รายงานวิจัยฉบับสมบูรณ์

โครงการ

The Effectiveness of Topical Tofacitinib VS Topical Minoxidil on Hair Growth in Mice

โดย

รศ.ดร.พญ. จิตรลดา มีพันแสน

15 สิงหาคม 2562

CONTENTS

ABSTRACT

Background

Tofacitinib is a janus kinase 3 (JAK3) inhibitor that promotes hair growth; however, the efficacy and mechanism of this effect are not yet understood.

Objective

This study aimed to evaluate the efficacy and mechanism of topical tofacitinib on hair growth in mice.

Methods

Eight-week-old male C57BL/6 mice were divided equally into four groups and treated topically with tofacitinib, minoxidil, or vehicle once daily for 21 days. Weekly photographs were taken to determine the area and rate of hair growth, and tissue samples were collected for histopathological evaluation. mRNA and protein expression of anagen-maintaining growth factors, including vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), were determined via RT-PCR and ELISA, respectively.

Results

Tofacitinib-treated mice exhibited more hair regrowth than either minoxidil-treated or control mice did between days 7 and 21 ($P<0.05$). Topical tofacitinib also promoted more rapid hair growth rate than topical minoxidil or control did ($P<0.001$). Histopathology showed a distinct increase in the number of hair follicles, mostly in the anagen phase, in the tofacitinib-treated group. Hair follicles in the minoxidil- and vehicle-treated groups were more often classified as catagen and anagen. VEGF mRNA and protein expression in the tofacitinib-treated group was significantly greater than those in the other groups ($P<0.05$). IGF-1 mRNA expression was not upregulated in tofacitinib-treated mice.

Conclusions

Topical tofacitinib is effective in promoting hair growth, and the possible mechanism involves increased VEGF levels and lowered inflammation. This study will help develop a new therapeutic option for non-scarring alopecia.

Keywords: JAK3 inhibitor, non-scarring alopecia, hair growth, VEGF, IGF-1

บทคัดย่อ

บทนำ

ได้มีการศึกษามาก่อนหน้านี้ว่า Tofacitinib ซึ่งเป็นยาในกลุ่ม Pan-JAK inhibitor สามารถกระตุ้นการงอกของเส้นผมได้ แต่อย่างไรก็ตาม กลไกการกระตุ้นผมยังไม่ทราบเป็นที่แน่ชัด

วัตถุประสงค์

การศึกษานี้เป็นการศึกษาประสิทธิผลของยา Tofacitinib ชนิดทาเทียบกับ Minoxidil ชนิดทาต่อการงอกของเส้นขนในสัตว์ทดลอง

วิธีการทดลอง

ทำการทดลองในหนูสายพันธ์ C57BL/6 อายุ 8 สัปดาห์ โดยแบ่งออกเป็นสี่กลุ่ม แต่ละกลุ่ม ทายา Tofacitinib, Minoxidil, DMSO และ Ethanol เป็นระยะเวลา 21 วัน วัดผลโดยวิธี การถ่ายภาพ เพื่อเปรียบเทียบสัดส่วนระหว่างบริเวณที่ขนงอกต่อบริเวณที่ไม่มีขนงอกการตัดชิ้นเนื้อ เพื่อดูลักษณะทางจุลกายวิภาคศาสตร์เนื้อเยื่อ ศึกษาลักษณะเส้นขนที่งอกใหม่ ขนาดเส้นผ่านศูนย์กลางของเส้นขน เส้นเลือดที่มาเลี้ยง ตลอดจนการอักเสบของกระเปาะรากขน การตรวจวัดปริมาณ VEGF และ IGF-1 protein โดยวิธี ELISA ซึ่ง VEGF และ IGF-1 มีคุณสมบัติเป็น cell growth factor อันจะบ่งถึงการงอกของเส้นขน

ผลการศึกษา

หนูในกลุ่มที่ทายา Tofacitinib มีการงอกของขนที่มากกว่าและรวดเร็วกว่าหนูในกลุ่มอื่น ๆ อย่างมีนัยสำคัญทางสถิต ลักษณะทางจุลชีววิทยาพบว่า ในกลุ่มที่ทายา Tofacitinib มีการเพิ่มขึ้นของกระเปาะรากผม ผมส่วนใหญ่อยู่ในระยะเจริญเติบโต และเซลล์อักเสบต่าง ๆ ลดลง การแสดงออกของ ยีนส์และโปรตีน VEGF ในกลุ่มที่ทาด้วยยา Tofacitinib สูงกว่ากลุ่มอื่นๆ ในขณะที่ การแสดงออกของยีนส์ IGF-1 มีมากกว่าในกลุ่มของหนูที่ทาด้วยยา Minoxidil

การสรุปผล

ยา Tofacitinib ชนิดทามีประสิทธิภาพที่ดีในการกระตุ้นการงอกของผม โดยกลไกในการกระตุ้นผมนั้น น่าจะมาจากการกระตุ้น VEGF และการลดการอักเสบ การศึกษานี้ช่วยในการพัฒนายาตัวใหม่ที่ใช้ในการรักษาผมร่วงผมบางชนิด non-scarring alopecia ในมนุษย์ต่อไป

Keywords: JAK3 inhibitor, non-scarring alopecia, hair growth, VEGF, IGF-1

REVIEW OF LITERATURE

Janus kinase 3 (JAK3) inhibitors, a new class of immunomodulatory agents, provides immunosuppressive, anti-inflammatory, anti-allergic, anti-thrombotic and anti-leukemic effects (1).

Tofacitinib (former CP-690,550), a drug of the janus kinase (JAK) inhibitor class, inhibits JAK3 and JAK1 with lesser effects on JAK2 (2)(3). Among JAK inhibitors family, tofacitinib has high specificity compared to others. It has crucial role in driving autoimmunity on Type I/II cytokines receptors in γc family : IL-2, IL-4, IL-7, IL-9, IL-15 (2)(4), as well as cytokines receptors that signal via JAK1. Accordingly, tofacitinib disrupts Th1 and Th2 differentiation and also impaired inflammatory Th17 cells production(4). The drug has been widely used in clinical trials in many settings of rheumatoid arthritis (2)(5)(6)(7), inflammatory bowel disease (8), psoriasis (9), organ transplantation (1)(10) to corneal inflammation and dry eyes (11)(12). Subsequently, tofacitinib is approved by Food and Drug Administration (FDA) for the treatment of rheumatoid arthritis since 2012 and currently in clinical trials for treatment of psoriasis (13), vitiligo (14) and alopecia areata (AA) (15)(16).

Recently, Xing *et al*. demonstrated that pharmacological inhibition of the Janus kinase signal transducer and activator of transcription (STAT) pathway promotes hair regrowth in AA. JAK inhibitors could block IFN-γ, IL-2, and IL-15Rβ prevented the development of AA, decreasing the $CD8^{+}NKG2D^{+}$ T cells accumulation in the skin and the response of dermal IFN in AA mice model (15). Accordingly, Harel *et al*. demonstrated that JAK inhibitors treatment resulted in rapid onset of hair growth in mice by the process of activates Wnt and Shh signaling pathways (3) which mimics the mechanisms of normal anagen initiation (17)(18). Moreover, JAK-STAT inhibition causes hair follicle progenitor cells activate (3) from the evidence that hair germ compartment (P-cadherin+) of drug-treated hair follicles was proliferated (19). And also enhanced pathways, such as Rho and integrin signaling, involved in cell motility and migration which responsible in the transition between telogen and anagen (3). Furthermore, they also examined the effect of JAK inhibition on hair growth in human tissue (grafted human fetal scalp skin onto severe combined immunodeficient mice). The results suggested that tofacitinib treatment promotes hair elongation rate and enhances effect on inductivity of human dermal papilla. These were followed from the process evidence from repressed in receptors that involved in the modulation of dermal papilla inductivity, such as fibroblast growth factor receptor 1 (FGFR1), activin A receptor like type 1 (ACVRL1), insulin-like growth factor 1

receptor (IGFR1), oncostatin M receptor (OSMR) and prostaglandin F receptor (PTGFR) (20)(21)(22)(23) collocate with down-regulated of proapoptotic genes included Bcl2-associated X protein (BAX), Bcl2-like11 (Bcl2L11) and Caspase12 (CASP12). In addition, genes up-regulated by tofacitinib treatment involved the members of the transforming growth factor–beta (TGF-β) pathway and the bone morphogenetic protein (BMP) pathway, which formerly shown to have a critical role in dermal papilla (DP) inductivity (23)(24)(25)(26). Besides, lymphoid enhancer-binding factor 1 (LEF1), another key regulators of the WNT pathway, which control dermal-epidermal interactions (27)(28) together with the NOTCH pathway members, which control hair follicle fate (29) were also overexpression in tofacitinib treatment (3).

In summary, from their findings can be concluded that JAK-STAT signal inhibition stimulates the activation and/or proliferation of hair follicle stem cells, resulting in promotion of hair growth (3).

Despite the aspect of successful therapeutic options for alopecia areata and variants with tofacitinib and ruxolitinib (JAK1,2 inhibitors), another aspect is the potential risks of these medications if taken in oral form, Craiglow *et al.* were concerned about this matter. They decided to pursue a trial of topical ruxolitinib on a failure treatment alopecia universalis (variant of alopecia areata) patient. Then finally reported a first case of successful treatment of alopecia universalis with topical ruxolitinib as JAK inhibitor. While further studies are needed to confirm the efficacy, safety and tolerability (30).

Nowadays, the problem of hair loss has been increasingly concerned by patients, which can be categorized into scarring and non-scarring alopecia. Examples of diseases organised in scarring alopecia group are lichen planopilaris (LPP), frontal fibrosing alopecia (FFA) and discoid lupus erythematosus (DLE). Meanwhile, androgenetic alopecia (AGA), alopecia areata (AA) and telogen effluvium (TE) are in non-scarring alopecia group.

AA is a non-scarring loss of hair on scalp or other area of the body, affects approximately up to 2% of population. Its pathogenesis is involved multifactorial autoimmune associated with unknown etiology. AA is characterized by one or more well-defined hair-less non-scarring patches, which it could be progressed to more severe form as involved whole scalp or body hair. In spite of multiple treatments available including steroids, photochemotherapy and immunotherapy, AA could be spontaneously resolved within 1 year, whereas up to 25% developed to severe form.

TE, defined by loss of telogen hair, results from abnormal hair cycling. Possible causes of TE including systemic illness, drug induced, loss of weight, nutritional deficiency, delivery,

oral contraceptives interruption and scalp inflammation. As same as AA, TE could spontaneously be resolved, after treated underlying causes (31).

AGA is known to be the most common form of non-scarring alopecia. According to a previous study in Thailand reported the prevalence of baldness reaching 61.78% at 70 years of age (32). It mainly results from androgen-dependent process and genetic transmission, in associated with suspected pathogenic factors such as microbial flora, stress (endogenous and exogenous), and microinflammation (33). The hallmark characteristics of AGA is vellus-like hair transformation in correspond to miniaturization of hair follicles during repeated hair cycles with premature termination of anagen (34). In male AGA, commonly known to be associated with increasing in 5-alpha reductase activity propagate to an increase in dihydrotestosterone (DHT) production, which may be results in hair follicle loss, yet the mechanism is not clearly known (35).

Apart from genetic predisposition and androgens main theories, suspected noticeably coincidence from scalp biopsy has shown that the miniaturization of terminal hairs is oftenly associated with perifollicular lymphocytic infiltration, and fibrosis (36). Subsequently, this may be one of the credible reasons encourages microscopic follicular inflammation theory.

In the focus on hair follicle cycling, AGA implies a process of shortened anagen phase associated with premature catagen phase. Catagen has been advocated to occur as a consequence of decreased in anagen maintaining factors expression, including insulin-like growth factor 1 (IGF-1) (37), basic fibroblast growth factor (bFGF) (38) and vascular endothelial growth factor (VEGF) (39), associated with increasing in cytokines promoting apoptosis expression, including transforming growth factor beta 1 (TGF-β1) (34)(25), interleukin-1 alpha (IL-1α) and tumor necrosis factor alpha (TNF-α) (33)(40).

The goal of treatment in AGA is to increase in hair number on the scalp and to delay progression of hair thinning. Current available treatment modalities for AGA with proven efficacy (FDA approved) are oral finasteride (dose 1 mg/day) and topical solution of minoxidil (dose 2% for women, 5% for men).

Finasteride acts as a potent competitive inhibitor of type II 5α-reductase and inhibits the conversion of testosterone to DHT (41)(42), which is involved in miniaturization process of hair follicle in AGA. In the focus on drug efficacy, according to a systematic review of twelve studies showed moderate-quality evidence that daily use of oral finasteride increased the mean hair count from baseline in comparison to placebo treatment, reported as a percentage of the initial count in each patient, at short term (mean difference (MD) 9.42% [95% CI, 7.95%-10.90%]; I^2, 50%) and at long term (MD 24.3% [95% CI, 17.92%-30.60%]; I^2, 0%) and also

increased in the proportion of patients reported as improved by investigator assessment in the short term (relative risk (RR), 1.80 [95% CI, 1.43-2.26]) (43). In contrast to its efficacy, adverse effects of finasteride are increasingly concerned especially on sexual functions (reported in clinical trials at rates of 2.1% to 38%) (44), which leads to poor compliance problem. Moreover, Thompson *et al.* reported that taking finasteride may associate with increased risk of high-grade prostate cancer (6.4 % and 5.1 % in the finasteride treated group compared to placebo group respectively, P<0.001; RR, 1.67 [95% CI, 1.44%-1.93%]) due to it prevented or delayed the onset of cancer (45), but long term follow-up of the same subjects proved no significant difference in the rates of overall survival or survival after the diagnosis of prostate cancer (the 10-year survival rates were 73.0% [95% CI, 68.1%-78.0%] and 73.6% [95% CI, 68.3%-78.9%] in the finasteride treated group compared to placebo group respectively, among those with high-grade prostate cancer) (46). Nevertheless, further study is required to conclude the association between the use of finasteride and prostate cancer. Another limitation of finasteride is its slow onset and must be in long term use which may increase risk of adverse effects. Finasteride is contraindicated in who are or may potentially be pregnant because of the risk in impair virilization of a male fetus (47).

Topical minoxidil, an adenosine-triphosphate-sensitive potassium channel opener and vasodilator (33), which feasible mechanism is to extend the duration of anagen phase of hair follicles by induces cell growth factors including VEGF, hepatocyte growth factor (HGF), IGF-1 and activates uncoupled sulfonylurea receptor on dermal papilla plasma membrane which leads to enhance HGF and IGF-1 actions, as well as inhibits TGF-β which acts as hair matrix cells apoptosis inducer, and lastly dilates hair follicle arteries and increases blood flow in dermal papilla (48). In the aspect of clinical use, Olsen *et al.* reported that 5% topical minoxidil was significantly greater in efficacy to 2% topical minoxidil and placebo in increasing hair regrowth in AGA patient (5% topical minoxidil treated group showed 45% more hair regrowth than 2% topical minoxidil treated group at week 48, P 0.025). The adverse events using topical minoxidil are dose-dependent, commonly from dermatologic nature such as pruritus, itching, burning and other symptoms of scalp dermatitis, but no systemic effects has been reported (49). Another important adverse event of patient using topical minoxidil is that it provokes a transitory TE. TE is characterized by increased hair shedding, in case of minoxidil use, the condition caused by mechanism of fast reentry to anagen phase of the hair follicles. Although acute TE from minoxidil treatment is reversible and transient, but the condition causes psychoemotional stress which leads to the main cause of concern and the reason for seeking medical advice (50).

Since clinical success rate of the treatment of AGA with modulators of androgen metabolism or hair growth promoters is still limited. In spite of the standard treatment of AGA which are oral finasteride and topical minoxidil, still some problems on adverse events which leads to drop out of patient. Thus, we are interested in doing research on a new group of medication in purpose of improved alternative modalities of treatment for non-scarring alopecia patient. As mentioned above, tofacitinib as JAK3 inhibitor, a new class of immunomodulatory agent, provides multiple therapeutic effects. In the focus on hair promoting effect, inhibitory of JAK-STAT signal could leads to initiate anagen phase of hair cycle, and also activates hair follicle stem cells. In considering, another possible mechanism in promoting hair growth of tofacitinib maybe take part in the process of hair follicle microinflammation, due to its anti-inflammatory effect. However, the mechanism of tofacitinib is still not completely clear, hence further study should be done. In this research, we are interested in the treatment of tofacitinib on its efficacy in promoting hair growth in mice, in comparable to topical minoxidil which has been approved as standard treatment for AGA, the most common form of non-scarring alopecia. The process of determining therapeutic mechanism of tofacitinib in the study which involved clinical, histopathology and cytokine protein expression would lead to further understanding in the mechanism of tofacitinib and probably be beneficial in developing an alternative group of drug for patient with non-scarring alopecia in the near future.

RESEARCH METHODOLOGY

1 Materials

1.1 Animals

Seven-week-old male C57BL/6 mice procure from National Laboratory Animal Center. A total of studied mice will be randomly assigned to tofacitinib-treated group (N=7), minoxidil-treated group (N=7) and control group (N=7). During the experiments, the animals will be housed under strict hygienic conventional standard, maintain under controlled environmental conditions (12-hour light/dark cycle, temperature approximately 23°C), and provided with standard laboratory food and water ad libitum. Study protocol was approved by Thammasat University's Animal Ethical Committee and conducted according to Ethical Principals and Guidelines for the Use of Animals for Scientific Purpose.

1.1.2 Sample size

A total of 21 eight-week-old male C57BL/6 mice are recruited for this study.

▪ Program G* Power 3.1.7 (162) :

Effect size f = 0.8

α error probability = 0.05

Power (1-β error probability) = 0.80

Number of groups = 3

Total sample size = 21

1.1.3 Inclusion criteria

Male C57BL/6 mice aged 8 weeks.

1.1.4 Exclusion criteria

(1) Mice are in seriously ill condition, weight loss >20%, moan with pain or soundless, reject food or water.

(2) Death during the experiment

1.2 Drugs

Tofacitinib was purchased from Abmole Bioscience (catalog no.477600-75-2), and dissolved in dimethyl sulfoxide (DMSO) into 2% concentration. 5% topical minoxidil was purchased from drug industry, dissolved in ethanol base (60-65%), was used in compare with topical tofacitinib.

2 Research design

2.1 Animal experiment

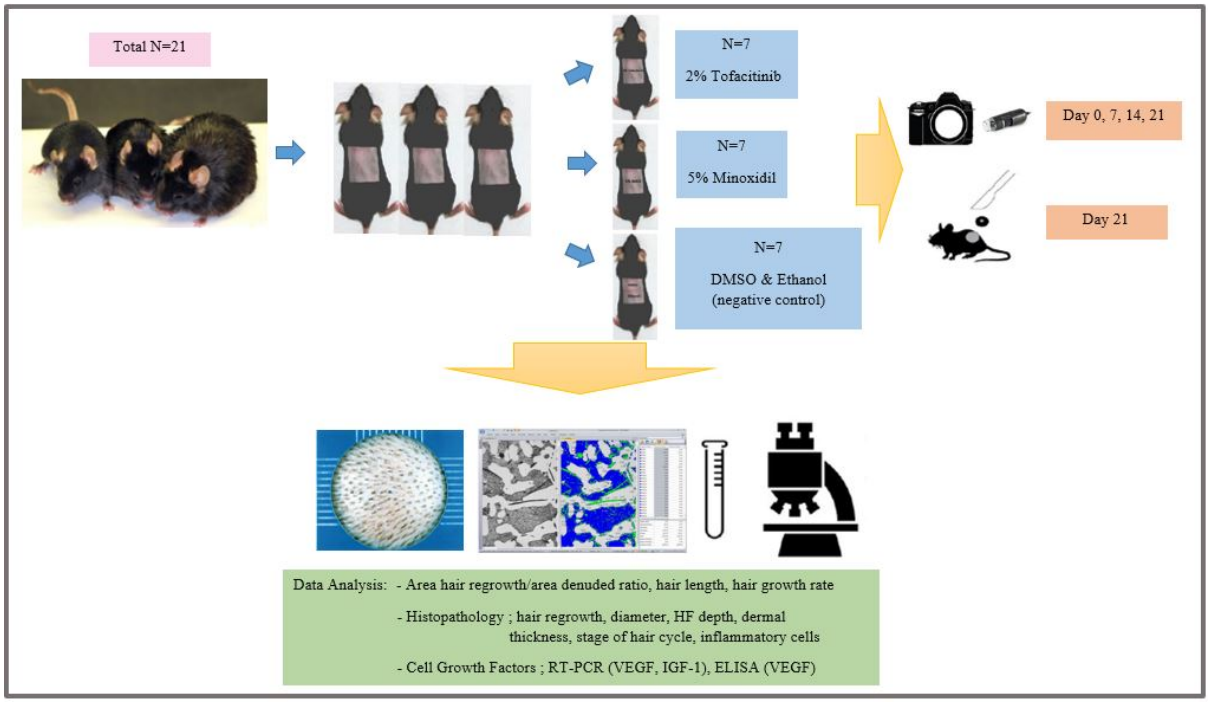

Figure 1 Flow chart of research methodology

Mice were anesthetized with inhaled isoflurane (Aerrane, Baxtar, USA) one day before the procedure. Hair on the dorsal skin (size 2.5x3.6 cm position just below to scapula) of all mice were gently coated under sterile conditions using an electric shaver to avoid injury.

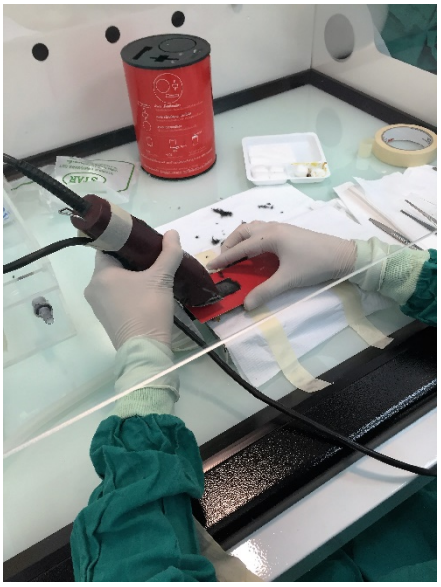

Figure 2 Mice were gently coated under sterile conditions using an electric shaver.

After coating, the mice were randomly divided into group of seven, then numbering and labelling.

Figure 3 Numbering and labelling mice.

Each labelled mouse was digitally photographed using digital camera and microscope, focused on the dorsal back coated skin. On the day on which the reagents were to be applied (day 1-21), each group were treated topically 0.1 mL/area once daily with 2% tofacitinib or 5% minoxidil. A digital image of the coated area was weekly recorded until three weeks. In case of minimized variations, we calculated area hair regrowth relatively from each mouse baseline. For hair regrowth study area, a window template size 2.5x3.6 cm was

used to standardize the size of calculated area. And for microscopic area photographed by digital microscope, the mark point on a window template helped focusing on the same position while taking photo in each week.

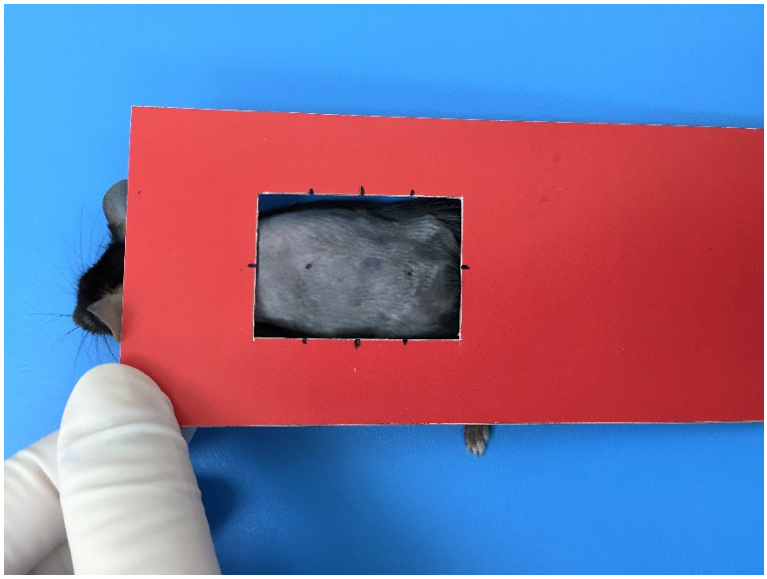

Figure 4 A digital image of the coated area was recorded at the same position.

At the experimental endpoint, tissues from the dorsal back coated area were collected from each treated mouse using sterile punch biopsy (6 mm in diameter) for histopathological study and cell growth factors analysis. Finally, mice were humanely euthanized with carbon dioxide (CO_2).

Figure 5 Tissues from the dorsal back coated area were collected using punch biopsy.

2.2 Outcome measurements

2.2.1 Hair growth observation

Hair growth was determined weekly by digital photograph using a digital camera (DSC-RX100M3, Sony Corporation, Japan). The photographic data was analysed using Adobe Photoshop 6.0 software (Adobe Systems Incorporated, United States), performed by the same independent computer graphic administrator, without bias from recognizing the previous treatment information, to calculate the ratio of the area showing hair regrowth to the area denuded of hair. Hair growth rate was evaluated using digital microscope (Dino-Lite AM7013MZT(R4), AnMo Electronics Corporation, Taiwan) at 65x magnification.

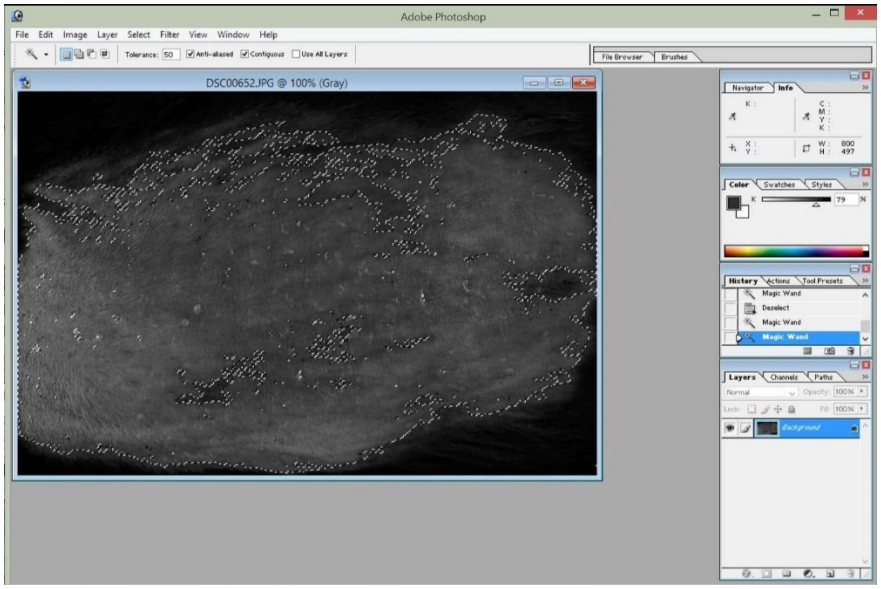

Figure 6 An example of the photographic data that was analysed using Adobe Photoshop 6.0 software to calculate the ratio of the area showing hair regrowth to the area denuded of hair.

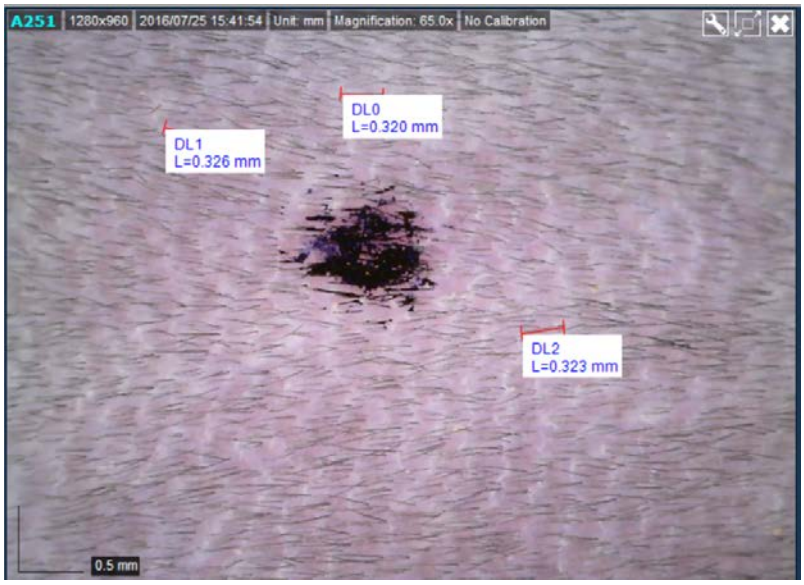

Figure 7 An example of mean hair growth measurement using digital microscope in order to evaluate hair growth rate.

2.2.2 Histopathological analysis

Tissues from the coated area were collected on day 21 using punch biopsy (6 mm in diameter) under inhaled isoflurane anaesthesia. The samples were fixed in 10% buffered formalin for 24 hours, embedded in paraffin wax, and stained with haematoxylin and eosin (H&E), to evaluate:

(1) Hair re-growth by comparing mean number of hair follicles in the examined area of 6 mm between control and treated groups.

(2) Dermal thickness and hair follicle depth were measured using the scale bar tool of the microscope, then compared among the treated and control groups.

(3) The hair follicle of the examined area was classified according to the stages of the hair growth cycle (anagen, catagen and telogen).

(4) The inflammatory cells were categorized and evaluated using an optical microscope comparing among the treated and control groups.

All histopathological analysis was done by veterinary pathologists under 4x and 10x magnification using optical microscopy.

2.2.3 Cell growth factors analysis

2.2.3.1 Real-time polymerase chain reaction (RT-PCR)

The collected tissue samples were stored as fresh frozen at -80°c until use. VEGF and IGF-1, hypothesized important cell growth factors on hair growth promotion induced by tofacitinib, were analysed by RT-PCR. RNeasy® Mini Kit (QIAGEN, Valencia, California, USA) was used for total RNA purification from the collected tissues. RNA isolation was performed according to the manufacturer’ s protocol.

Figure 8 RNeasy® Mini Kit for total RNA extraction.

(1) Tissue samples were removed from the -80°c storage. According to this experimental research, we used entire unstable cryopreserved tissues placing directly into a suitable sized vessel for disruption and homogenization process.

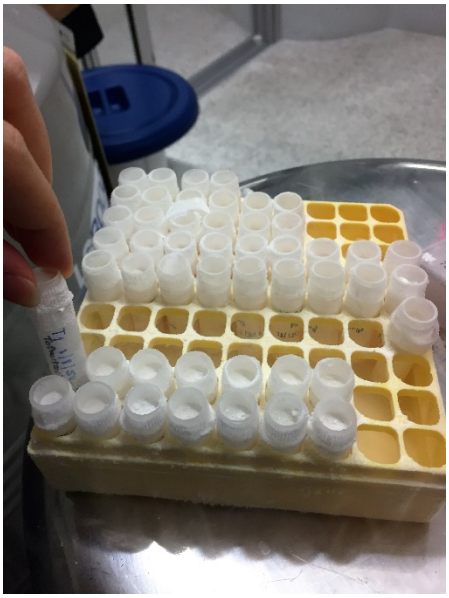

Figure 9 Tissue samples.

(2) Mortar and pestle were used to destruct tissues. The frozen tissues were placed in mortar and added liquid nitrogen, then grinded with pestle into small grain. After liquid nitrogen was evaporated, the tissue grain was gently transferred into an RNase-free micro-centrifuge tube.

Figure 10 Tissue samples were destructed using mortar and pestle.

(3) Homogenized the lysate with 600 μL of RLT buffer. Then, the lysate was pipetted into a 1.5 mL eppendorf tube and placed into provided shredder spin column. Centrifuged the lysate for 2 minutes at full speed. Next, carefully pipetting to transfer the supernatant into a new micro-centrifuge tube.

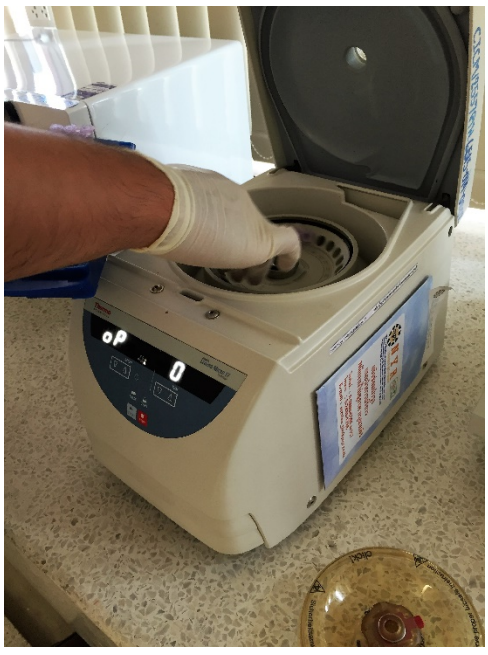

Figure 11 The lysate was centrifuged.

(4) Adding 600 µL of 70% ethanol into the lysate and immediately pipetted to mix it. Transferring 700 µL of the sample with any precipitate into the provided RNeasy spin column placed in a 2 mL collection tube, then, centrifuged for 15 seconds at 10,000 rpm, thereafter, discarding the flow-through.

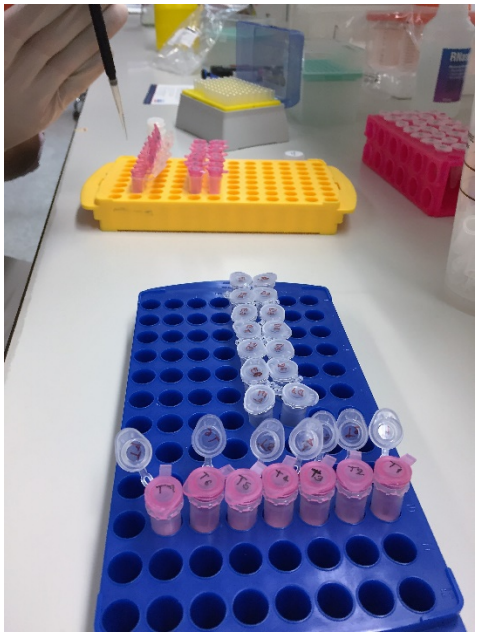

Figure 12 Transferred the sample with any precipitate into the provided RNeasy spin column placed in a 2 mL collection tube.

(5) The spin column membrane was washed 3 times with:

- 700 µL Buffer RW1, then centrifuged for 15 seconds at 10,000 rpm.
- 500 µL Buffer RPE, then centrifuged for 15 seconds at 10,000 rpm.
- 500 µL Buffer RPE, then centrifuged for 2 minutes at 10,000 rpm.

(6) Transferring the RNeasy spin column into a new 1.5 mL collection tube. RNase-free water was then added directly to the spin column membrane in order to elute the RNA, and centrifuged for 1 minute at 10,000 rpm.

(7) The previous step was repeated using another 15 µL of RNase-free water to elute the RNA.

(8) RNA concentration was measured by NanoDrop spectrophotometer in order to evaluate the RNA quantity and purity.

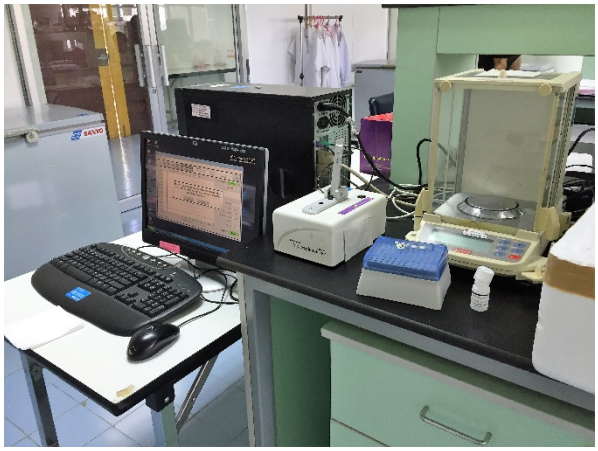

Figure 13 RNA concentration was measured using NanoDrop spectrophotometer.

Due to the concentration of RNA was too low or maybe error in some samples, we have to exclude those off, left over 22 samples (tofacitinib 7 samples, minoxidil 5 samples, DMSO 7 samples, and ethanol 3 samples) to further analysed by reverse transcription process.

In order to synthesis complementary DNA (cDNA), reverse transcription was performed using ImProm-II™ Reverse Transcription System (Promega Madison, USA). Before mixing with other reagents, RNA was heated at 70°c for 5 minutes, and left on ice 1-2 minutes, then the following components were added and mixed.

Figure 14 RNA was heated at 70°c for 5 minutes, before mixing with other reagents.

Finally, the mixture was placed in T100™ Thermal Cycler (BIO-RAD, Hercules, California, US) running the process of cDNA synthesis under control temperature at 25°c for 5 minutes, 42°c for 1 hour, and 70°c for 15 minutes.

- Oligo dT 1 µL
- dNTP mix 1 µL
- Buffer 4 µL
- Reverse transcriptase enzyme 1 µL
- Recombinant Rnasin 0.5 µL
- $MgCl_2$ 1.6
- RNA 0.582-10 µL (volume used is according to RNA concentration in each sample; 30-100 ng of RNA)
- RNase free water (titrate until total volume reach 20 µL)

Total volume = 20 µL

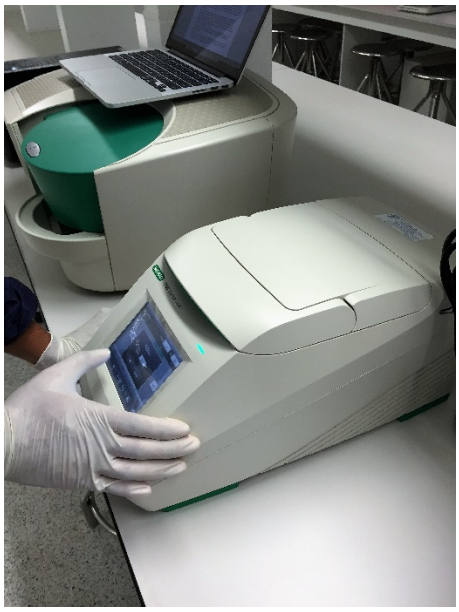

Figure 15 T100™ Thermal Cycler

We evaluated the mRNA expression of VEGF, IGF-1, and beta 2-microglobulin (as an internal control). The following primers were used:

- Mm.282184 for VEGF
- Mm.268521 for IGF-1
- Mm.163 for beta 2-microglobulin

One step RT-PCR was performed following the manufacturer's protocol using the ImProm-II™Reverse Transcription System. The reverse transcription reaction mixture was prepared by combining the following components in a sterile 1.5 mL microcentrifuge tube on ice.

- 2x Probe RT-PCR Master mix 10 µL
- QN Probe RT mix 0.2 µL
- Primer 1-FAM 1 µL
- Primer 2-VIC 1 µL
- RNA (7 ng)
- RNase-free water

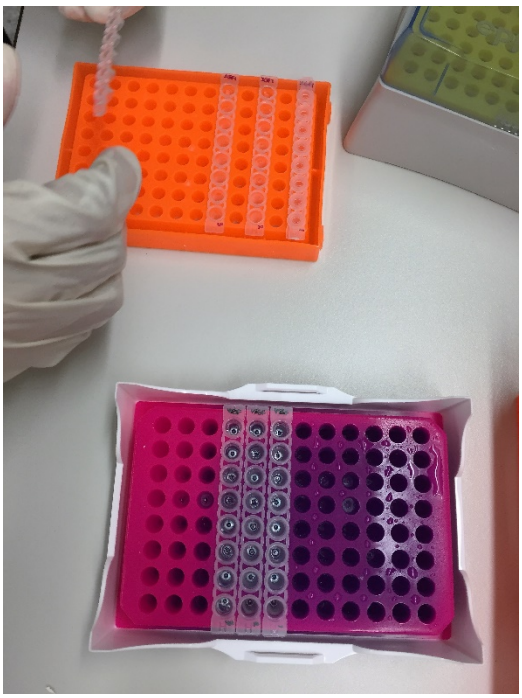

Figure 16 The reverse transcription reaction mixture.

The reactions were placed in a C1000 Touch™ thermal cycler BIO-RAD CFX96™ Real-Time System (BIO-RAD, Hercules, California, US) that has been preheated to 95°C. An optimized program for amplification using the Upstream and Downstream Control Primers provided as 95°c for 10 minutes, and 40 cycles of 95°c for 15 seconds denaturing, and 60°c for 1 minute annealing. Fluorescence dye was detected. The relative ratio of gene expression for each gene was determined by standard exponential curves. The internal control gene (beta 2-microglobulin) was used to normalize the target gene ratio.

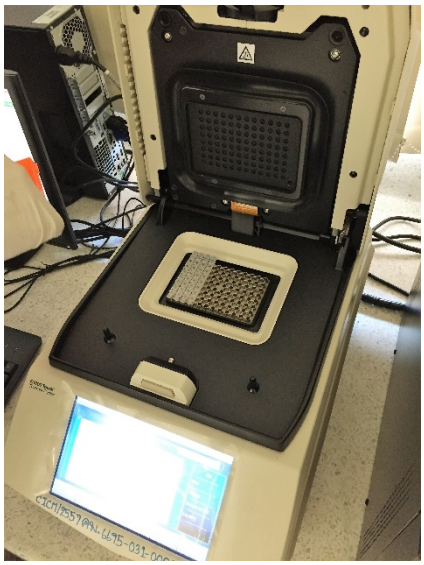

Figure 17 The reaction mixture plate was transferred in the thermal cycler, then RT-PCR was run.

2.2.3.2 Enzyme-linked immunosorbent assay (ELISA)

Quantitative measurement of VEGF protein was done by ELISA technique using Abcam's VEGF Mouse ELISA kit (ab100752, Abcam, UK). Dermal levels of VEGF were determined in the biopsy tissue sample. The skin tissues were minced and homogenized in a polytron-type homogenizer using 2 mL of 1N acetic acid. Then the tissue homogenates were centrifuged (3,000 × g, 10 minutes, 4°C). The resulting supernatants were measured by Bradford method before using for ELISA.

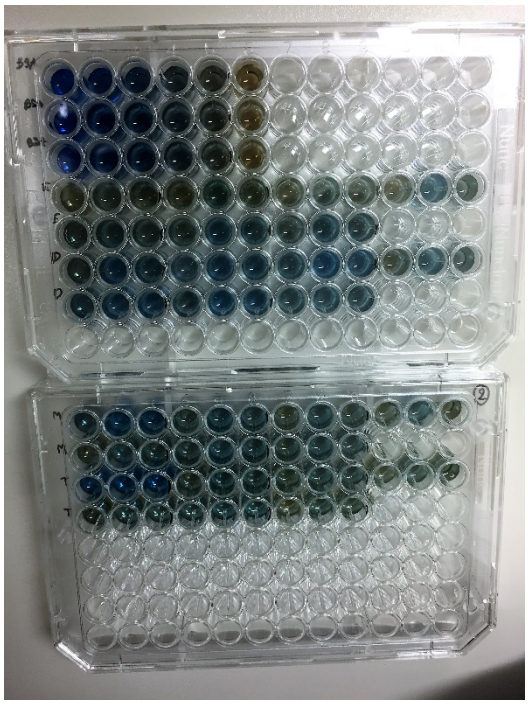

Figure 18 The supernatants were measured by Bradford method.

The concentration of VEGF protein in the supernatants was measured using an Abcam's VEGF Mouse ELISA kit according to the manufacturer's instructions.

(1) Reagent Preparation

Sample diluent buffer was diluted 5-fold, cell lysate buffer was diluted 2-fold, and assay diluent was diluted 5-fold with deionized or distilled water before use. Wash solution was diluted into 400 mL of 1-fold wash solution with deionized or distilled water. The Biotinylated VEGF anti-Mouse vial was spinned before use. Then 1-fold assay diluent 100 μL was added into the vial to prepare a detection antibody concentrate. Gently mixed with pipette. Before using in the assay procedure, the detection antibody concentrate was diluted to 80-fold with 1-fold sample diluent buffer. Prior to use in the assay procedure, the HRP-Streptavidin concentrate vials were spinned and diluted to 160-fold with 1-fold sample diluent buffer. All reagents were equilibrated to room temperature (18-25°C) before using.

(2) Standard Preparations

The vial of VEGF standard was grossly spinned, then added 400 μL of 1-fold assay diluent or 1-fold sample diluent buffer into the vial to prepare a 25 ng/mL VEGF stock standard. After gently mixed, the standards were prepared into 8 concentration. Standard #1 was prepared by adding 40 μL of 25 ng/mL stock standard to 960 μL of 1-fold assay diluent or

1-fold assay diluent into tube #1, then the tube was thoroughly and gently mixed. 300 µL of 1-fold assay diluent was pipetted into each tube. Standard #2 was prepared by transferring 200 µL of the mixture from tube #1 to #2, then mixed rigorously. As same as standard #2, other standards were prepared in serial as shown in the standard dilution preparation table below. 1-fold assay diluent or 1-fold sample diluent buffer was served as the zero standard (0 pg/mL).

Table 1 Standard dilution preparation table.

Standard #	Volume to dilute (µL)	Diluent (µL)	Total volume (µL)	Starting conc. (pg/mL)	Final conc. (pg/mL)
1	40	960	1,000	25,000	1,000
2	200	300	500	1,000	400
3	200	300	500	400	160
4	200	300	500	160	64
5	200	300	500	64	25.6
6	200	300	500	25.6	10.2
7	200	300	500	10.2	4.1
8	0	300	300	0	0

(3) Sample Preparation

Tissue lysate sample was diluted to 5-fold with 1-fold sample diluent buffer. Final concentration in each sample was not exceed 1,000 pg/mL.

(4) Assay Procedure

Before using, equilibrate all materials and prepared reagents to room temperature (18-25°C). 100 µL of each standard and sample were added into the appropriate wells, then incubate for 2.5 hours at room temperature or 4°C overnight with gentle shaking. Next, the wells were washed by filling each well with 300 µL of 1-fold wash solution for 4 times. After the last wash, any remaining solution was removed from wells by aspirating. The plate was inverted and blotted against clean paper towels. Then, 100 µL of 1-fold biotinylated VEGF detection antibody was added to each well and incubated at room temperature for 1 hour with gentle shaking. After that, the 4-time wash process with 1-fold wash solution was repeated again. 100 µL of 1-fold HRP-Streptavidin solution was added to each well prior to 45 minutes incubation at room temperature with gentle shaking. After the solution was discarded, the 4-time wash process was repeated again. 100 µL of TMB one-step substrate reagent was added to each well prior to 30 minutes incubation in the dark at room temperature with gentle shaking. Finally, 50 µL of stop solution was added to each well. The results was read at 450 nm immediately.

(5) Calculations

The mean absorbance for each set of duplicate standards, controls and samples was calculated, and subtracted the average zero standard optical density. Eventually, the standard curve was plotted in order to indicate VEGF concentration relating from the absorbance.

3 Data analysis

ANOVA with post-hoc Bonferroni test and independent t-test were run to compare the data obtained from the treatment groups, and all measured values were reported in means. In case of the data was not scattered in normal distribution, the values were presented as median (range: min - max) and p-value was corresponded to Mann-Whitney U test. All statistical analyses were performed using Stata version 13 (StataCorp LP, USA). Statistical significance in all cases was considered to have a p-value <0.05.

RESULTS

The total of 21 C57BL/6 male mice were enrolled in this research and all remained healthy until the experimental endpoint.

1 Hair growth

1.1 Significant hair regrowth in the topical tofacitinib-treated group compared to that in the topical minoxidil-treated group

On day 7 of treatment, mice treated with tofacitinib showed initiation of hair regrowth on the coated area, while minoxidil-treated mice were still denuded with higher statistical significance for the tofacitinib-treated group (25.36%) as compared to minoxidil-treated group (18.23%) with 95% CI 7.13 (3.1-11.16), P=0.002. On day 14, more distinct hair regrowth was observed with tofacitinib treatment (64.62%) than with minoxidil (30.31%), with 95% CI 34.32 (14.5-54.13), P=0.004. At experimental endpoint on day 21, hair regrowth continued, and we could macroscopically observe almost complete regrowth of hair in the tofacitinib-treated group (98.5%) compared to a partial regrowth seen in the minoxidil-treated group (61.58%) with a statistically significant 95% CI 36.91 (11.35-62.48), P=0.012. As the data shown in Table 1 and Figure 1.

Table 1 Comparison of area hair growth (%) at day 0, 7, 14, and 21 between tofacitinib-treated group and minoxidil-treated group.

	Tofacitinib (n=7)	**Minoxidil (n=7)**	**Mean difference (95%CI)**	**p-value (t-test)**
	Mean (95%CI)	**Mean (95%CI)**		
Area hair growth (%)				
Day 0	15.22 (11.86, 18.58)	14.42 (11.55, 17.29)	0.8 (-3.13, 4.73)	0.666
Day 7	25.36 (21.66, 29.06)	18.23 (15.63, 20.83)	7.13 (3.1, 11.16)	0.002**
Day 14	64.62 (45.08, 84.16)	30.31 (23, 37.62)	34.32 (14.5, 54.13)	0.004**
Day 21	98.5 (96.8, 100.2)	61.58 (36.02, 87.14)	36.91 (11.35, 62.48)	0.012**

Values presented as mean (95% confident interval of mean). P-value corresponds to Independent t-test.

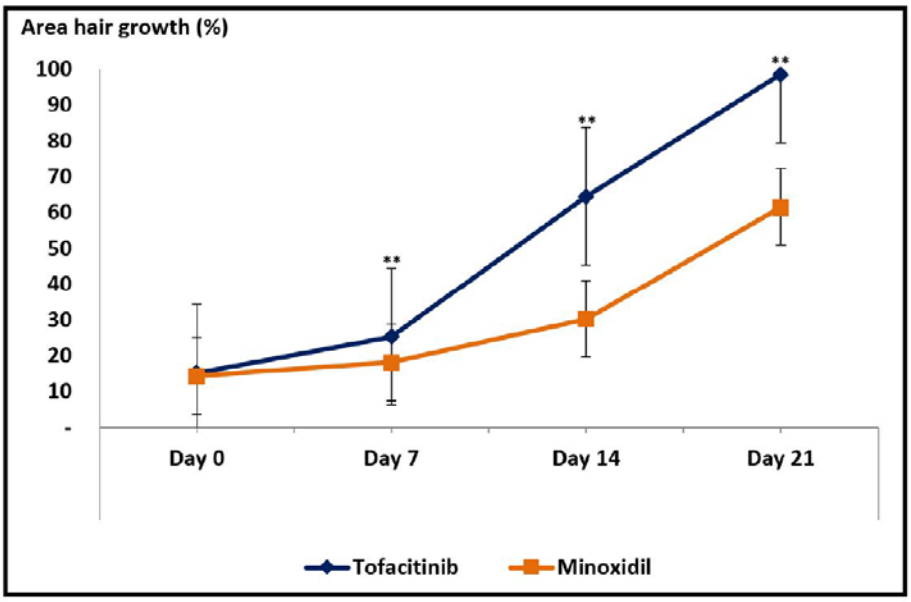

Figure 1 Comparison of area hair growth (%) at day 0, 7, 14, and 21 between tofacitinib-treated group and minoxidil-treated group. Mean ± SEM, **P<0.05

In the same way, on day 14, tofacitinib showed more significant hair regrowth on the coated area (64.62%) when compared to DMSO-treated group (31.21%) and ethanol-treated group (31.48%), P<0.001. On day 21, tofacitinib also showed distinct hair regrowth on the coated area (98.5%) when compared to DMSO-treated group (66.59%) and ethanol-treated group (36.62%), P<0.001. Whereas minoxidil-treated group demonstrated no significant hair regrowth when compared to both vehicle-treated groups. Noticeably, on day 21, DMSO-treated group (66.59%) showed significant hair regrowth as compared to ethanol-treated group (36.62%), P<0.001. As the data shown in Table 2 and Figure 2.

Although, at baseline (day 0), the data showed significant difference of area hair growth between tofacitinib-treated versus (VS) ethanol-treated group and minoxidil-treated VS ethanol-treated group which this resulted from the error of hair coated process, but in this study, we evaluated area hair regrowth relatively from each mouse baseline, thus would compromise the results error.

Table 2 Comparison of area hair growth (%) at day 0, 7, 14, and 21 among tofacitinib-treated group, minoxidil-treated, DMSO-treated and ethanol-treated group.

	Tofacitinib (n=7)	Minoxidil (n=7)	Vehicle-DMSO (n=7)	Vehicle-ethanol (n=7)	p-value	
					ANOVA test	Bonferroni test
Area hair growth (%)						
Day 0	15.22 (11.86, 18.58)	14.42 (11.55, 17.29)	21.62 (17.77, 25.47)	27.22 (17.42, 37.02)	0.002*	c,e
Day 7	25.36 (21.66, 29.06)	18.23 (15.63, 20.83)	28.25 (22.28, 34.22)	28.47 (20.41, 36.53)	0.012*	d,e
Day 14	64.62 (45.08, 84.16)	30.31 (23, 37.62)	31.21 (24.38, 38.04)	31.48 (23.63, 39.33)	<0.001**	a,b,c
Day 21	98.5 (96.8, 100.2)	61.58 (36.02, 87.14)	66.59 (43.2, 89.98)	36.62 (30.77, 42.47)	<0.001**	a,b,c,f

Values presented as mean (95% confident interval of mean). P-value corresponds to ANOVA test and Post hoc test by Bonferroni.

(a) Tofacitinib VS Minoxidil, (b) Tofacitinib VS Vehicle-DMSO, (c) Tofacitinib VS Vehicle-ethanol, (d) Minoxidil VS Vehicle-DMSO, (e) Minoxidil VS Vehicle-ethanol,

(f) Vehicle-DMSO VS Vehicle-ethanol

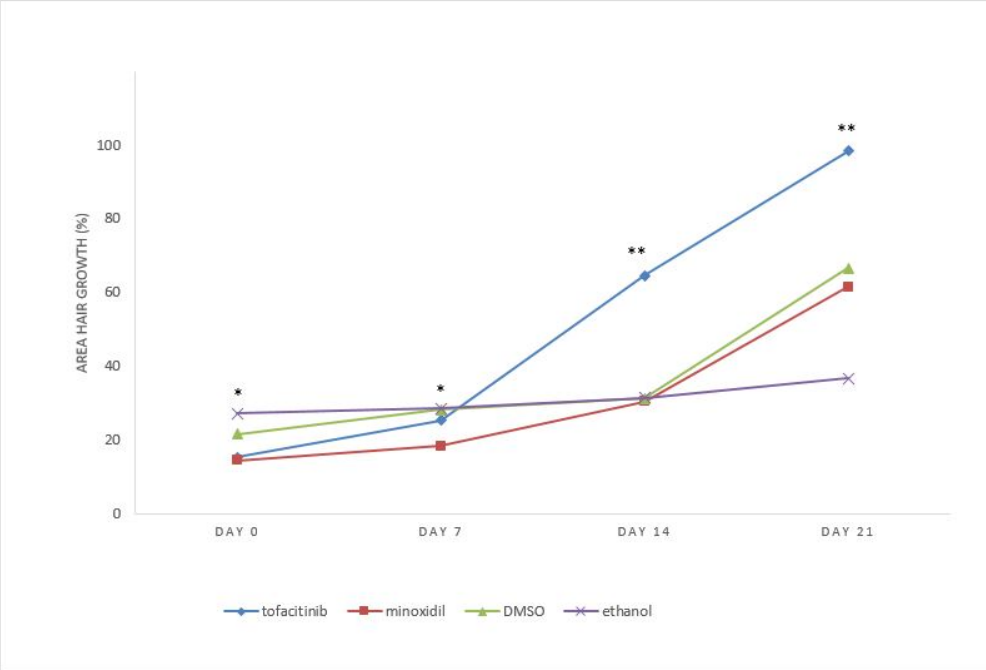

Figure 2 Comparison of area hair growth (%) at day 0, 7, 14, and 21 among tofacitinib-treated group, minoxidil-treated, DMSO-treated and ethanol-treated group. *P<0.05, ** P<0.001

From observation, we could grossly see thinned-quality hair regrowth initiated on the dorsal back coated area of mice treated with tofacitinib approximately since day 7 after treatment, despite still denuded in minoxidil-treated group. With slower onset, we could observe hair regrowth initiation from minoxidil-treated group on day 11, and DMSO-treated group on day 14. Despite, no distinct hair regrowth observed from ethanol-treated group. Hair regrowth in mice treated with tofactinib, minoxidil, and DMSO continue up to the end of the study (21 days) with gradually larger in hair diameter and density. Another unexpected findings recognized from the study was that mice in tofacitinib-treated group and DMSO-treated group developed dry erythematous skin on the dorsal back coated area after applying those reagents since day 7, but after continue to apply, erythema was gradually diminished, then the skin turned grey and replaced with regrown hair in tofacitinib-treated group following by DMSO-treated group. As the results of the hair regrowth model were represented in Figure 3.

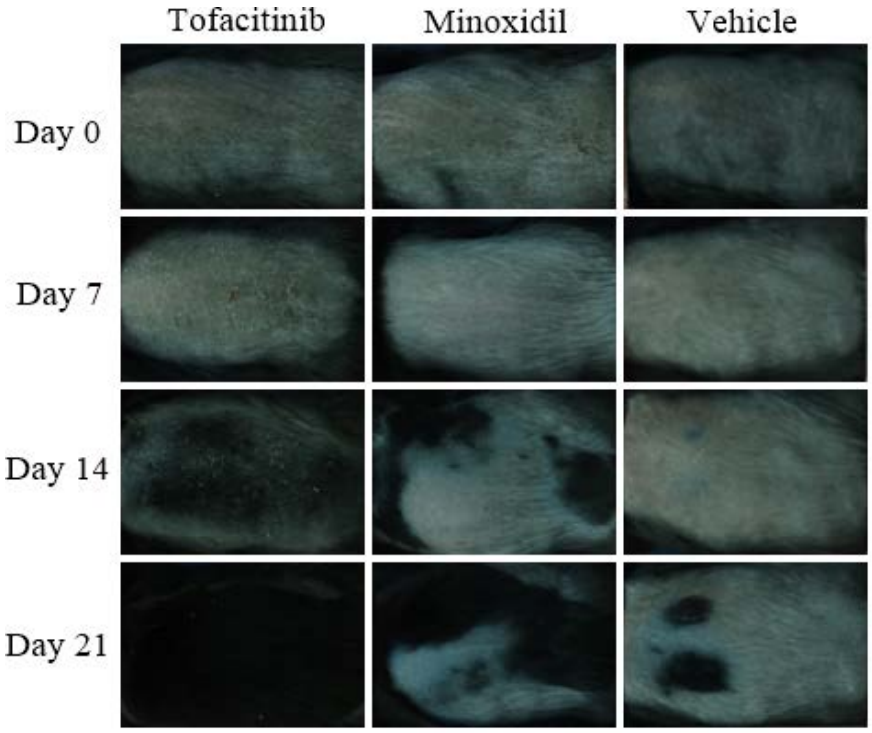

Figure 3 Hair regrowth model on day 0, 7, 14, and 21 after topical application of tofacitinib (left), minoxidil (middle), and vehicle (right). Tofacitinib-treated group displayed faster onset of hair regrowth since day 7, whereas minoxidil-treated and vehicle-treated group still denuded. On day 21, despite almost full area of hair regrowth showed in tofacitinib-treated group, partial hair regrowth observed in minoxidil-treated and vehicle-treated group.

Thus, the results suggest that tofacitinib promotes significantly more hair regrowth coupled with rapid onset than minoxidil and vehicle.

1.2 Mice treated with topical tofacitinib exhibited rapid rate of hair growth

We examined hair growth rate, calculated from the mean hair length (mm) measured using a digital microscope from day 0 until day 14 of treatment (on day 21, most hairs in tofacitinib-treated group were greater in length and their length were beyond the microscopic field so that it could not be measured), and found that mice treated with topical tofacitinib had a significant increase in the rate of hair growth compared with that of topical minoxidil at day 14, with 95% CI 0.03 (0.01-0.04), P=0.003, as shown in Table 3 and Figure 4.

Table 3 Comparison of average rate of hair growth between tofacitinib-treated and minoxidil-treated groups calculated from mean hair length (mm) measured using digital microscope from day 0 until day 14 of treatment.

	Tofacitinib (n=7)	**Minoxidil (n=7)**	**Mean difference**	**p-value**
	Mean (95%CI)	**Mean (95%CI)**	**(95%CI)**	**(t-test)**
Mean hair length (mm)				
Day 0	0.29 (0.25, 0.33)	0.28 (0.25, 0.31)	0.01 (-0.03, 0.05)	0.522
Day 7	0.33 (0.29, 0.37)	0.32 (0.29, 0.35)	0 (-0.04, 0.05)	0.818
Day 14	0.8 (0.56, 1.04)	0.41 (0.33, 0.49)	0.39 (0.16, 0.61)	0.003**
Hair growth rate (mm/day)				
Day 0-14	0.04 (0.02, 0.06)	0.01 (0, 0.02)	0.03 (0.01, 0.04)	0.003**

Values presented as mean (95% confident interval of mean). P-value corresponds to Independent t-test.

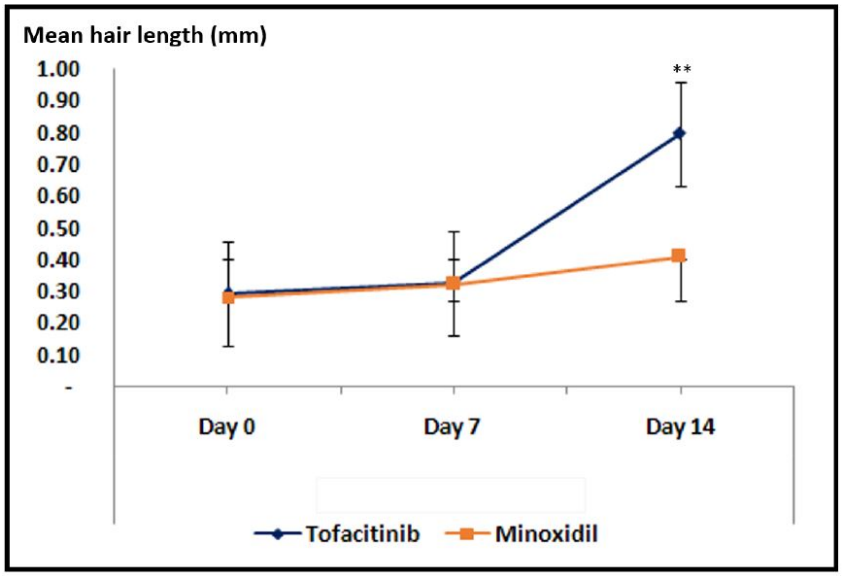

Figure 4 Comparison of mean hair length (mm) from day 0 until day 14 after treatment between tofacitinib-treated group and minoxidil-treated group. Mean ± SEM, **P<0.05

Overall, mice treated with topical tofacitinib had a significant increase in the rate of hair growth compared with that of minoxidil and vehicle at day 14, with P<0.001. Mean hair growth rate of tofacitinib-treated group, minoxidil-treated group, DMSO-treated group, and ethanol-treated group were 0.04, 0.01, 0.01, and 0 mm/day, respectively, as shown in Table 4 and Figure 5.

Table 4 Comparison of average rate of hair growth among tofacitinib-treated, minoxidil-treated, DMSO-treated, and ethanol-treated groups calculated from mean hair length (mm) measured using digital microscope from day 0 until day 14 of treatment.

	Tofacitinib (n=7)	**Minoxidil (n=7)**	**Vehicle-DMSO (n=7)**	**Vehicle-ethanol (n=7)**	**p-value**	
					ANOVA test	**Bonferroni test**
Mean hair length (mm)						
Day 0	0.29 (0.25, 0.33)	0.28 (0.25, 0.31)	0.33 (0.31, 0.35)	0.37 (0.34, 0.4)	<0.001**	c,d,e
Day 7	0.33 (0.29, 0.37)	0.32 (0.29, 0.35)	0.38 (0.35, 0.41)	0.39 (0.35, 0.43)	0.001*	c,d,e
Day 14	0.8 (0.56, 1.04)	0.41 (0.33, 0.49)	0.44 (0.35, 0.53)	0.41 (0.37, 0.45)	<0.001**	a,b,c
Hair growth rate (mm/day)						
Day 0-14	0.04 (0.02, 0.06)	0.01 (0, 0.02)	0.01 (0, 0.02)	0 (0, 0)	<0.001**	a,b,c

Values presented as mean (95% confident interval of mean). P-value corresponds to ANOVA test and Post hoc test by Bonferroni.

(a) Tofacitinib VS Minoxidil, (b) Tofacitinib VS Vehicle-DMSO, (c) Tofacitinib VS Vehicle-ethanol, (d) Minoxidil VS Vehicle-DMSO, (e) Minoxidil VS Vehicle-ethanol,

(f) Vehicle-DMSO VS Vehicle-ethanol

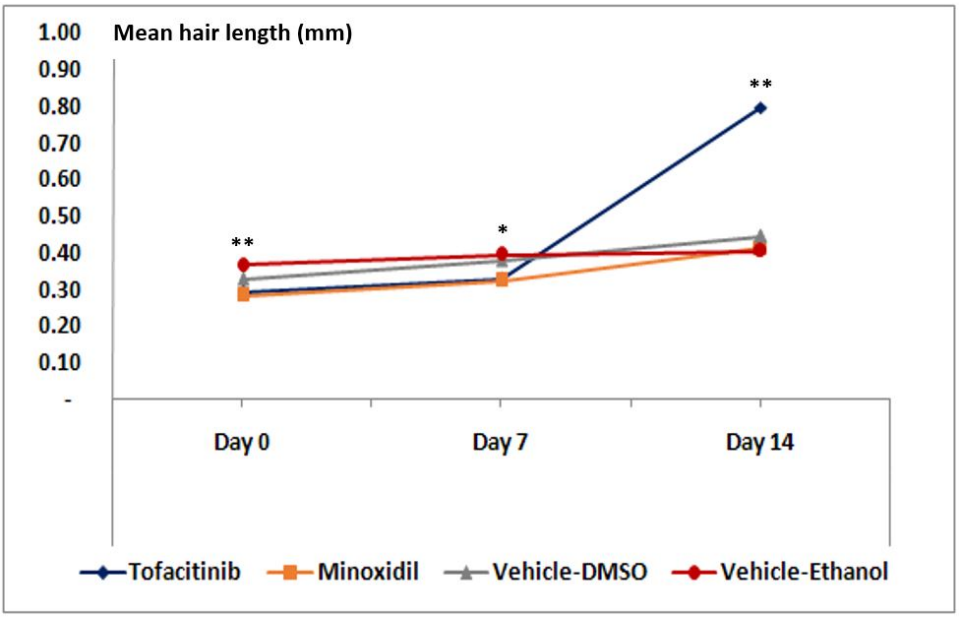

Figure 5 Comparison of mean hair length (mm) from day 0 until day 14 after treatment among tofacitinib-treated group, minoxidil-treated group, DMSO-treated group, and ethanol-treated group. *P<0.05, **P<0.001

2 Histopathology

2.1 Significant increase in the mean number of hair follicles in anagen phase observed in tofacitinib-treated group under optical microscopic observations with H & E staining

At the experimental endpoint (day 21), the number of hair follicles in tofacitinib-treated group (95% CI 43.43 (33.29-53.57)) was significantly more in number than that in minoxidil-treated group (95% CI 28.43 (20.42-36.44)), P=0.015, as shown in Table 5 and Figure 6.

Table 5 Comparison of mean number of hair follicle per 6 mm at day 21 between tofacitinib-treated group and minoxidil-treated group.

	Tofacitinib (n=7)	**Minoxidil (n=7)**	**p-value**
Mean number of hair follicle	43.43 (33.29, 53.57)	28.43 (20.42, 36.44)	0.015**

Values presented as mean (95% confident interval of mean). P-value corresponds to Independent t test.

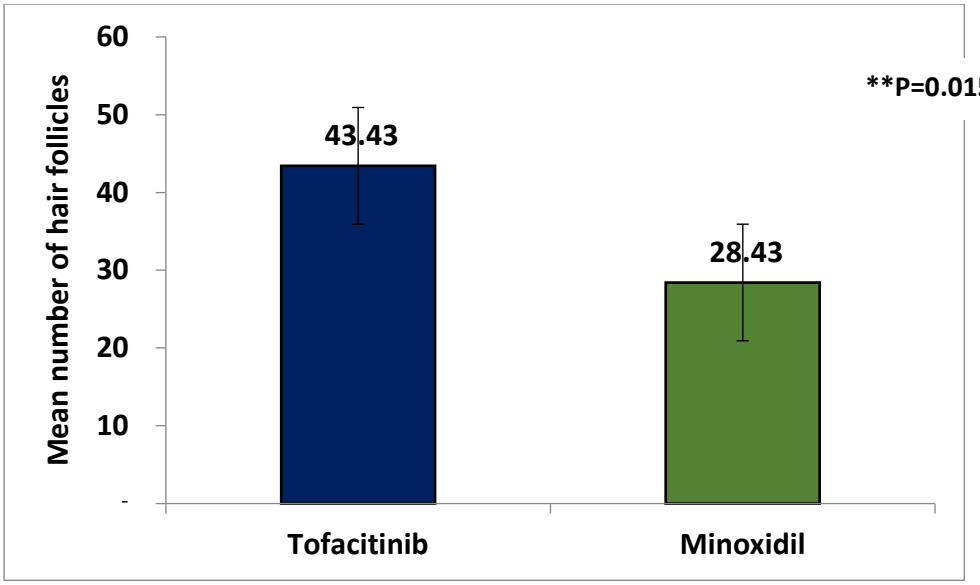

Figure 6 Comparison of mean number of hair follicle per 6 mm at day 21 between tofacitinib-treated group and minoxidil-treated group. Mean ± SEM, **P<0.05

2.2 Hair follicles in tofacitinib-treated group mainly found in late anagen stage, while hair follicles minoxidil-treated group were partly found to be categorized in catagen and some were in anagen.

It was observed that the number of hair follicles mainly increased in the mature/late anagen phase in hypodermis in the tofacitinib-treated group, followed by some early anagen hair follicles in superficial dermis, but rarely catagen or telogen hair follicles were found. Meanwhile, the number of hair follicles in the catagen phase in deep dermis and the late anagen in deep dermis and hypodermis were approximately resemble in the minoxidil-treated group. Interestingly, the number of hair follicles in DMSO-treated group were largely found to be in late anagen in deep dermis and hypodermis, followed by catagen in deep dermis and telogen in superficial dermis. Whereas hair follicles in ethanol-treated group were mainly revealed to be in telogen phase in superficial dermis and catagen phase in deep dermis. As expected, anagen hairs were scarcely reported in ethanol-treated group.

2.3 Minoxidil-treated group revealed to be increasing in new capillaries number.

Observations demonstrated of new capillaries found in minoxidil-treated group with slightly dilation, while in tofacitinib-treated group showed lessen in new capillaries. As expected, ethanol-treated group also had less new capillaries sprouting, in contrast to DMSO-treated group which found some new capillaries.

2.4 Tofacitinib-treated group demonstrated the least inflammatory cells infiltration.

Among all treated groups, tofacitinib-treated seemed having the least inflammatory cells infiltrated in tissues, correlated to clinical which its sign of inflammation lessened after continued applying tofacitinib, and having hair regrowth instead. In minoxidil-treated group, slight mononuclear inflammatory cells were found to be infiltrated. DMSO-treated group also revealed to have mononuclear inflammatory cells infiltration, while ethanol-treated group had relatively less inflammatory cells infiltration.

The model results of H&E staining of the dorsal skin tissue after 21 days of topical tofacitinib, minoxidil, DMSO, and ethanol application are shown in Figure 7. All tissues were well epithelialization with keratinization. The granulation was seen well organized with abundant collagen bundles and fibroblast infiltration.

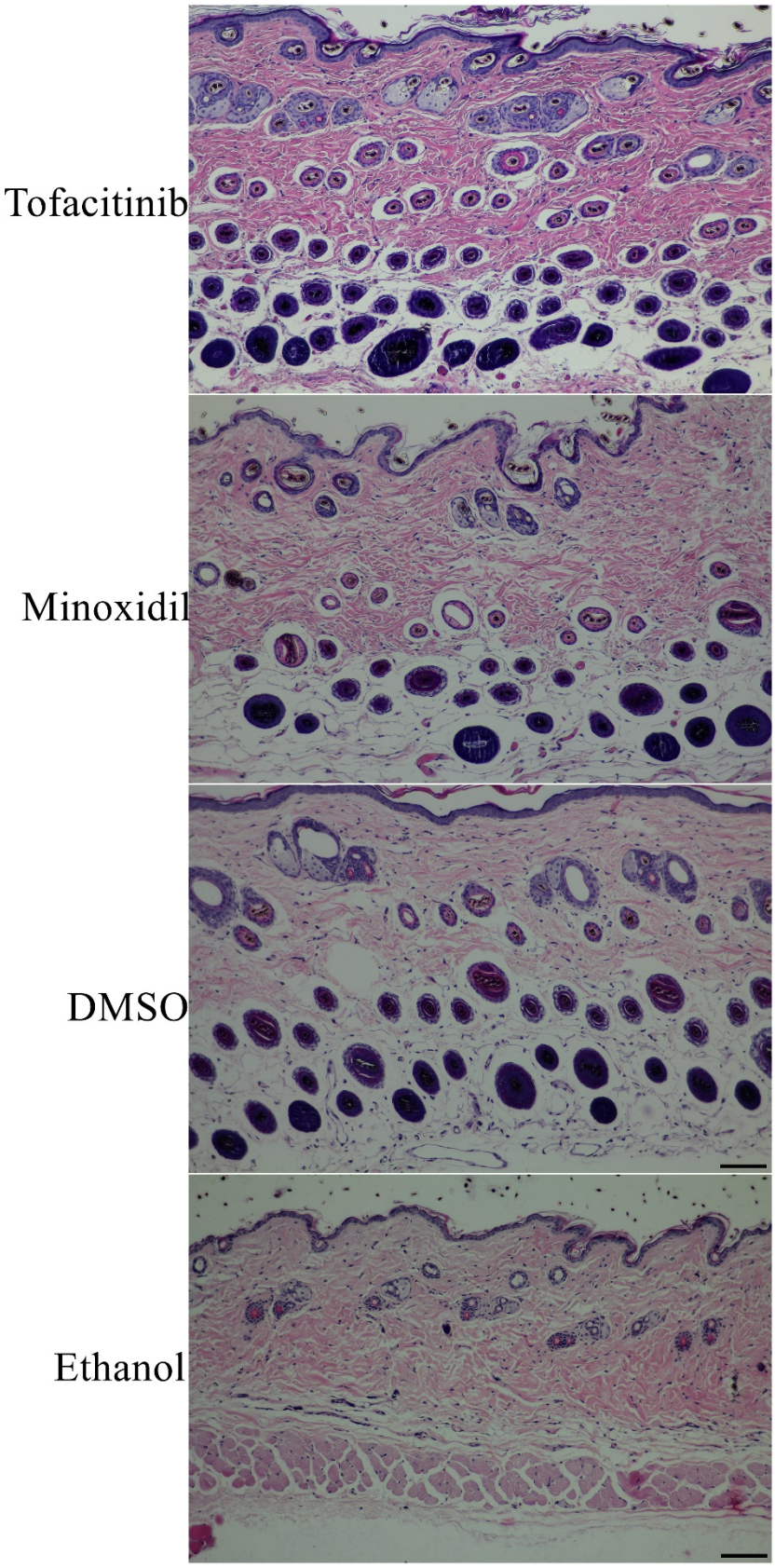

Figure 7 The model results of H&E staining of the dorsal skin tissue after 21 days of topical tofacitinib, minoxidil, DMSO, and ethanol application.

3 Cell growth factors

3.1 RT-PCR

3.1.1 VEGF

According to either laboratory error or the quantity of cell growth factor expression was too low, hence, some values were not be able to evaluate and to be odded out. 6 samples of tofacitinib-treated group were left to be calculated for the VEGF mRNA expression, while 4, 7, and 3 samples of minoxidil-treated, DMSO-treated, and ethanol-treated groups, respectively, were to be included in calculation for the VEGF mRNA expression.

The median expression of the VEGF mRNA in tofacitinib-treated group (117.45) showed significantly higher in value when comparing to minoxidil-treated group (25.415), P=0.033. As well as vehicle-treated group, the median expression of the VEGF mRNA in tofacitinib-treated group (117.45) was markedly higher as compared to DMSO-treated group (28.33), P=0.032, and to ethanol-treated group (9.15), P=0.02. In contrast, although minoxidil-treated group demonstrated quite large quantity of the median expression of the VEGF mRNA as compared to vehicle-treated group, but still not that high to be statistically significant. As shown in Table 6 and Figure 8.

Table 6 The median expression of the VEGF mRNA evaluated from RT-PCR.

Treated group	n	Median	Min	Max	p-value		
Tofacitinib	6	117.45	20.01	189.13	Ref		
Minoxidil	4	25.415	10.32	63.21	0.033*	Ref	
Vehicle-DMSO	7	28.33	4.32	45.01	0.032*	1	Ref
Vehicle-ethanol	3	9.15	5.17	13.39	0.02*	0.157	0.087

Values presented as median (range: min - max). P-value corresponds to Mann-Whitney U test. Ref, reference group. *P<0.05

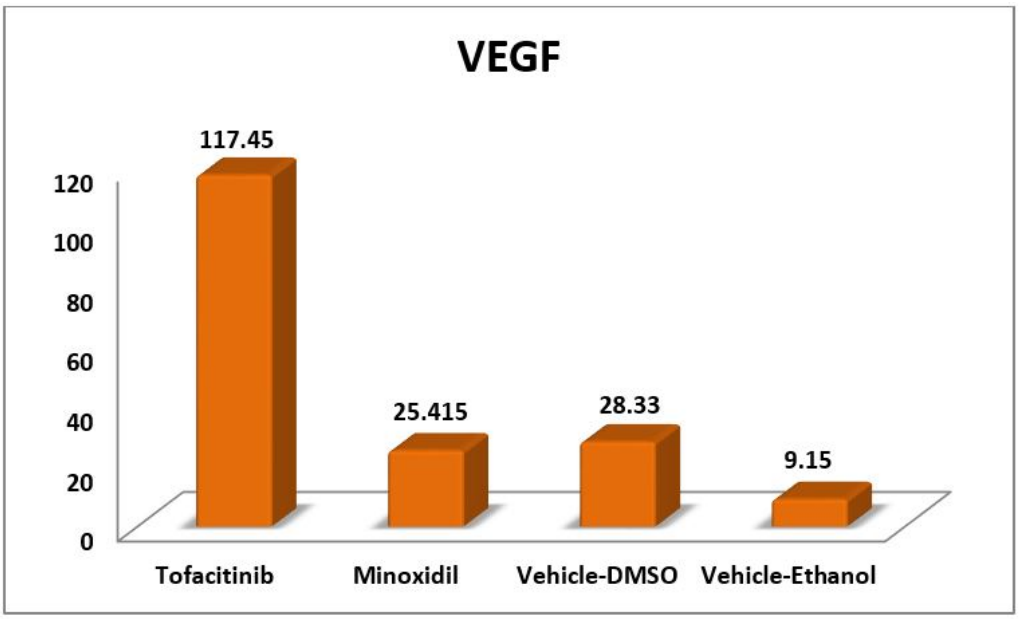

Figure 8 Comparison of the median expression of the VEGF mRNA evaluated from RT-PCR.

3.1.2 IGF-1

Similarly to the VEGF mRNA evaluation, in case of the IGF-1, either laboratory error or the quantity of cell growth factor expression was too low, thus, some values were not be able to evaluate and to be odded out. 6 samples of tofacitinib-treated group were left to be calculated for the IGF-1 mRNA expression, whereas 3, 7, and 3 samples of minoxidil-treated, DMSO-treated, and ethanol-treated groups, respectively, were to be included in calculation for the IGF-1 mRNA expression.

The median expression of the IGF-1 mRNA in minoxidil-treated group (175.46) showed the greatest value as compared to others with statistically significant with P=0.039, 0.03, and 0.037 for tofacitinib-treated, DMSO-treated, and ethanol-treated reference groups, respectively. The median expression of the IGF-1 mRNA in tofacitinib-treated group (15.23) was significantly higher when comparing to ethanol-treated group (too low to be evaluated, imputation as 1), P=0.043. DMSO-treated group (27.13) also had significantly higher value of the median expression of the IGF-1 mRNA as compared to ethanol-treated group (too low to be evaluated, imputation as 1), P=0.015. Surprisingly, DMSO-treated group (27.13) revealed higher value of the median expression of the IGF-1 mRNA when comparing to tofacitinib-treated group (15.23), but yet was not that high to be statistically significant. As shown in Table 7 and Figure 9.

Table 7 The median expression of the IGF-1 mRNA evaluated from RT-PCR.

Treated group	n	Median	Min	Max	p-value		
Tofacitinib	6	15.23	1	81.81	Ref		
Minoxidil	3	175.46	73.76	236.09	0.039*	Ref	
Vehicle-DMSO	7	27.13	1.04	129.38	0.317	0.03*	Ref
Vehicle-ethanol	3	1	1	1	0.043*	0.037*	0.015*

Values presented as median (range: min - max). P-value corresponds to Mann-Whitney U test.

1, too low to be evaluated. Ref, reference group. *P<0.05

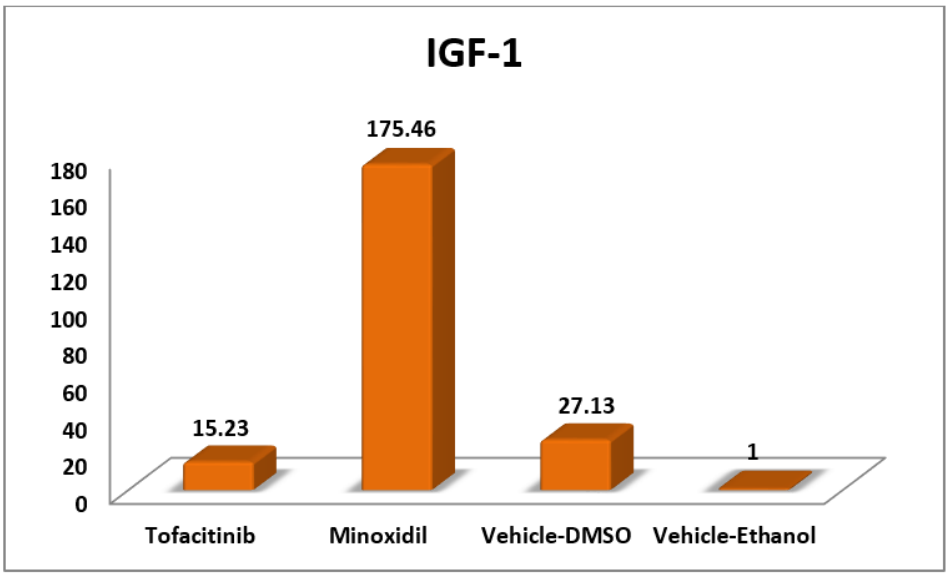

Figure 9 Comparison of the median expression of the IGF-1 mRNA evaluated from RT-PCR.

DISCUSSION AND RECOMMENDATIONS

1 Discussion

Currently approved medication for AGA includes oral finasteride and topical minoxidil, both still limited in efficacy and many have untoward side effects. Tofacitinib has been proven of its stimulatory effect towards hair growth (3)(15). Treatment of JAK inhibitors in topical form resulted in more robust hair growth than did systemic form, possibly because it increases the local drug concentration in the hair follicle microenvironment, allowing interactions to occur (3) as well as lesser side effect achieved in topical treated. Thus, this study demonstrates the efficacy in promoting hair growth of topical tofacitinib compare with topical minoxidil, conducted in mice model.

From our data, topical application of tofacitinib led to hair regrowth initiation since day 7 after treatment, whereas minoxidil and DMSO also led to hair regrowth but with slower onset, on day 11 and 13 respectively, despite no hair growth initiation observed in ethanol-treated group until entire experiment. Moreover, area hair regrowth observed from tofacitinib-treated group demonstrated almost full area of the dorsal back previously coated, despite partial hair regrowth area observed in minoxidil-treated and DMSO-treated group, even though till day 21 which was the experimental endpoint. Correspond with clinical, histopathological observation showed significantly more increase in number of hair follicles in tofacitinib-treated group compared with others. Another interesting outcome, we measured mean hair length using Dino-Lite microscope on day 0 until day 14 (the hair length of tofacitinib-treated group on day 21 was too long to be evaluated, thus we used the data till day 14) and used to calculate average hair growth rate. The results showed that mice treated with topical tofacitinib had significantly more rapid rate of hair regrowth compared to minoxidil and DMSO. Overall, this could ascertain the previous study findings that application of topical tofacitinib led to hair growth promotion, following from the mechanism of WNT and SHH signalling pathways activation, even though the process of stimulation has still not be known, together with stimulatory effect of hair follicle stem cells activation and/or proliferation (3). Whereof the processes are evidenced from the repression of receptors that involved in the modulation of dermal papilla inductivity, such as FGFR1, ACVRL1, IGFR1, OSMR, and PTGFR (20)(21)(22)(23) collocate with down-regulation of proapoptotic genes included BAX, Bcl2L11 and CASP12. In addition, genes up-regulation induced by treatment of tofacitinib involved the members of the TGF-β

and BMP pathway, which formerly shown to play a critical role in dermal papilla inductivity (23)(24)(25)(26). Besides, LEF1, another key regulators of the WNT pathway, together with the NOTCH pathway members are also overexpressed during the treatment with tofacitinib (3). Minoxidil induces cell growth factors, such as VEGF, HGF, IGF-1, and activates uncoupled sulfonylurea receptor on dermal papilla plasma membrane, leading to enhanced activity of HGF and IGF-1. It also inhibits TGF-β, which induces hair matrix cells apoptosis, and dilates hair follicle arteries, thereby increasing blood flow to the dermal papillae, resulting in an anagen phase extension (163)(48)(164).

Interestingly, from the study, we found that one of the possible potential mechanisms leading to hair growth of tofacitinib might be that of the expression of the VEGF mRNA and protein which demonstrated in the highest level within tofacitinib-treated group as compared to others. Although, we previously known that the VEGF expression level has shown high in minoxidil treatment, resulting in angiogenesis, tofacitinib could even induce more. Correlatively, the histopathological results also proved the angiogenic process by the finding of some new capillaries in tofacitinib-treated tissue, meanwhile in minoxidil-treated tissue found plenty of new slightly dilated capillaries. These demonstrated that although JAK-STAT inhibitory effect of tofacitinib could induce greater amount of the VEGF expression, despite lower amount in minoxidil, but the new capillaries which reflected the process of angiogenesis were fewer found. The explanation may be that of the inhibitory of JAK-STAT can downstream lead to the BMP7 expression inhibition (165) resulting in extracellular signal-regulated protein kinase (ERK) 1/2 suppression via leukemia inhibitory factor (LIF) inhibition cascade (166) and finally VEGF-induced angiogenic signalling is blocked (167), hence, even though the VEGF expression demonstrated high level in tofacitinib-treated group, the angiogenesis may not concordantly be inflated. Another explanation is that in case of the angiogenic mechanism, VEGF is not the only factors promoting angiogenesis, but bFGF, angiopoietins, interleukin-8 (IL-8), placental growth factor (PlGF), and PDGF also play an important role as the angiogenic factors (168)(169), which apart from VEGF, minoxidil may up-regulated the angiogenic mechanism via promoting other cell growth factors that support the production of the angiogenic factors for example HGF. Thus, according to those probable explanation, we could see some new capillaries in tofacitinib-treated tissue from the histopathological section, but not that much as compared to minoxidil. In conclusion, we suggest that inhibitory effect of JAK-STAT could induce high amount of the VEGF mRNA and protein expression which probably plays another important role in hair growth promotion. Though the possible inhibitory effect of tofacitinib resulting in angiogenesis, but in order to promote hair growth, VEGF does not

just only have a crucial role in angiogenesis, but it also has direct action on hair dermal papilla cells as an autocrine growth factor which may be another beneficial mechanism in hair growth promotion from tofacitinib application (170).

Apart from VEGF, tofacitinib has previously been proved of its stimulatory effect on multiple hair follicle growth factors and inhibitory effect on apoptotic signal inducers which plenty of those factors are enrolled in greater volume resulting in better hair growth outcome.

In contrast to the VEGF expression level that found high in tofacitinib application, but the expression of the IGF-1 mRNA was demonstrated to be low suggesting that the IGF-1 gene expression cascade does not involve in the mechanism in promoting hair growth of tofacitinib.

According to the histopathological results, most hair follicles in tofacitinib-treated group were classified as anagen, while nearly half of the hair follicles in minoxidil-treated group were classified as catagen, which indicates and reaffirms that tofacinib could accelerate transition to anagen phase (3)(15) in greater magnitude. An additional evidence was that after continue treating with tofacitinib, we observed skin darkening which indicated the migration of melanin to the skin surface during telogen to anagen conversion (171).

Furthermore, another feasible effect of tofacitinib that supports the results is that of its anti-inflammatory effect (2)(4) confirming by the experimental evidence that during the first week after the application of tofacitinib and DMSO, the dorsal skin of mice turned erythema with dry scales due to the exorbitant concentration of DMSO (100% concentration using as tofacitinib's vehicle and control), but after continue applying, the erythema and scales were lessened with new hair regrowth recognized instead in tofacitinib-treated group, whereas still some dry scaly erythematous skin observed in DMSO-treated group. Concurrently, according to the histopathological results, tofacitinib-treated group revealed the least inflammatory cells infiltration, while still observed the mononuclear inflammatory cells infiltration in other groups. We suggest that an anti-inflammatory effect of tofacitinib may be an additional potential in promotion of hair growth by inhibiting the process of hair follicle micro-inflammation, which can lead to apoptosis in hair follicles and play a crucial role in the pathogenesis of follicle miniaturization (172)(173). Whereas minoxidil alone has limited in anti-inflammatory effect (174).

Moreover, from the observation, we surprisingly recognized hair regrowth from DMSO-treated group, although tofacitinib-treated group still revealed more significant hair regrowth area, but at the experimental endpoint, mice treated with DMSO demonstrated that the percentage of hair regrowth area was quite high nearly to that of the minoxidil-treated

group. The explanation was that during the application of DMSO, the dorsal coated skin of mice showed the characteristic signs of contact dermatitis by turning erythema with dry scales. According to previous knowledge, the protein kinase C (PKC)-α inducing by skin irritation or allergic contact dermatitis has been implicated in the growth of mouse hair (175)(176), thus, in this setting, DMSO application in mouse skin resulting in contact dermatitis might lead to hair growth according to the same condition. Even though DMSO was used as vehicle for tofacitinib and we could also observe sign of dermatitis reaction in tofacitinib-treated mice skin, but as continue treating, the inflammatory sign lessened and hair regrowth was observed instead. The percentage of area hair growth in tofacitinib-treated group revealed strikingly highest among all treated groups, this might be implicate that though dermatitis reaction could more or less influenced hair regrowth in tofacitinib-treated group, but other mechanisms that has been discussed above in hair growth induction of tofacitinib still be mostly determined.

In conclusion, according to macroscopic, histopathologic, and immunologic results underscored the statistically significant of tofacitinib effect as hair growth promoter with greater in efficacy as compare to topical minoxidil which has been approved as therapeutic agent and generally use in non-scarring alopecia.

Tofacitinib has been approved in rheumatoid arthritis treatment by the US FDA and currently in clinical trials for treatment of psoriasis (13), vitiligo (14), and AA (15)(16). The successful treatment of AU with topical ruxolitinib as JAK inhibitor (177) together with the report of significantly hair regrowth regarding the use of oral tofacitinib in AA, AT, and AU (178)(161) has been demonstrated.

Our study raises another crucial therapeutic effect in addition to immune-driven conditions, tofacitinib proved to be beneficial in having significantly greater in hair growth promoting efficacy comparing to topical minoxidil with faster onset, more rapid rate of hair regrowth and better outcome. These findings would probably benefit in open access to apply in human clinical trials in further study.

2 Recommendations

2.1 Further studies with using vary concentration of tofacitinib in order to determine its dose dependent effect.

2.2 Further studies in long term duration are pursued in order to examine tofacitinib adverse events and to determine whether its therapeutic effect on hair growth can be maintain throughout after treatment cessation or not.

2.3 Further studies with initiating age of mice variation to achieve the data on reproducibility of hair growth resulting from tofacitinib treatment on hair stage variation, whether tofacitinib can abrogate the quiescence-promoting microenvironment at the early stages of telogen or not.

2.4 Further studies in other cell growth factors expression according to tofacitinib treatment which will benefit in providing further information on hair growth promoted mechanism.

2.5 Further studies in human clinical trials.

REFERENCES

1. Cetkovic-Cvrlje M TH. Therapeutic potential of Janus kinase 3 (JAK3) inhibitors. Curr Pharm Des. 2004;10(15):1767–84.
2. John J. O'Shea, Apostolos Kontzias, Kunihiro Yamaoka, Yoshiya Tanaka AL. Janus kinase Inhibitors in autoimmune diseases. Ann Rheum Dis. 2013;72(2):ii111–ii115.
3. Sivan Harel, Claire A. Higgins, Jane E. Cerise et al. Pharmacologic inhibition of JAK-STAT signaling promotes hair growth. Sci Adv. 2015;1(9):e1500973.
4. Kamran Ghoreschi, Michael I. Jesson, Xiong Li et al. Modulation of Innate and Adaptive Immune Responses by Tofacitinib (CP-690,550). J Immunol. 2011;186(7):4234–4243.
5. Fleischmann R, Kremer J, Cush J et al. Placebo-Controlled Trial of Tofacitinib Monotherapy in Rheumatoid Arthritis. N Engl J Med. 2012;367:495–507.
6. Boyce EG, Vyas D, Rogan EL, Valle-Oseguera CS OK. Impact of tofacitinib on patient outcomes in rheumatoid arthritis - review of clinical studies. Patient Relat Outcome Meas. 2016;14(7):1–12.
7. K Migita, Y Izumi, Y Jiuchi et al. Effects of Janus kinase inhibitor tofacitinib on circulating serum amyloid A and interleukin-6 during treatment for rheumatoid arthritis. Clin Exp Immunol. 2014;175(2):208–14.
8. Sandborn WJ, Ghosh S, Panes J et al. Tofacitinib, an oral Janus kinase inhibitor in active ulcerative colitis. New Engl J Med. 2012;367(7):616–24.
9. Boy MG, Wang C, Wilkinson BE et al. Double-blind, placebo-controlled, dose-escalation study to evaluate the pharmacologic effect of CP-690,550 in patients with psoriasis. J Invest Dermatol. 2009;129(9):2299–302.
10. Van Gurp E, Weimar W, Gaston R et al. Phase 1 dose-escalation study of CP-690550 in stable renal allograft recipients: preliminary findings of safety, tolerability, effects on lymphocyte subsets and pharmacokinetics. Am J Transpl. 2008;8:1711–1718.
11. Sakimoto T IA. Anti-inflammatory effect of topical administration of tofacitinib on corneal inflammation. Exp Eye Res. 2015;12(145):110–7.
12. Liew SH, Nichols KK, Klamerus KJ, Li JZ, Zhang M FG. Tofacitinib (CP-690550), a Janus Kinase Inhibitor for Dry Eye Disease: Results from a Phase 1/2 Trial. Ophthalmology. 2012;119(7):1328–35.
13. Bachelez H., van de Kerkhof P. C., Strohal R. et al. Tofacitinib versus etanercept or

placebo in moderate-to-severe chronic plaque psoriasis: A phase 3 randomised non-inferiority trial. Lancet. 2015;386:552–561.

14. Craiglow B. G. KBA. Tofacitinib citrate for the treatment of vitiligo: A pathogenesis-directed therapy. JAMA Dermatol. 2015;151(10):1110–2.
15. Xing L., Dai Z., Jabbari A. et al. Alopecia areata is driven by cytotoxic T lymphocytes and is reversed by JAK inhibition. Nat Med. 2014;20:1043–1049.
16. Craiglow B. G. KBA. Killing two birds with one stone: oral tofacitinib reverses alopecia universalis in a patient with plaque psoriasis. J Invest Dermatol. 2014;134(12):2988–2990.
17. Paladini R. D., Saleh J., Qian C., Xu G.-X. RLL. Modulation of hair growth with small molecule agonists of the hedgehog signaling pathway. J Invest Dermatol. 2005;125:638–646.
18. Alonso L. FE. The hair cycle. J Cell Sci. 2006;119:391–393.
19. Greco V., Chen T., Rendl M., Schober M., Pasolli H. A., Stokes N. et al. A two-step mechanism for stem cell activation during hair regeneration. Cell Stem Cell. 2009;4:155–169.
20. Kawano M., Komi-Kuramochi A., Asada M. et al. Comprehensive analysis of FGF and FGFR expression in skin: FGF18 is highly expressed in hair follicles and capable of inducing anagen from telogen stage hair follicles. J Invest Dermatol. 2005;124:877–885.
21. Kamp H., Geilen C. C., Sommer C. B-PU. Regulation of PDGF and PDGF receptor in cultured dermal papilla cells and follicular keratinocytes of the human hair follicle. Exp Dermatol. 2003;12(5):662–672.
22. Rendl M., Lewis L. FE. Molecular dissection of mesenchymal–epithelial interactions in the hair follicle. PLOS Biol. 2005;3:e331.
23. Rendl M., Polak L. FE. BMP signaling in dermal papilla cells is required for their hair follicle-inductive properties. Genes Dev. 2008;22:543–557.
24. Paus R., Foitzik K., Welker P., Bulfone-Paus S. ES. Transforming growth factor-β receptor type I and type II expression during murine hair follicle development and cycling. J Invest Dermatol. 1997;109:518–526.
25. Foitzik K., Paus R., Doetschman T. DGP. The TGF-β2 isoform is both a required and sufficient inducer of murine hair follicle morphogenesis. Dev Biol. 1999;212:278–289.
26. Oshimori N. FE. Paracrine TGF-β signaling counterbalances BMP-mediated repression in hair follicle stem cell activation. Cell Stem Cell. 2012;10:63–75.
27. Zhou P., Byrne C., Jacobs J. FE. Lymphoid enhancer factor 1 directs hair follicle

patterning and epithelial cell fate. Genes Dev. 1995;9:700–713.

28. Kratochwil K., Dull M., Farinas I., Galceran J. GR. LEF-1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. Genes Dev. 1996;10:1382–1394.
29. Ambler CA WF. Expression of Notch pathway genes in mammalian epidermis and modulation by β-catenin. Dev Dyn. 2007;236:1595–1601.
30. Craiglow BG, Tavares D KB. Topical Ruxolitinib for the Treatment of Alopecia Universalis. JAMA Dermatol. 2016;152(4):490–1.
31. Katherine A Gordon and Antonella Tosti. Alopecia: evaluation and treatment. Clin Cosmet Investig Dermatol. 2011;4:101–6.
32. Pathomvanich D, Pongratananukul S, Thienthaworn P MS. A random study of Asian male androgenetic alopecia in Bangkok, Thailand. Dermatol Surg. 2002;28:804–7.
33. Ralph M. Tru¨eb. Mini-Review Molecular mechanisms of androgenetic alopecia. Exp Gerontol. 2002;37:981–90.
34. Shigeki Inui SI. Molecular basis of androgenetic alopecia: From androgen to paracrine mediators through dermal papilla. J Dermatol Sci. 2011;61(1):1–6.
35. C. J. Androgenetic alopecia. Ann Dermatol Venereol. 2002;129(5 Pt 2):801–3.
36. Jaworsky, C., Kligman, A.M., Murphy GF. Characterisation of inflammatory infiltrates in male pattern alopecia: implication for pathogenesis. Br J Dermatol. 1992;127:239–246.
37. Rudman SM, Philpott MP, Thomas GA KT. The role of IGF1 in human skin and its appendages-morphogen as well as mitogen. J Invest Dermatol. 1997;109:770–7.
38. Ozeki M TY. Promoted growth of murine hair follicles through controlled release of basic fibroblast growth factor. Tissue Eng. 2002;8(3):359–66.
39. Wei Li, Chunming Li ZL. Vascular endothelial growth factor and its receptor may be one molecular mechanism for androgenetic alopecia. Med Hypotheses. 2009;72(3):366–7.
40. Stenn KS PR. Controls of hair follicle cycling. Physiol Rev. 2001;81(1):449–94.
41. E O. Finasteride (1 mg) in the treatment of androgenetic alopecia in men. Aust J Dermatol. 1997;38:A316.
42. Rishi Raj Chhipa, Danny Halim, Jinrong Cheng et al. The Direct Inhibitory Effect of Dutasteride or Finasteride on Androgen Receptor Activity is Cell Line Specific. Prostate. 2013;73(14):1483–1494.
43. Mella JM, Perret MC, Manzotti M, Catalano HN GG. Efficacy and safety of finasteride

therapy for androgenetic alopecia: A systematic review. Arch Dermatol. 2010;146:1141–50.

44. Erdemir F, Harbin A HW. 5-alpha reductase inhibitors and erectile dysfunction: The connection. J Sex Med. 2008;5:2917–24.
45. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG et al. The influence of finasteride on the development of prostate cancer. N Engl J Med. 2003;17(349):215–24.
46. Thompson IM Jr, Goodman PJ, Tangen CM et al. Long-term survival of participants in the prostate cancer prevention trial. N Engl J Med. 2013;369:603–10.
47. Keith D. Kaufman, Elise A. Olsen, David Whiting, Ronald Savin, Richard DeVillez, Wilma Bergfeld et al. Finasteride in the treatment of men with androgenetic alopecia. JAAD. 1998;39:578–89.
48. Li M, Marubayashi A, Nakaya Y, Fukui K AS. Minoxidil-induced hair growth is mediated by adenosine in cultured dermal papilla cells: possible involvement of sulfonylurea receptor 2B as a target of minoxidil. J Invest Dermatol. 2001;117(6):1594–600.
49. Elise A. Olsen, Frank E. Dunlap, Toni Funicella, Judith A. Koperski, James M. Swinehart EHT et al. A randomized clinical trial of 5% topical minoxidil versus 2% topical minoxidil and placebo in the treatment of androgenetic alopecia in men. JAAD. 2002;47:377–385.
50. Michelangelo La Placa, Riccardo Balestri, Federico Bardazzi CV. Scalp Psoriasiform Contact Dermatitis with Acute Telogen Effluvium due to Topical Minoxidil Treatment. Ski Appendage Disord. 2016;1(3):141–143.
51. Brown MP. Cytokines, Jaks, Stats, health and disease. Aust NZ J Med. 1999;29(1):73–8.
52. Warren J. Leonard and John J. O'Shea. JAKS AND STATS: Biological Implications∗. Annu Rev Immunol. 1998;16:293–322.
53. Lai KS, Jin Y, Graham DK, Witthuhn BA, Ihle JN LE. A kinase-deficient splice variant of the human JAK3 is expressed in hematopoietic and epithelial cancer cells. J Biol Chem. 1995;270(42):25028–36.
54. Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zürcher G ZA. Two novel protein-tyrosine kinases, each with a second phosphotransferase-related catalytic domain, define a new class of protein kinase. Mol Cell Biol. 1991;11(4):2057–65.
55. Feng J, Witthuhn BA, Matsuda T, Kohlhuber F, Kerr IM IJ. Activation of Jak2 catalytic

activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol. 1997;17(5):2497–501.

56. Lindauer K, Loerting T, Liedl KR KR. Prediction of the structure of human Janus kinase 2 (JAK2) comprising the two carboxy-terminal domains reveals a mechanism for autoregulation. Protein Eng. 2001;14(1):27–37.
57. Hornakova T, Chiaretti S, Lemaire MM et al. ALL-associated JAK1 mutations confer hypersensitivity to the antiproliferative effect of type I interferon. Blood. 2010;115(16):3287–95.
58. Funakoshi-Tago M, Tago K, Kasahara T, Parganas E IJ. Negative regulation of Jak2 by its auto-phosphorylation at tyrosine 913 via the Epo signaling pathway. Cell Signal. 2008;20(11):1995–2001.
59. Zhou YJ, Chen M, Cusack NA, Kimmel LH, Magnuson KS, Boyd JG et al. Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases. Mol Cell. 2001;8(5):959–69.
60. Pellegrini S D-FI. The structure, regulation and function of the Janus kinases (JAKs) and the signal transducers and activators of transcription (STATs). Eur J Biochem. 1997;248:615–33.
61. Funakoshi-Tago M, Pelletier S, Matsuda T, Parganas E IJ. Receptor specific downregulation of cytokine signaling by autophosphorylation in the FERM domain of Jak2. EMBO J. 2006;25:4763–72.
62. Darnell JEJ, Kerr IM SG. Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science (80-). 1994;264:1415–21.
63. Witthuhn BA, Quelle FW S, O, Yi T, Tang B, Miura O IJ. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell. 1993;74:227–36.
64. Argetsinger LS, Campbell GS YX, Witthuhn BA, Silvennoinen O, Ihle JN et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell. 1993;74:237–44.
65. Stahl N, Boulton TG, Farruggella T I, NY, Davis S, Witthuhn BA et al. Association and activation of Jak-Tyk kinases by CNTF-LIF-OSMIL- 6 beta receptor components. Science (80-). 1994;263:92–5.
66. Quelle FW, Sato N, Witthuhn BA I, RC, Eder M, Miyajima A et al. JAK2 associates with the beta c chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol Cell Biol.

1994;14:4335–41.

67. Russell SM, Johnston JA NM, Kawamura M, Bacon CM, Friedmann M et al. Interaction of IL-2R beta and gamma c chains with Jak1 and Jak3: implications for XSCID and XCID. Science (80-). 1994;266:1042–45.
68. Colamonici OR, Uyttendaele H D, P, Yan H KJ. p135tyk2, an interferon-alpha-activated tyrosine kinase, is physically associated with an interferon-alpha receptor. J Biol Chem. 1994;269:3518–22.
69. Zou J, Presky DH, Wu CY GU. Differential associations between the cytoplasmic regions of the interleukin- 12 receptor subunits beta1 and beta2 and JAK kinases. J Biol Chem. 1997;272:6073–7.
70. Baker SJ, Rane SG RE. Hematopoietic cytokine receptor signaling. Oncogene. 2007;26(47):6724–37.
71. Shashidhar S. Jatiani, Stacey J. Baker, Lewis R. Silverman and EPR. JAK/STAT Pathways in Cytokine Signaling and Myeloproliferative Disorders. Genes Cancer. 2010;1(10):979–993.
72. Schindler C SI. Cytokines and STAT signaling. Adv Pharmacol. 2000;47:113–74.
73. S.J Rodig, M.A Meraz, J.M White, P.A Lampe, J.K Riley, C.D Arthur et al. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. Cell. 1998;93:373–83.
74. C.B Ware, M.C Horowitz, B.R Renshaw, J.S Hunt, D Liggitt, S.A Koblar et al. Targeted disruption of the low-affinity leukemia inhibitory factor receptor gene causes placental, skeletal, neural and metabolic defects and results in perinatal death. Development. 1995;121:1283–99.
75. U von Freeden-Jeffry, P Vieira, L.A Lucian, T McNeil, S.E Burdach RM. Lymphopenia in interleukin (IL)-7 gene-deleted mice identifies IL-7 as a nonredundant cytokine. J Exp Med. 1995;181:1519–26.
76. Kisseleva T, Bhattacharya S, Braunstein J SC. Signaling through the JAK/STAT pathway, recent advances and future challenges. Gene. 2002;285(1–2):1–24.
77. H Neubauer, A Cumano, M Mueller, H Wu, U Huffstadt KP. Jak2 deficiency defines an essential developmental checkpoint in definitive hematopoiesis. Cell. 1998;93:397–409.
78. John J O'Shea, Massimo Gadina RDS. Cytokine Signaling in 2002: New Surprises in the Jak/Stat Pathway. 2002;109(2):121–31.
79. L Velazquez, M Fellous, G.R Stark SP. A protein tyrosine kinase in the Interferon α/β signaling pathway. Cell. 1992;70:313–22.

80. M Karaghiosoff, H Neubauer, C Lassnig, P Kovarik, H Schindler, H Pircher et al. Partial impairment of cytokine responses in Tyk2-deficient mice. Immunity. 2000;13:549–60.
81. C Bogdan, M Rollinghoff AD. The role of nitric oxide in innate immunity. Immunol Rev. 2000;173:17–26.
82. PJ M. The JAK-STAT signaling pathway: input and output integration. J Immunol. 2007;178(5):2623–9.
83. Schaefer TS, Sanders LK ND. Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. Proc Natl Acad Sci USA. 1995;92:9097–101.
84. Lin JX, Mietz J, Modi WS, John S LW. Cloning of human Stat5B: reconstitution of interleukin-2-induced Stat5A and Stat5B DNA binding activity in COS-7 cells. J Biol Chem. 1996;271:10738–44.
85. Claudia Mertens, Minghao Zhong, Ravi Krishnaraj, Wenxin Zou, Xiaomin Chen, and James E. Darnell J. Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain. Genes Dev. 2006;20(24):3372–81.
86. A K. The role of Janus kinases in haemapoiesis and haematological malignancy. Br J Haematol. 2006;134:366–84.
87. Horvath CM DJ. The state of STATs: recent developments in the study of signal transduction to the nucleus. Curr Opin Cell Biol. 1997;9:233–9.
88. Ihle J. The Stat family in cytokine signaling. Curr Opin Cell Biol. 2001;13:211–7.
89. V Shankaran, H Ikeda, A.T Bruce, J.M White, P.E Swanson, L.J Old R. S. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature. 2001;410:1107–11.
90. Y.E Chin, M Kitagawa, K Kuida, R.A Flavell X. F. Activation of the STAT signaling pathway can cause expression of caspase 1 and apoptosis. Mol Cell Biol. 1997;17:5328–37.
91. A Kumar, M Commane, T.W Flickinger, C.M Horvarth G. S. Defective TNF-α induced apoptosis in Stat1 null cells due to low constitutive levels of caspases. Science (80-). 1997;278:1630–2.
92. C Park, S Li, E Cha CS. Immune response in Stat2 knockout mice. Immunity. 2000;13:795–804 a.
93. Akira S. Roles of STAT3 defined by tissue-specific gene targeting. Oncogene. 2000;19:2607–11.
94. S Sano, Y Takahama, T Sugawara, H Kosaka, S Itami, K Yoshikawa et al. Stat3 in

thymic epithelial cells is essential for postnatal maintenance of thymic architecture and thymocyte survival. Immunity. 2001;15:261–73.

95. A.L Wurster, T Tanaka M. G. The biology of Stat4 and Stat6 . Oncogene. 2000;19:2577–84.
96. T Chitnis, N Najafian, C Benou, A.D Salama, M.J Grusby, M.H Sayegh S. K. Effect of targeted disruption of STAT4 and STAT6 on the induction of experimental autoimmune encephalomyelitis. J Clin Invest. 2001;108:739–47.
97. J.D Farrar, J.D Smith, T.L Murphy, S Leung, G.R Stark K. M. Selective loss of type I interferon-induced STAT4 activation caused by a minisatellite insertion in mouse Stat2. Nat Immunol. 2000;1:65–9.
98. K.D Bunting, H.L Bradley, T.S Hawley, R Moriggl, B.P Sorrentino J. I. Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood. 2002;99:479–87.
99. J.W Snow, N Abraham, M.C Ma, N.W Abbey, B Herndier M. G. STAT5 promotes multilineage hematolymphoid development in vivo through effects on early hematopoietic progenitor cells. Blood. 2002;99:95–101.
100. M Kieslinger, I Woldman, R Moriggl, J Hofmann, J.C Marine, J.N Ihle et al. Antiapoptotic activity of Stat5 required during terminal stages of myeloid differentiation. Genes Dev. 2000;14:232–44.
101. H Okamura AR. Transcriptional regulation in lymphocytes. Curr Opin Cell Biol. 2001;13:239–43.
102. M Terabe, S Matsui, N Noben-Trauth, H Chen, C Watson, D.D Donaldson et al. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol. 2000;1:515–20.
103. J Irie-Sasaki, T Sasaki, W Matsumoto, A Opavsky, M Cheng, G Welstead et al. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signaling. Nature. 2001;409:349–54.
104. X Cao, E.W Shores, J Hu-Li, M.R Anver, B.L Kelsall, S.M Russell et al. Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain. Immunity. 1995;223–38.
105. K Saijo, S.Y Park, Y Ishida, H Arase TS. Crucial role of Jak3 in negative selection of self-reactive T cells. J Exp Med. 1997;185:351–6.
106. O'Shea JJ, Holland SM SL. JAKs and STATs in immunity, immunodeficiency, and cancer. N Engl J Med. 2013;368(2):161–70.

107. John J. O'Shea, Daniella M. Schwartz, Alejandro V. Villarino, Massimo Gadina, Iain B. McInnes and AL. The JAK-STAT Pathway: Impact on Human Disease and Therapeutic Intervention*. Annu Rev Med. 2015;66:311–28.
108. Aniseh Samadi, Saman Ahmad Nasrollahi, Ashkan Hashemi M, Firooz, Nassiri-Kashani A. Janus kinase (JAK) inhibitors for the treatment of skin and hair disorders: A review of literature. J Dermatolog Treat. 2016;1–32.
109. K. Ortiz-Ibáñez, M.M. Alsina CM-S. Tofacitinib and Other Kinase Inhibitors in the Treatment of Psoriasis. Actas Dermosifiliogr. 2013;104:304–10.
110. Harrison C, Kiladjian JJ, Al-Ali HK, Gisslinger H, Waltzman R, Stalbovskaya V et al. JAK inhibition with ruxolitinib versus best available therapy for myelofibrosis. N Engl J Med. 2012;366:787–98.
111. RA M. Ruxolitinib, a selective JAK1 and JAK2 inhibitor for the treatment of myeloproliferative neoplasms and psoriasis. IDrugs. 2010;13(6):394–403.
112. Mackay-Wiggan J, Jabbari A, Nguyen N, Cerise JE, Clark C, Ulerio, Grace et al. Oral ruxolitinib induces hair regrowth in patients with moderate-to-severe alopecia areata. JCI Insight. 2016;1(15):e89790.
113. Papp KA, Menter MA, Raman M, Disch D, Schlichting DE, Gaich C et al. A randomized phase 2b trial of baricitinib, an oral Janus kinase (JAK) 1/JAK2 inhibitor, in patients with moderate-to-severe psoriasis. Br J Dermatol. 2016;174(6):1266–76.
114. Changelian PS, Flanagan ME, Ball DJ, Kent CR, Magnuson KS, Martin WH et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. Science (80-). 302AD;875–8.
115. Meyer DM, Jesson MI, Li X, Elrick MM, Funckes-Shippy CL, Warner JD et al. Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis. J Inflamm. 2010;7:41.
116. Villarino AV, Tato CM, Stumhofer JS, Yao Z, Cui YK, Hennighausen L et al. Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. J Exp Med. 2007;204:65–71.
117. Kudlacz E, Conklyn M, Andresen C, Whitney-Pickett C CP. The JAK-3 inhibitor CP-690550 is a potent anti-inflammatory agent in a murine model of pulmonary eosinophilia. Eur J Pharmacol. 2008;582:154–61.
118. Ghoreschi K, Laurence A, Yang XP, Tato CM, McGeachy MJ, Konkel JE et al. Generation of pathogenic T(H)17 cells in the absence of TGF-beta signalling. Nature. 2010;467:967–71.

119. Boy MG, Wang C, Wilkinson BE, Chow VF, Clucas AT, Krueger JG et al. Double-blind, placebo-controlled, dose-escalation study to evaluate the pharmacologic effect of CP-690,550 in patients with psoriasis. J Invest Dermatol. 2009;129(9):2299–302.
120. Papp KA, Menter A, Strober B, Langley RG, Buonanno M, Wolk R et al. Efficacy and safety of tofacitinib, an oral Janus kinase inhibitor, in the treatment of psoriasis: a Phase 2b randomized placebo-controlled dose-ranging study. Br J Dermatol. 2012;167(3):668–77.
121. Bachelez H, van de Kerkhof PC, Strohal R, Kubanov A, Valenzuela F, Lee JH et al. Tofacitinib versus etanercept or placebo in moderate-to-severe chronic plaque psoriasis: a phase 3 randomised non-inferiority trial. Lancet. 2015;386(9993):552–61.
122. Bissonnette R, Iversen L, Sofen H et al. Tofacitinib withdrawal and retreatment in moderate-to-severe chronic plaque psoriasis: a randomized controlled trial. Br J Dermatol. 2015;172:1395–406.
123. Papp KA, Krueger JG, Feldman SR, Langley RG, Thaci D, Torii H et al. Tofacitinib, an oral Janus kinase inhibitor, for the treatment of chronic plaque psoriasis: Long-term efficacy and safety results from 2 randomized phase-III studies and 1 open-label long-term extension study. J Am Acad Dermatol. 2016;74(5):841–50.
124. Ports WC, Feldman SR, Gupta P, Tan H, Johnson TR BR. Randomized Pilot Clinical Trial of Tofacitinib Solution for Plaque Psoriasis: Challenges of the Intra-Subject Study Design. J Drugs Dermatol. 2015;14(8):777–84.
125. Kim A. Papp, Robert Bissonnette, Melinda Gooderham, Steven R. Feldman, Lars Iversen, Jennifer Soung et al. Treatment of plaque psoriasis with an ointment formulation of the Janus kinase inhibitor, tofacitinib: a Phase 2b randomized clinical trial. BMC Dermatol. 2016;16:15.
126. Brittany G. Craiglow BAK. Tofacitinib Citrate for the Treatment of Vitiligo A Pathogenesis-Directed Therapy. JAMA Dermatol. 2015;151(10):1110–2.
127. Bissonnette R, Papp KA, Poulin Y, Gooderham M, Raman M, Mallbris L et al. Topical tofacitinib for atopic dermatitis: a phase IIa randomized trial. Br J Dermatol. 2016;175(5):902–11.
128. Kao L, Chung L FD. Pathogenesis of dermatomyositis: role of cytokines and interferon. Curr Rheumatol Rep. 2011;13(3):225–32.
129. O'Shea JJ, Schwartz DM, Villarino AV, Gadina M, McInnes IB LA. The JAK-STAT pathway: impact on human disease and therapeutic intervention. Annu Rev Med. 2015;66:311–28.

130. Morgan BA. The Dermal Papilla: An Instructive Niche for Epithelial Stem and Progenitor Cells in Development and Regeneration of the Hair Follicle. Cold Spring Harb Perspect Med. 2014;4(7):a015180.
131. Westgate GE, Tobin NVB and DJ. Review Article The biology of hair diversity. Int J Cosmet Sci. 2013;35(4):329–36.
132. Jiro Kishimoto, Robert E. Burgeson BAM. Wnt signaling maintains the hair-inducing activity of the dermal papilla. Genes Dev. 2000;14(10):1181–5.
133. Botchkarev VA PR. Molecular biology of hair morphogenesis: development and cycling. J Exp Zool B Mol Dev Evol. 2003;298(1):164–80.
134. Lavker RM, Sun TT, Oshima H, Barrandon Y, Akiyama M, Ferraris C et al. Hair follicle stem cells. J Investig Dermatol Symp Proc. 2003;8(1):23–8.
135. Andl T, Ahn K, Kairo A, Chu EY, Wine-Lee L, Reddy ST et al. Epithelial Bmpr1a regulates differentiation and proliferation in postnatal hair follicles and is essential for tooth development. Development. 2004;131(10):2257–68.
136. Mak KK CS. Epidermal Growth Factor as a Biologic Switch in Hair Growth Cycle. J Biol Chem. 2003;278(28):26120–6.
137. Schmidt-Ullrich R PR. Molecular principles of hair follicle induction and morphogenesis. Bioassays. 2005;27(3):247–61.
138. Foitzik K, Lindner G, Mueller-Roever S, Maurer M, Botchkareva N, Botchkarev V et al. Control of murine hair follicle regression (catagen) by TGF-beta1 in vivo. FASEB J. 2000;14(5):752–60.
139. G. Lindner, V. A. Botchkarev, N. V. Botchkareva, G. Ling, C. van der Veen RP. Analysis of apoptosis during hair follicle regression (catagen). Am J Pathol. 1997;151(6):1601–17.
140. Hideo Oshima, Ariane Rochat, Cécile Kedzia, Koji Kobayashi YB. Morphogenesis and Renewal of Hair Follicles from Adult Multipotent Stem Cells. Cell. 2001;104(2):233–45.
141. Tumbar T, Guasch G, Greco V, Blanpain C, Lowry WE, Rendl M FE. Defining the epithelial stem cell niche in skin. Science (80-). 2004;303(5656):359–63.
142. Holger Kulessa, Gail Turk BLMH. Inhibition of Bmp signaling affects growth and differentiation in the anagen hair follicle. EMBO J. 2000;19(24):6664–74.
143. Mu S, Handjiski B, Veen C Van Der, Eichmu S, Foitzik K, Mckay IA, et al. Murine Hair Follicles in Distinct Hair Cycle Stages. 2001;3–15.
144. Kos L CJ. An update on alopecia areata. Curr Opin Pediatr. 2009;21(4):475–80.

145. MacDonald Hull SP, Wood ML, Hutchinson PE, Sladden M MA. Guidelines for the management of alopecia areata. Br J Dermatol. 2003;149(4):692–9.
146. Friedli A, Labarthe MP, Engelhardt E, Feldmann R, Salomon D SJ. Pulse methylprednisolone therapy for severe alopecia areata: an open prospective study of 45 patients. J Am Acad Dermatol. 1998;39(4 Pt 1):597–602.
147. Alkhalifah A, Alsantali A, Wang E, McElwee KJ SJ. Alopecia areata update: part II. Treatment. J Am Acad Dermatol. 2010;62(2):191–202.
148. Tosti A, Bellavista S IM. Alopecia areata: a long term follow-up study of 191 patients. J Am Acad Dermatol. 2006;55(3):438–41.
149. Gurcharan Singh ML. Topical Immunotherapy in Alopecia Areata. Int J Trichology. 2010;2(1):36–9.
150. Yalcin Bas, Havva Yildiz Seckin, Göknur Kalkan, Zennure Takci, Riza Citil, Yalcin Önder et al. Prevalence and types of androgenetic alopecia in north Anatolian population: A community-based study. J Pak Med Assoc. 2015;65(8):806–9.
151. Nuwaihyd R, Redler S, Heilmann S, Drichel D, Wolf S, Birch P et al. Investigation of four novel male androgenetic alopecia susceptibility loci: no association with female pattern hair loss. Arch Dermatol Res. 2014;306(4):413–8.
152. Stefanie Heilmann, Amy K. Kiefer, Nadine Fricker, Dmitriy Drichel, Axel M. Hillmer, Christine Herold et al. Androgenetic Alopecia: Identification of Four Genetic Risk Loci and Evidence for the Contribution of WNT Signaling to Its Etiology. J Invest Dermatol. 2013;133(6):1489–96.
153. Rinaldi S, Bussa M MA. Update on the treatment of androgenetic alopecia. Eur Rev Med Pharmacol Sci. 2016;20(1):54–8.
154. Sherrie J Divito TSK. Inhibiting Janus kinases to treat alopecia areata. Nat Med. 2014;20:989–90.
155. Geyfman M, Plikus MV, Treffeisen E, Andersen B PR. Resting no more: re-defining telogen, the maintenance stage of the hair growth cycle. Biol Rev Camb Philos Soc. 2015;90(4):1179–96.
156. R. Schmidt-Ullrich, D. J. Tobin, D. Lenhard, P. Schneider, R. Paus CS. NF-kB transmits Eda A1/EdaR signalling to activate Shh and cyclin D1 expression, and controls post-initiation hair placode down growth. Development. 2006;133:1045–57.
157. A. J. Kellenberger MT. Mammalian target of rapamycin complex 1 (mTORC1) may modulate the timing of anagen entry in mouse hair follicles. Exp Dermatol. 2013;22:77–80.

158. J.M.D. Legrand, E. Roy, J.J. Ellis, M. Francois, A.J. Brooks KK. STAT5 activation in the dermal papilla is important for hair follicle growth phase induction. J Invest Dermatol. 2016;136:1781–91.
159. K. S. Stenn RP. Control of hair follicle cycling. Physiol Rev. 2001;81(1):449–94.
160. M. V. Plikus, J. A. Mayer, D. de la Cruz, R. E. Baker, P. K. Maini, R. Maxson C-MC. Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature. 2008;451:340–4.
161. Jabbari A, Nguyen N, Cerise JE et al. Treatment of an alopecia areata patient with tofacitinib results in regrowth of hair and changes in serum and skin biomarkers. Exp Derm. 2016;25(8):642–3.
162. Franz Faul, Edgar Erdfelder A-GL and AB. G*Power 3: A flexible statistical power analysis program for the social, behavioural, and biomedical sciences. Behav Res Methods. 2007;39(2):175–91.
163. Otomo S. Hair growth effect of minoxidil. Nippon Yakurigaku Zasshi. 2002;119(3):167–74.
164. Messenger AG, Rundegren J. Minoxidil: mechanisms of action on hair growth. Br J Dermatol. 2004;150(2):186–94.
165. Pramod Darvin, Youn Hee Joung YMY. JAK2-STAT5B pathway and osteoblast differentiation. JAKSTAT. 2013;2(4):e24931.
166. Kodama H, Fukuda K, Pan J, Sano M, Takahashi T, Kato T et al. Significance of ERK cascade compared with JAK/STAT and PI3-K pathway in gp130-mediated cardiac hypertrophy. Am J Physiol Hear Circ Physiol. 2000;279(4):H1635-44.
167. Na HJ, Hwang JY, Lee KS, Choi YK, Choe J, Kim JY et al. TRAIL negatively regulates VEGF-induced angiogenesis via caspase-8-mediated enzymatic and non-enzymatic functions. Angiogenesis. 2014;17(1):179–94.
168. Napoleone Ferrara RSK. Review Article Angiogenesis as a therapeutic target. Nature. 2005;438:967–74.
169. Zhao Y AA. Targeting Angiogenesis in Cancer Therapy: Moving Beyond Vascular Endothelial Growth Factor. Oncologist. 2015;20(6):660–73.
170. Souad Lachgar, Hafida Moukadiri, Frédéric Jonca, Marie Charveron, Nadia Bouhaddioui, Yvon Gall et al. Vascular endothelial growth factor is an autocrine growth factor for hair dermal papilla cells. J Invest Dermatol. 1996;106(1):17–23.
171. Eun Jin Kim, Joo Yeon Choi, Byung Cheol Park B-HL. Platycarya strobilacea S. et Z. Extract Has a High Antioxidant Capacity and Exhibits Hair Growth-promoting Effects

in Male C57BL/6 Mice. Prev Nutr Food Sci. 2014;19(3):136–144.

172. Ramos PM, Brianezi G, Martins AC, da Silva MG, Marques ME MH. Apoptosis in follicles of individuals with female pattern hair loss is associated with perifollicular microinflammation. Int J Cosmet Sci. 2016;38(6):651–4.
173. Yann F. Mahe´, Jean-Franc¸ois Michelet, Nelly Billoni, Franc¸oise Jarrousse, Bruno Buan, Stephane Commo et al. Androgenetic alopecia and microinflammation. Int J Dermatol. 2000;39(8):576–84.
174. Farouk M Sakr, Ali MI Gado, Haseebur R Mohammed ANIA. Preparation and evaluation of a multimodal minoxidil microemulsion versus minoxidil alone in the treatment of androgenic alopecia of mixed etiology: a pilot study. Drug Des Devel Ther. 2013;7:413–23.
175. Li LF, Guo J GZ. Overexpression of skin protein kinase C-alpha in anagen hair follicles during induced growth of mouse hair. Clin Exp Dermatol. 2003;28(4):429–33.
176. Li LF, Fiedler VC KR. The potential role of skin protein kinase C isoforms alpha and delta in mouse hair growth induced by diphencyprone-allergic contact dermatitis. J Dermatol. 1999;26(2):98–105.
177. Brittany G. Craiglow, Daniel Tavares BAK. Topical Ruxolitinib for the Treatment of Alopecia Universalis. JAMA Dermatology. 2016;152(4):490–1.
178. Gupta AK, Carviel JL, Abramovits W. Efficacy of tofacitinib in treatment of alopecia universalis in two patients. J Eur Acad Dermatol Venereol. 2016;30(8):1373–8.

APPENDICES