BDNF, NGF, NT-3 and NT-4 neutralizing antibodies determined by MTT assay. The results are expressed as a percentage of cell number compared to control.

Growth factor	Percent of (mean of DP cells) control	
	Day 2	Day 4
Control	0	0
BDNF 1 ng/mL	11.7%	-18.1%
anti-BDNF 0.5 µg/mL	2.0%	-14.1%
BDNF + anti-BDNF	7.7%	-16.5%
NGF 1 ng/mL	12.8%	-9.7%
Anti-NGF 0.1 µg/mL	9.27%	6.7%
NGF + Anti-NGF	-2.8%	-7.7%
NT-3 1 ng/mL	-12.9%	-19.2%
anti-NT-3 0.1 µg/mL	-1.5%	3.9%
NT-3 + anti-NT-3	2.6%	1.8%
NT-4 1 ng/mL	-16.4%	-20.2%
anti-NT-4 1 µg/mL	8.9%	4.%
NT-4 + anti-NT-4	-5.3%	-10.3%

Recombinant BDNF

Balding DP cells were cultured in absence (group 1, 3) or presence (group 2, 4) of 1 ng/mLBDNF with 0.5 µg/mL anti-BDNF Ab (group 3, 4). Decrease in cell viability was observed in all study groups of cultured DP cells compared to control at 4-day culture. In group 2 which BDNF1 ng/mL was added alone, the cell number decrease 614.3 cells (18.1%) compared to control. In the presence of anti-BDNF Ab (group 3 and 4), the number of DP cells increase higher than group 2 which BDNF was added alone. In group 3, which only anti-NGF was added, the number of cells was 135.7 cells higher than group 2 (NGF alone). In group 4, the number of cells was 56.6 cells (2%) higher than group 2. However, anti-BDNF Ab failed to demonstrate statistically significant reversal of the BDNF-induced DP cells growth inhibition.

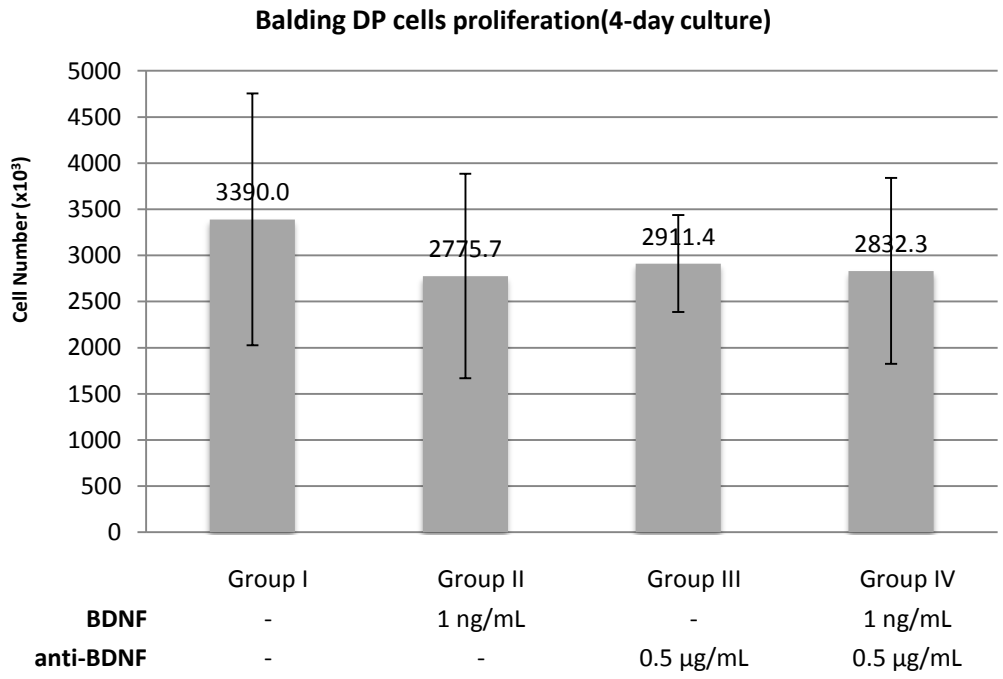


Figure 40 Number of DP cells after treatment with anti-BDNF determined by MTT assay. Balding DP cells were cultured for 4 days in the absence of Ab (lane 2, BDNF1ng/mL), presence of anti-BDNF Ab 0.5 µg/mL (lane 3), presence of both (lane 4) and control (lane 1). Values are mean ± SD of hair shaft growth from baseline (micron).

Recombinant NGF

Balding DP cells were cultured in absence (group 1, 3) or presence (group 2, 4) of 1 ng/mL NGF with 0.1µg/mL anti-NGF Ab (group 3, 4). At day 4 of culture, NGF at 1ng/ mL inhibited the proliferation of cultured DP cells compared to control with 9.7%decrease in cell number. In the presence of anti-BDNF Ab (group 3 and 4), the number of DP cells is higher compared to 2nd group which BDNF was added alone. The cell growth was highest in group 3, which only anti-NGF was added (226 cells (6.7%) increase compared to control and 552 cells (18.1%) increase compared to group 2 (NGF alone)). In group 4, the number of cells was 192.5 cells (6.3%) higher than group 2.However, this reversal effect was not statistically significant.

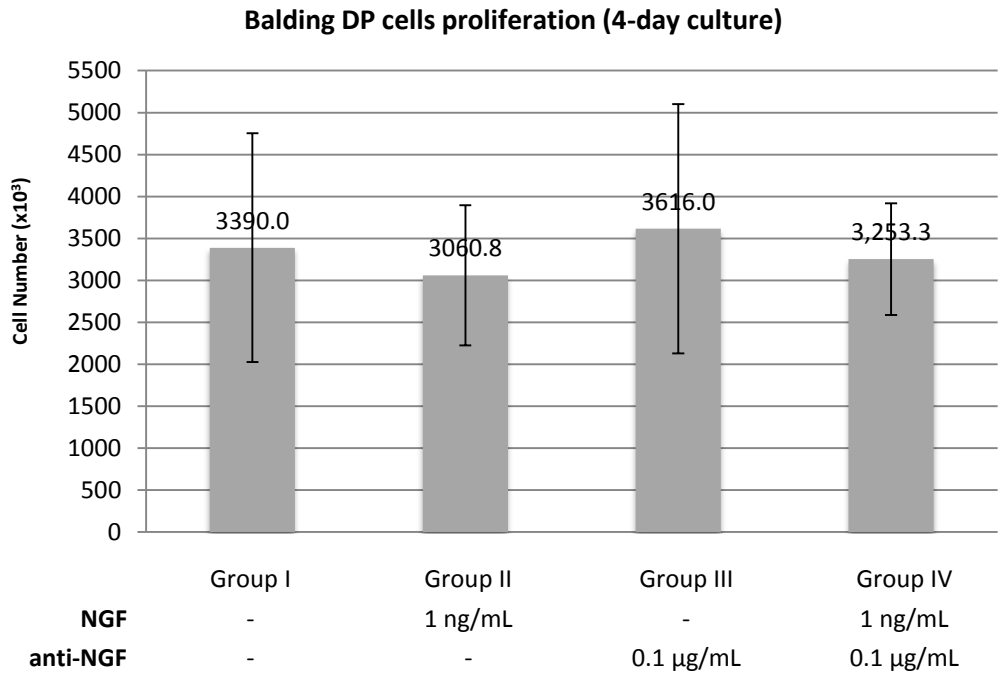


Figure 41 Effect of neutralizing antibody against NGF on the proliferation of cultured DP cells. Balding DP cells were cultured for 4 days in the absence of Ab (lane 2, NGF1ng/mL), presence of anti-NGF Ab 0.1 µg/mL (lane 3), presence of both (lane 4) and control (lane 1). Values are mean ± SD of hair shaft growth from baseline (micron).

Recombinant NT-3

Balding DP cells were cultured in absence (group 1, 3) or presence (group 2, 4) of 1 ng/mL NT-3 with 0.1µg/mL anti-NT-3 Ab (group 3, 4). At day 4 of culture, NT-3could inhibit the proliferation of cultured DP cells compared to control with19.2%decrease in cell number. In the presence of anti-NT-3 Ab (group 3 and 4), the number of DP cells is higher compared to control and 2nd group which NT-3 was added alone. The cell growth was highest in group 3, which only anti-NT-3 was added at133.3 cells (3.9%) higher than control and 782.6 cells (28.6%) higher than group 2 (NT-3 alone). Group 4, which both NT-3 and anti-NT-3 were added in media, the number of cells was 708.6 cells (25.9%) higher than group 2 and 59.3 cells (1.7%) higher than control. However, this reversal effect was not statistically significant.

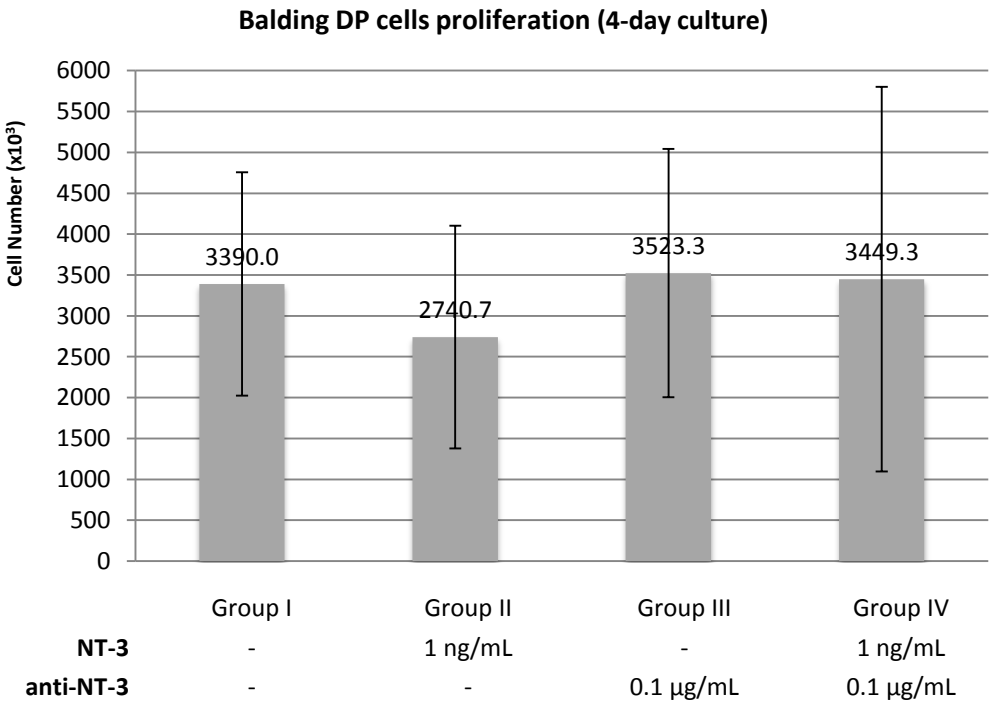


Figure 42 Effect of neutralizing antibody against NT-3 on the proliferation of cultured DP cells. Balding DP cells were cultured for 4 days in the absence of Ab (lane 2, NT-31ng/mL), presence of anti-NT-3 Ab 0.1 µg/mL (lane 3), presence of both (lane 4) and control (lane 1). Values are mean ± SD of hair shaft growth from baseline (micron).

Recombinant NT-4

Balding DP cells were cultured in absence (group 1, 3) or presence (group 2, 4) of 1 ng/mL NT-4 with 1µg/mL anti-NT-4 Ab (group 3, 4). At day 4 of culture, NT-4 at 1ng/ mL inhibited the proliferation of cultured DP cells compared to control with 20.2% decrease in cell number. In the presence of anti-NT-4 Ab (group 3 and 4), the number of DP cells is higher compared to 2nd group which NT-4 was added alone. The cell growth was highest in group 3, which only anti-NT-4 was addedat 135.9 cells (4%) higher than control and 820.9 cells (30.3%) higher than group 2 (NT-4 alone).The number of cells in group 4 was 334.9 cells (12.4%) higher than group 2. Nevertheless, this reversal effect was not statistically significant.

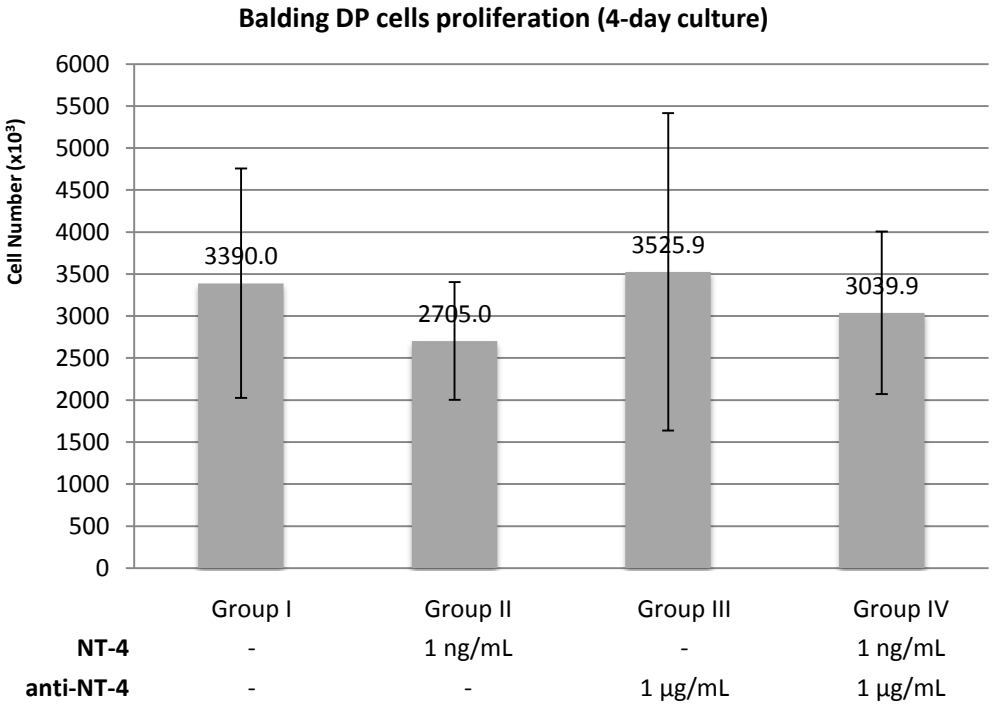


Figure 43 Effect of neutralizing antibody against NT-4 on the proliferation of cultured DP cells. Balding DP cells were cultured for 4 days in the absence of Ab (lane 2, NT-41ng/mL), presence of anti-NT-4 Ab 1 µg/mL (lane 3), presence of both (lane 4) and control (lane 1). Values are mean ± SD of hair shaft growth from baseline (micron).

Table 16 Summary of effect of neutralizing antibody against BDNF, NGF, NT-3 and NT-4 on the proliferation of cultured balding DP cells

Group	NTs	Anti-NTs	DP cells number	
I	-	-	Slightly increase the number	Control
II	+	-	Decrease less than control (lowest)	↓
III	-	+	Marked increase (highest)	↑↑
IV	+	+	Slightly increase or near control	↑

4.4.2 Hair shaft elongation study in hair organ culture system

To evaluate the effect of neutralizing antibody to NTs on the hair follicle growth, hair shaft length of each hair follicle was measured by Olympus DP2-BSW picture software at 10-day organ culture.

Effect of NTs-neutralizing antibody on NTs-induced inhibition of cultured DP cell proliferation

We next treated cultured human hair follicles with the recommended dose of recombinant human NTs. We found that BDNF and NGF at concentration of 1 ng/ mL significantly inhibited hair shaft growth by 482.03 μm,-39.3% (P=0.007) and 343.49μm,-30% (P=0.048) at 10-day culture, respectively. However neutralizing antibody had trend to reverse the NTs-induce hair growth inhibition of hair organ culture but the figures didn't show statistically significant. There were 4 study groups: control, NTs alone, anti-NT Ab and both NTs and anti-NT Ab. The hair growth length in each NT group was shortest of all the four other groups, while HFs treated with anti-NT Ab alone showed the highest hair shaft length. When both NTs and anti-NT Ab were added into media, the inhibitory effect of NTs decrease as it shown by increasing of hair shaft length compared to those with NTs alone, however, the length was not as high as control group. (Table 17-, Figure 44-)

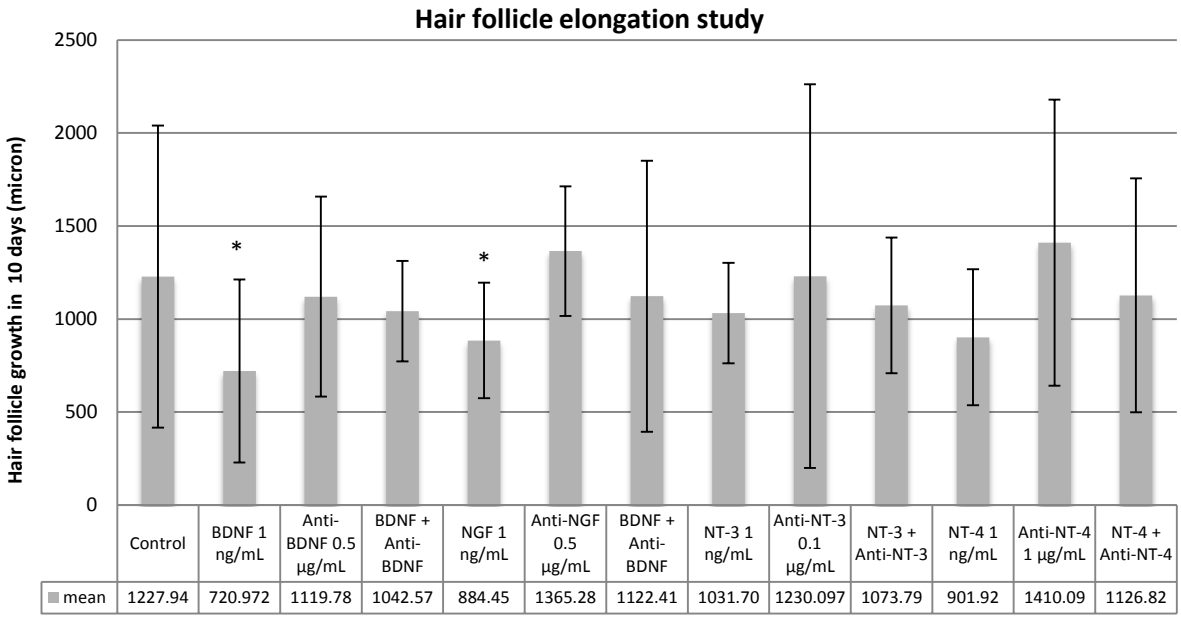


Figure 44 Mean lengths of hair shafts after treatment with anti-NTs antibodies in hair follicle organ culture. Isolated hair follicles were cultured for 10 days in the absence (control) or presence of BDNF, NGF, NT-3, NT-4 and their neutralizing antibodies. Values are mean ± SD of hair shaft growth from baseline (micron). (* $P< 0.05$ compared with control)

Table 17 Mean lengths of hair shafts after treatment with anti-BDNF antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days (* $P< 0.05$ compared with control)

BDNF	Mean length of hair growth (micron)	SD	Count (N)	*P* value
I Control	1227.94	812.3	21	-
II BDNF 1 ng/mL	742.91	514.4	22	0.007*
III anti-BDNF 0.5 µg/mL	1119.78	537.5	20	0.545
IV BDNF + anti-BDNF	1042.57	269.7	20	0.301

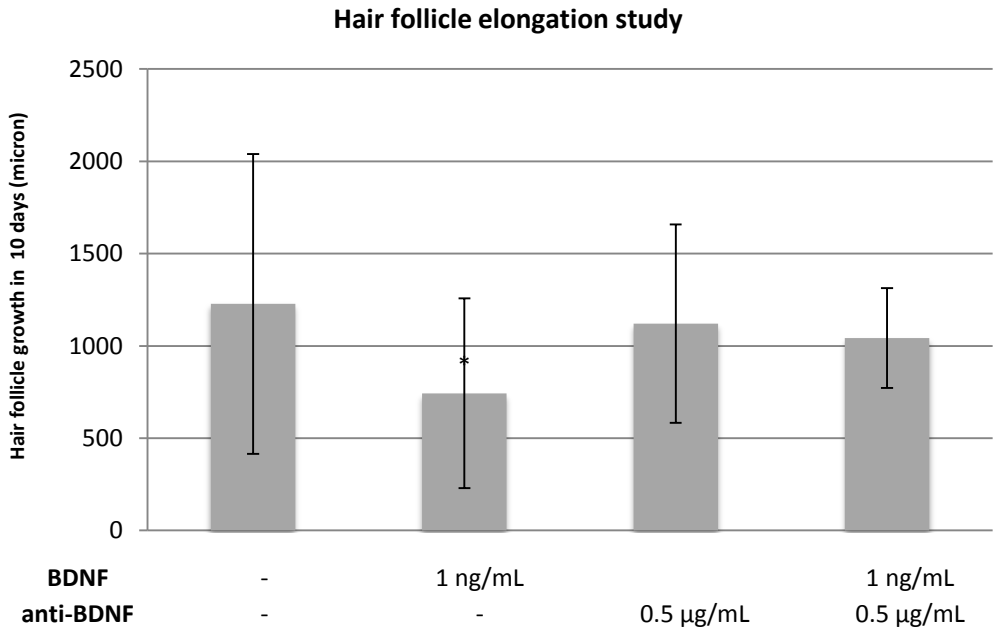


Figure 45 Mean lengths of hair shafts after treatment with anti-BDNF antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days in the absence of Ab (lane II, BDNF1ng/mL), presence of anti-BDNF Ab 0.5 µg/mL (lane III), presence of both (lane IV) and control (lane I). Values are mean ± SD of hair shaft growth from baseline (micron). (* $P< 0.05$)

Table 18 Mean lengths of hair shafts after treatment with anti-NGF antibody in hair follicle organ culture.Isolated hair follicles were cultured for 10 days (* $P< 0.05$ compared with control)

NGF	Mean length of hair growth (micron)	SD	Count (N)	*P* value
I Control	1227.94	812.3	21	-
II NGF 1 ng/mL	884.45	269.3	21	0.048*
III Anti-NGF 0.5 µg/mL	1365.28	311.8	22	0.420
IV BDNF + Anti-BDNF	1122.41	646.9	20	0.545

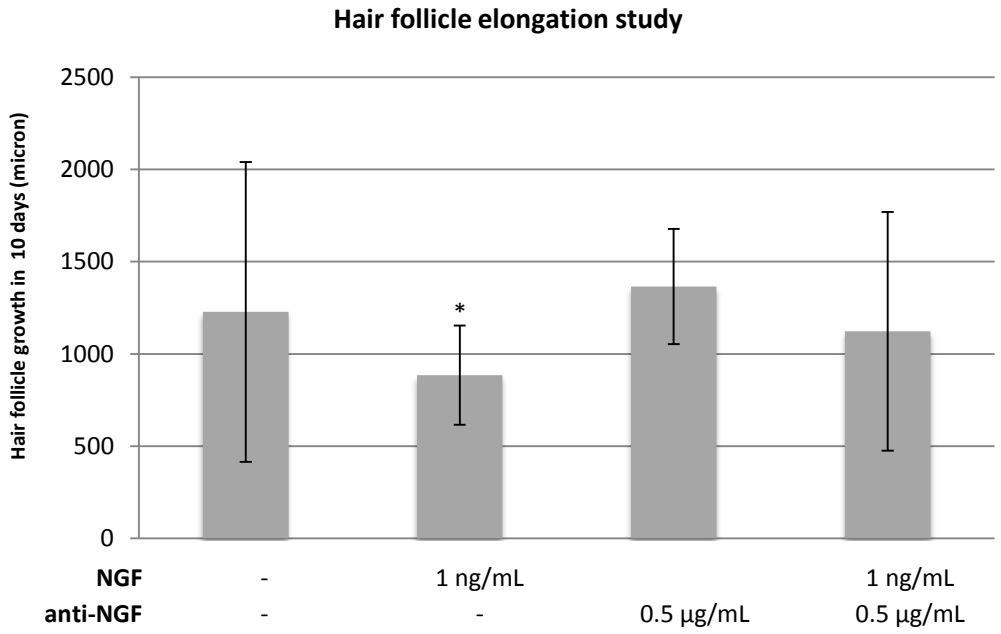


Figure 46 Mean lengths of hair shafts after treatment with anti-NGF antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days in the absence of Ab (lane II, NGF 1ng/mL), presence of anti-BDNF Ab 0.5 µg/mL (lane III), presence of both (lane IV) and control (lane I). Values are mean ± SD of hair shaft growth from baseline (micron). (* $P< 0.05$)

Table 19 Mean lengths of hair shafts after treatment with anti-NT-3 antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days

NT-3	Mean length of hair growth (micron)	SD	Count (N)	*P* value
I Control	1227.94	812.3	21	-
II NT-3 1 ng/mL	1031.7	270.1	18	0.386
III anti-NT-3 0.1 µg/mL	1230.1	1031.4	18	0.992
IV NT-3 + anti-NT-3	1073.79	364.5	18	0.495

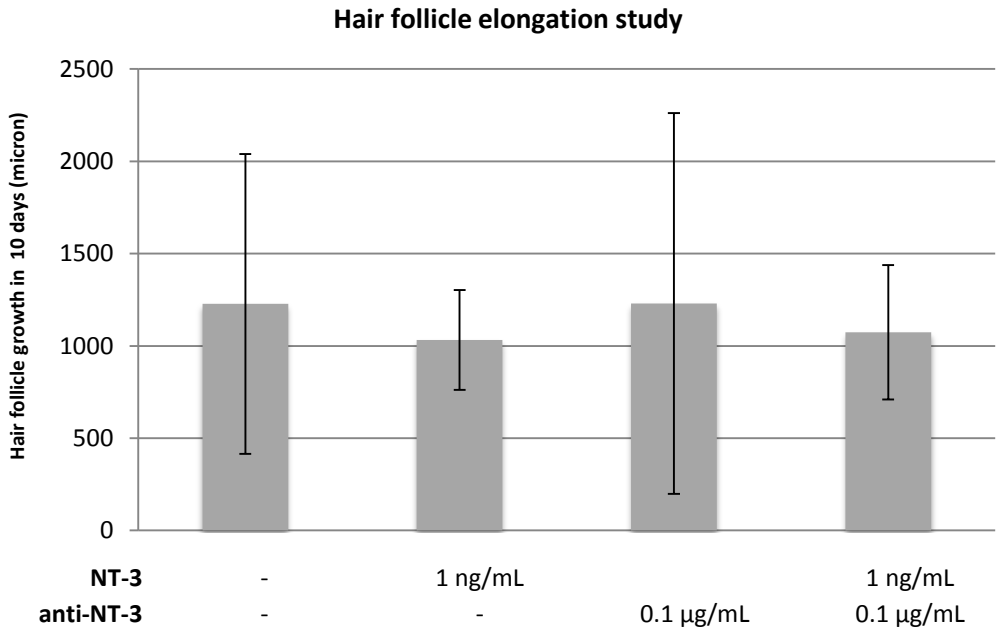


Figure 47 Mean lengths of hair shafts after treatment with anti-NT-3 antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days in the absence of Ab (lane II, NT-3 1 ng/mL), presence of anti NT- 3Ab 0.1 µg/mL (lane III), presence of both (lane IV) and control (lane I). Values are mean ± SD of hair shaft growth from baseline (micron). (* $P< 0.05$)

Table 20 Mean lengths of hair shafts after treatment with anti-NT-4 antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days

NT-4	Mean length of hair growth (micron)	SD	Count (N)	*P* value
I Control	1227.94	812.3	21	-
II NT-4 1 ng/mL	901.92	366.3	21	0.117
III anti-NT-4 1 µg/mL	1410.09	768.4	21	0.379
IV NT-4 + anti-NT-4	1126.82	628.6	20	0.629

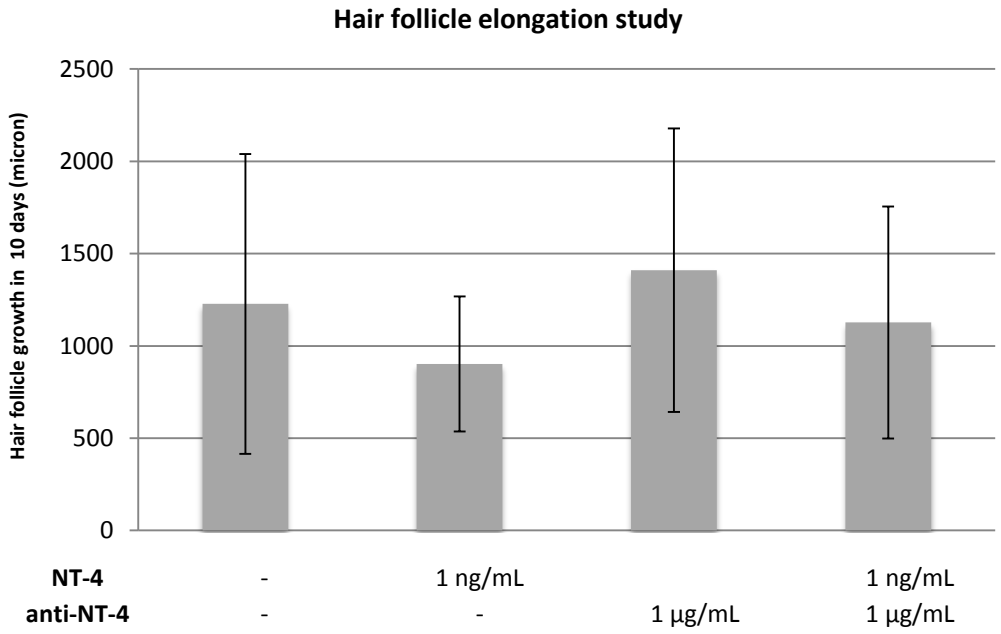


Figure 48 Mean lengths of hair shafts after treatment with anti-NT-4 antibody in hair follicle organ culture. Isolated hair follicles were cultured for 10 days in the absence of Ab (lane II, NT-41ng/mL), presence of anti-NT-4Ab1µg/mL (lane III), presence of both (lane IV) and control (lane I). Values are mean ± SD of hair shaft growth from baseline (micron). (* $P< 0.05$)

Table 21 Summary of effect of neutralizing antibody against BDNF, NGF, NT-3 and NT-4 on the elongation of human hair shafts in culture hair follicles

Group	NTs	Anti-NTs	Hair shaft length	
I	-	-	Increase in length	Control
II	+	-	Decrease shorter than control (lowest)	↓
III	-	+	Marked increase in length (highest)	↑↑
IV	+	+	Slightly increase or near control	↑

4.5 Discussion

4.5.1 Discussion of Phase 1 study

The hair follicle is a small but dynamic organ which undergoes cyclic growth from growing (anagen) phase through regression (catagen) and to a resting (telogen) phase. Highly specialized mesenchymal (dermal papilla) cells, that reside at the base of the hair follicles, are thought to provide the signals that control the terminal differentiation, initiate anagen and control the hair cycle by secretion of various growth factors (24).

AGA is caused by a shortening of the anagen period and miniaturization of the hair follicles, which results in the formation of progressively thinner, lighter colored, shorter hair. Although etiological studies(78) have proved that genetic predisposition and androgen are the main causes of this disease, the molecular events in AGA downstream from AR activation are not fully understood. Well-reported cytokines/ paracrine factors that are known to be involved include IGF-1, DKK-1,bFGF and VEGF (9).

IGF-1, arguably the best studied, plays an important role in maintaining anagen in cultured human scalp hair follicles in vitro (79) and causes an abnormal pattern of growth and differentiation of hair follicles when its effects are blocked in IGF-1 receptor deficient knockout mice (80). Furthermore, Itami et al. (8) have identified the mRNA expression for IGF-1 in beard DP cells and confirmed its importance by blocking the mitogenic effect of DP cells on outer root sheath cells cocultured with an IGF-1 antibody. IGF-1 has also been shown to be upregulated in DP cells of patients with AGA who respond to finasteride (81). In line with previous reports, we demonstrated that significantly lower levels of IGF-1 and its binding proteins (IGFBP-2, IGFBP-4) are expressed by DP cells obtained from balding vs. nonbalding scalps.

The novel findings in our study are that BDNF, NT-3 and b-NGF were the three most upregulated factors in supernatants from balding DP cells. Specifically, BDNF is increased by more than 12-fold. These three factors are members of the neurotrophic factors, which are known to promote survival of neurones by preventing them from programmed cell death and also induce the differentiation of progenitor cells to form neurones. Increasing evidence

suggests that neurotrophic factors not only influence the neurones, but also exert multiple effects on non-neuronal tissues, including the hair follicles. Nervous system and hair follicle epithelium share a common ectodermal origin and some NTs can modulate keratinocyte proliferation and apoptosis. NT-3 and NGF stimulate hair follicle development during morphogenesis (5, 6). However, several lines of experiments have shown that these neurotrophic factors also accelerate catagen development (7, 75, 77, 82).

Botchkarev et al. have shown that expression of BDNF and NT-4 is tightly coupled with the hair cycle and is likely to be involved in the transformation of active growth during anageninto the regressing catagen phase. Specifically, these two NTs, together with their specific receptor, TrkB, are upregulated during the catagen phase (75). This is not merely an epiphenomenon, as upon BDNF and NT-4 addition to organ-cultured murine skin, catagen was indeed accelerated (75) These NTs are thus regarded as apoptosis-inducible factors in the hair matrixcells. Apart from accelerating catagen transformation, BDNF also inhibits hair shaft elongation, at least in mice (77). Surprisingly, NT-4 was expressed at significantly lower levels by balding DP cells in our experiments.

The fact that some NTs possess dual functions, exerting antiapoptotic properties in the neural tissues while inducing apoptosis in other systems such as hair follicles, is not at all surprising (77). Different roles of various NTs and their respective receptors on hair morphogenesis have been extensively studied and reviewed (73).

Human scalp hair follicles are a source and target of BDNF (77). We propose that this neurotrophic factor may be one of the important links between androgens and hair follicles, serving as a negative regulatory control signal. In fact, BDNF expression has been shown to be under the control of androgens in several neural target tissues, at least in mice (83).

Osteoprogerin (OPG) was found to be secreted by non-balding DP cells in the highest level and its level was significantly different to that of by balding DP cells. OPG or osteoclastogenesis inhibitory factor (OCIF) is a member of the tumor necrosis factor receptor superfamily. It is a soluble secreted peptide produced by osteoblasts in bone resorption

process. OPG-/- KO mice develop severe early onset osteoporosis (84) and also exhibit medial calcification of the aorta and renal arteries (85). OPG acts as decoy receptors which binds to RANK ligand (RANKL) and blocks RANKL interaction with RANK and then inhibit osteoclast development (86). The published data on global gene expression profiling revealed that there were abundant transcripts of OPG in murine bulge cells (87). Furthermore, recent study found that RANK–RANKL–OPG also functions in HF formation, hair cycling and epidermal renewal (88).

We demonstrated that VEGF-D and EG-VEGF were significantly downregulated in the balding scalp. VEGF-D is a member of the PDGF/ VEGF family and is involved in angiogenesis and endothelial cell growth. VEGF, essential for angiogenesis and vascular permeability, was found to be expressed by DP cells during anagen phase. It may be responsible for maintaining proper vasculature around the hair follicles during the anagen growth phase (89, 90) demonstrated that improved follicle vascularization promoted hair growth and increased sizes of hair follicles and hair shafts in mice. As an autocrine growth factor for hair DP cells, VEGF can also stimulate the proliferation and migration of DP cells (15, 91). Gold-man, et al. demonstrated that the expression of VEGF inhuman hair follicles with alopecia was significantly decreased when compared with normal follicles (91). Furthermore, Lachgar et al. (65) found that the production of VEGF was enhanced by minoxidil in cultured DP cells, and concluded that minoxidil promotes hair growth, at least in part, via this induction. We also demonstrated that PLGF is downregulated in balding DP cell supernatant. Being a member of the VEGF subfamily, PLGF might act in synergism with VEGF in promoting anagen angiogenesis. Previous studies reported that PLGF is upregulated in murine anagen hair follicles immediately before the maximum peak of perifollicular angiogenesis and it was concluded that PLGF plays a role in anagen-associated angiogenesis (92). The downregulation of these stimulatory factors might trigger development of catagen and miniaturization of hair follicles in AGA.

The FGF family plays different roles in hair follicles (93, 94).FGF-7, a glycoprotein secreted from mesenchymal DP cells, regulates epithelial development during hair morphogenesis (95).In addition, FGF-7 has also been shown to induce and maintain hair growth in the anagen phase by inhibiting the transition to catagen phase in hair follicle organ culture (79, 96), similar to FGF-18, which also stimulates anagen formation (97). In contrast, FGF-5 has been shown to induce catagen transformation in mice (94, 98).Our study demonstrated that FGF-4 expression is highly increased in the balding scalp while its expression was not detectable in the nonbalding counterpart.

4.5.2 Limitation of Phase 1 study

The limitations of our study are that the assays were cross-sectional, thus dynamic changes of the factors at different stages of AGA as well as different phases of the hair follicles could not be determined. The arrays allowed us only to investigate the protein expression of the factors of interest. Further investigations at the mRNA level as well as in situ expression of these cytokines may reveal more conclusive evidence. Moreover, the small sample size precludes statistically significant changes.

We suggest that the possible candidate downstream mediators are DKK1(9), interleukin-1α, tumour necrosis factor-α (67) and TGF-β, especially TGF-β2, a potent catagen inducer ((51, 99-101), which has been reported to play a role in retinoid-induced alopecia (102). Despite these limitations, our study is the first of its kind to compare the cytokine profiles of balding DP cells with those of nonbalding counterparts from the same individuals.

4.5.3 Summary of Phase 1 study

We have described novel cytokine/ growth factor profiles which may partly explain the balding pattern observed in AGA. The first phase is a cross sectional study to identify the remarkable factors secreted from balding compared to nonbalding DP cells from the same subject. Our results suggest that the NTs, BDNF, NGF, and NT-3 may be highly involved. The

roles of other factors of which expression was significantly changed, including GDF-15 and OPG, in hair follicle biology are not clear at this point. This will open doors to numerous future studies that may pave the way to new discoveries of therapeutic interventions especially in neurotrophins group.

Therefore, further phase 2 and 3 studies were designed to explore roles of these novel factors/ cytokines on downstream pathways. We evaluated the autocrine effects of four NTs (BDNF, NGF, NT-3 and NT-4) by culturing human dermal papilla cells with these NTs and also investigate their effects on anagen hair growth in organ culture system.

4.5.4 Discussion of Phase 2 study

There is increasing evidence supporting the important role of mesenchyme-derived dermal papilla which is probably the site that androgens act on by altering the regulatory autocrine/ paracrine factors produced by dermal papilla cells. Such factors not only act on adjacent epithelial cells in a paracrine manner but may also regulate the DP cells themselves in an autocrine manner. The size of a hair matrix and hair is thought to be determined by the volume of its DP. The volume of the DP depends on the number of cells it contains and on the volume of the extracellular matrix (103).This suggests that alterations within the DP itself play a key role in altering hair size in response to androgens. So in phase 2 study, we investigate the effect of these interesting secretory factors on the proliferation of cultured DP cells to evaluate the autocrine effect and explore the effect on hair follicle growth in human anagen hair organ culture system.

Among the upregulated factors secreted from balding DP cells, we focused on neurotrophin factors. From phase I study, we propose that these neurotrophic factors may be one of the important links between androgens and hair follicles, serving as a negative regulatory control signal. There is increasing evidence to suggest that NTs also play important role in hair follicle. NTs are involved in the control of hair follicle morphogenesis through KC proliferation and apoptosis (104-107) and hair cycling (75). However, most published studies were conducted in murine system.

In our study, exogenous BDNF did not show significant effect on the proliferation of balding DP cells themselves. However, in human anagen hair follicle culture BDNF showed significant inhibition of hair shaft elongation. These may suggest that BDNF does not have direct effect on DP cells itself or it means that BDNF does not act mainly as autocrine but more toward paracrine activities. It may affect hair epithelial cells including hair matrix cells, ORS and IRS KCs which are mainly responsible for hair growth in anagen phase. This is supported by the many previous studies. Botchkarev et al. explored the role of BDNF, NT-4 and their high-affinity receptor TrkB in the control of murine hair follicle cycling (75). The result showed a marked enhancement of BDNF mRNA expression in situ as well as of BDNF- and NT-IR in immunohistochimistry study in proximal hair matrix, ORS and IRS KCs when the HF entered the spontaneous, apoptosis-driven hair follicle regression (catagen phase), while TrkB mRNA and IR were seen in central and proximal ORS in anagen VI HFs and in hair matrix KCs and DPs in early catagen phase. Furthermore, the study of Peter et al. reported that BDNF both mRNA and protein were expressed in proximal IRS, distal ORS and DP of human anagen VI hair follicles while TrkB only expressed in the epithelium (hair matrix, IRS and ORS)(77).

We also found the involvement of BDNF in human hair growth control as showing in organ-cultured human anagen hair follicles that only1 ng/ mL BDNF significantly inhibit hair shaft elongation. When compared to the previous study (77), BDNF at higher dose (50 and 150 ng/ mL) did show significant reduction of human hair growth and cause premature catagen development while low concentration (1, 5, 25 ng/mL) BDNF did not show significant effect. Moreover, BDNF-overexpression mice displayed accerelation of catagen and significant shortening of hair length compared to the corresponding age-matched wild type animals (6, 75, 108).

Here, we can confirm that human DP cells secreted BDNF protein and from phase 1 quantitative growth factor assay(109), we found that DP cells from AGA affected hair follicles secreted more BDNF than normal follicles. Therefore, it is reasonable to speculate that BDNF

secreted from dermal papilla cells of balding anagen hair follicles may be one of the important negative signals sending to TrkB positive hair follicle keratinocytes, which results in hair growth inhibition.

NT-4, one of the members of neurotrophin family, shares the same high-affinity receptor to BDNF, TrkB and low affinity p75NTR (73). We found that NT-4 had the largest significant inhibitory effect on the proliferation of cultured balding DP cells in dose dependent manner but it failed to show significant effect on hair growth inhibition. Even though the result is not compatible with phase 1 result, but we are not surprised because many previous studies supported its negative effects on hair cycle control and quantitative growth factor array is cross-sectional type of study. The negative effect of NT-4 on hair follicles was supported by significant catagen retardation in NT-4 knockout mice. Furthermore, NT-4 also demonstrated significant stimulate catagen development in murine skin organ culture (6, 75, 108).

NGF, a prototype of NT, is mediated via high-affinity TrkA receptor. In DP cell culture, NGF demonstrated significant inhibitory effect on cell number in dose dependent manner. Up to now there is no study report of NGF receptors expressed by DP, to our surprise, our study shows NGF strongly inhibit DP cells. NGF and its receptor Trk A are prominently expressed in human scalp anagen HFs (anagen VI), while they were weakly expressed in catagen and telogen HFs. Within HF compartments, NGF expression was prominent in the ORS, IRS, DP and CTS inner while Trk A expression was prominent only in the ORS of the anagen HF. However, our data encourage one to consider that exogenous NGF is, at least in part, involved in DP cells proliferation inhibition.

In addition, NGF significantly inhibited the growth of human anagen hair follicles. Accordingly, our data suggests negative effect of NGF on dermal papilla cell proliferation and hair growth control. It can also be seen from the previous studies that neonatal transgenic mice wtih NGF, NT-3 and BDNF overexpressing displayed accelerated catagen development (9). In contrast, during morphogenesis NGF and NT-3/ TrkC were reported to stimulate hair

follicle development whereas $p75^{NTR}$played inhibitory roles and BDNF did not show any significant effects (4, 5, 73, 110). NGF is also found to stimulate the proliferation and inhibit apoptosis of cultured human epidermal KCs via the high-affinity (TrkA) NGF-receptors.

NT-3 is also a member of NT family. TrkC is a high-affinity receptor for NT-3. However, NT-3 may also bind with low-affinity to TrkA and TrkB receptors. We found that NT only at the highest dose (10 ng/ mL) significant decreased DP cells at 2-day culture compared to control but other concentrations didn't demonstrate significant outcome at both day 2 and 4 of culture. In hair organ culture, NT-3 did not show significant hair shaft elongation inhibition. This raises the possibility that NT-3 has inhibitory autocrine effect on DP cells. It is supported by previous study that NT-3 IR was prominent in ORS, IRS, DP and connective tissue sheath of human anagen IV of hair follicles while Trk C IR was prominent in all HF epithelial and mesenchymal compartments (111). Furthermore, NT-3 also binds with low affinity to TrkB receptors which are expressed by DP fibroblasts during early catagen (5, 6, 112). Regarding the involvement of NT-3 in regulation of hair follicle cycling, NT-3 stimulated catagen development in organ culture of normal C57BL/6 mouse skin (75). Nevertheless, there is no previous study of NT-3 effect in human hair organ culture.

Not only NTs have direct negative effect on the hair follicle, but they also regulate the expression of cytokines which are critical for the hair cycle control. In human HF organ culture, BDNF and NGF treatment upregulated TGF-β2, which initiates anagen-catagen transition (77).

In this context, it is crucial to understand that NTs action is complex. The NTs utilize more than one mode of interaction with their receptors (113): acting as high-affinity growth factors activated by low concentrations of NTs (69, 114, 115), activation of Trk receptors by high levels of NTs has been shown to induce apoptosis depending on receptor availability and receptor density (69). There are two types of cell surface receptors, the Trk family (TrkA, TrkB and TrkC) and the p75 neurotrophin receptor (p75NTR). While the high-affinity Trk receptors are responsible for most of the survival and growth properties of the NTs, the

actions of p75NTR fall into two groups. First, when p75NTR is co-expressed with the Trks, it can enhance or suppress neurotrophin-mediated Trk receptor activity. Second, in some cell types, p75NTR is the only neurotrophin receptor, it will activate signaling cascades that result in the induction of apoptosis or in the promotion of survival (69).

Our study demonstrated that OPG was secreted by DP cells and had stimulatory autocrine effect. However, the previous study in mice showed that RANK, RANKL and OPG were expressed by telogen bulge and SHG cells but not by DP. Nonetheless, the mammary gland stromal cells constitutively transcribe OPG. Because RANK, RANKL-null mice exhibit defects in mammary gland development and in the eruption of teeth which, like HFs, are skin appendages, it is possible that OPG may be induced in the DP in response to as-yet-unidentified stimuli. We also found that OPG was secreted in a smaller amount by balding DP cells compared nonbalding. In mice's hair cycle, RANK stimulation by RANKL induces anagen phase. As the hair cycle progresses from telogen to anagen, OPG expression decreases but RANKL increases (88). The underlying reasons for the changes in RANKL and OPG synthesis are currently unclear. One possibility is that the overexpression of OPG in nonbalding DP cells may be a positive feedback loop from RANK and RANKL.

4.5.5 Limitation of Phase 2 study

The limitations of our study are that we selected to study the effect of NTs only on DP cells but not on ORS keratinocytes because DP is the main target of androgen in androgenetic alopecia (116). The effects of some NTs on cultured KCs have been studied (117). NGF and NT-3 were reported to stimulate KC proliferation, where BDNF and NT-4 does not exert any effect (106, 107, 118). In situ, NGF may also induce KC apoptosis in epithelial strand or secondary germ via p75NTR- or even via TrkA- signaling pathways (22, 71, 105, 119). Furthermore, higher doses of BDNF inhibit hair follicle keratinocyte proliferation and stimulate expression of the catagen-promoting and keratinocyte proliferation-inhibiting factor TGFβ2.

We did explore the effect of NTs on hair follicle growth in hair organ culture system. However, functional study of NTs on human hair cycle and NTs transducing systems that mediate via two categories of NTs receptors were not explored here. Studying of hair cycle in human hair follicle is very challenging. Moreover, the small sample size precludes statistically significant changes. Even with these limitations, our study is the first of its kind to evaluate the effect of whole NT family on cultured balding human DP cells and the growth of human hair follicles.

4.5.6 Summary of Phase 2 study

In summary, we have described the inhibitory effects of 4 NTs: BDNF, NGF, NT-3 and NT-4. NGF, NT-3 and NT-4 are able to inhibit proliferation of balding DP cells, which may be considered as negative autocrine signals. So it is reasonable to speculate that these NTs play part in vellus transformation of AGA affected hair follicles via decreasing the number of DP cells which has been shown to determine the volume of dermal papilla, and thus the diameter of the hair shaft.

In human anagen hair organ culture model, BDNF and NGF can inhibit human anagen hair growth. It raises the possibility that the inhibition effect of BDNF and NGF on anagen hair growth may occur via NTs secretion of the dermal papilla, acting on TrkB and TrkA expressed hair follicle keratinocytes primarily in the inner and outer sheath and matrix in hair follicle.

Our data in this study provides early evidence that NTs produced by balding DP cells inhibit the proliferation of DP cells and human hair follicle growth in vitro. This data, together with the higher expression of NTs in balding compared with nonbalding DP cells, suggest that NTs may be one of the key factors involved in pathogenesis of AGA.

4.5.7 Discussion of Phase 3 study

Finally we examined the potential of NTs neutralizing antibody on both cultured balding DP cells and hair organ culture systems. We found that NGF, NT-3 and NT-4 significantly suppressed the proliferation of balding DP cells however these neutralizing

antibodies fail to reverse these effects. For human hair follicle growth study, BDNF and NGF at 1 ng/ mL also showed the significant inhibition effect as the results from phase 2 but again their antibodies failed to show significant neutralizing effects. However, we can observe the trend of our results. Group II that NT was added alone, it showed the smallest number of DP cells and significant hair shaft inhibition. In group III, which only anti-NTs antibody was added, it demonstrated the positive effect, both DP cells and hair shaft length showed marked increases in group III. Group IV, which both NT and anti-NT Ab were added in media, showed a slight to moderate increase or equal to control (group I).

It might be from the concentration of each NT and its neutralizing antibody, that we used, may not match. The concentration of anti-NTs antibody may not enough to neutralize NTs. Furthermore, the small sample size may preclude statistically significant changes.

This may suggest that BDNF, NGF, NT-3 and NT-4, which are one of the androgen-driven altered factors secreted from balding scalp dermal papilla cells, work in group like a cocktail. Neutralizing individual NT may not strong enough to reverse its negative effect.

4.5.8 Limitation of Phase 3 study

We selected only one concentration of NTs and anti-NTs antibody in this experiment according to the previous studies (6, 75, 106) and company recommendation. The varying in concentrations of both NTs and its neutralizing antibodies should be further investigated to endorse the significant inhibitory effects of NTs

4.5.9 Summary of Phase 3 study

This may suggest that NTs serve at least as one important negative cellular messenger system in the regressing human hair follicle apart from other possible downstream mediators including dickkopf(DKK)-1, interleukin-1α, tumour necrotic factor-α and TGF-β especially TGF- β2

4.6 Conclusion

In conclusion, NTs produced by balding DP cells were found to inhibit the proliferation of DP cells and human hair follicle growth in vitro. Altogether, our data suggests that NTs are one of the key factors involved in pathogenesis of AGA. However it still remains to be investigated whether our data translate into significant role for NTs in the hair growth cycle. Furthermore, OPG was found to be secreted by DP cells and at least, plays part in hair follicle growth control.

4.7 Suggestion

Future studies of the specific role of NTs and their receptors and transducing systems would shed light on molecular mechanism of androgenetic alopecia. Furthermore, the modulation of NTs and their receptors may pave the way to new discoveries of therapeutic interventions. Last but not least, the role of OPG in the hair cycle should be further investigated.

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