REGULATION OF TOPICAL TOFACITINIB ON BMP4/NOGGIN FOR PROMOTING HAIR GROWTH IN MICE

BY

MR. THANET PONGCHAROENSUK

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE (DERMATOLOGY)
CHULABHORN INTERNATIONAL COLLEGE OF MEDICINE
THAMMASAT UNIVERSITY
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ENTITLED

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was approved as partial fulfillment of the requirements for
the degree of Master of Science (Dermatology)

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ABSTRACT

Background. Recently hair loss disorders are highly concerning problem in generalized population. Many are cause by failure to entry the growth phase of hair follicle (anagen) and arrested in telogen phase including non-scaring alopecia. Androgenic alopecia (AGA) is the one form of non-scarring alopecia which characterized by pattern hair loss due to miniaturization of hair follicles result from hereditary and androgen dependent process. The clinical success rate of treatment in AGA with hair growth promoting agent and modulator of androgen metabolism is limited. Tofacitinib is a new oral disease modifying antirheumatic drug (DMARD) which mainly inhibit enzyme Janus kinase (JAK) JAK1 and JAK3 and, to a lesser extent, JAK2. It means that tofacitinib interfere JAK-STAT (Janus kinase-Signal transducer and activator of transcription) signaling pathway result in suppression of immune process. In human tofacitinib is approved for treatment of rheumatoid arthritis. Recently many clinical trials are focus on improvement of several hair loss disorders by tofacitinib. The previous studies have shown that tofacitinib can promote hair growth in both mice and human by promoting entry of anagen, hair follicle stem cells and anti-inflammatory process. However, many studies reported the efficacy of tofacitinib for promoting hair growth in both human and mice models by unknown exact mechanism.

Objective. The purpose of this study is to find the efficacy of topical tofacitinib for promoting hair growth in mice model and another possible mechanism which explains how topical tofacitinib promote hair growth.

Method. Topical tofacitinib was applied on shaved C57BL/6 male mice with telogen hair once daily for 21 consecutive days compare to Dimethyl sulfoxide (DMSO) as negative control. After day 7, 14, 21, we evaluated efficacy of topical tofacitinib by measure rate of hair regrowth, area of anagen hair and histopathology were collected on day 21 respectively. Tissues of mice were taken for evaluate expression of noggin and bone morphogenetic protein 4 (BMP4) mRNA by Real-time Polymerase Chain Reaction (RT-PCR).

Results. Topical tofacitinib-treated mice showed increase in anagen area and length of hair regrowth. Histopathology also show increase number of hair follicle, higher ratio of anagen hair and angiogenesis compare to vehicle-treated mice. In topical tofacitinib-treated mice, RT-PCR show significant increase expression of Noggin ($P < 0.05$) and BMP4 ($P < 0.05$) mRNA greater than vehicle controlled group. The previous studies have demonstrated that tofacitinib can promote hair growth in both mice and human models by promoting entry of anagen, increased hair follicle stem cells and anti-inflammatory process. This study helps us understanding further more on the efficacy and new possible mechanism of topical tofacitinib by stimulate expression of Noggin and BMP4, the important molecules that involve in onset of growth phase.

Keywords: Tofacitinib, JAK3 inhibitor, Hair growth, BMP4, Noggin,
Non-scarring alopecia

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Mr. Thanet Pongcharoensuk

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
AA	Alopecia areata
ACR20	American college of rheumatology 20 score
AGA	Androgenic alopecia
AT	Alopecia totalis
AU	Alopecia universalis
BAX	Bcl20associated X protein
BCLTL11	Bcl2-like11
BMPR	Bone morphogenetic protein receptor
BMP4	Bone morphogenetic protein four
BMP7	Bone morphogenetic protein seven
B2M	Beta-2-microglobulin
bFGF	Basic fibroblast growth factor
CASP12	Caspase12
CCCA	Central centrifugal cicatricial alopecia
CD	Cluster of differentiation
Da	Dalton
DLE	Discoid lupus erythromatosus
DMARD	Disease modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DPC	Dermal papilla cell
DHT	Dihydrotestosterone
Edu	5-Ethynyl-20 - deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
EPO-R	Erythropoietin receptor
FDA	Food and drug administration
FFA	Frontal fibrosing alopecia

FGF	Fibroblast growth factor
GH	Growth hormone
GH-R	Growth hormone receptor
HAQ-DI	Health assessment questionnaire-disability index score
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor one
IFN	Interferon
IFN-R	Interferon receptor
IGF	Insulin-liked growth factor
IGF-R	Insulin-liked growth factor receptor
IL	Interleukin
ISGF	Interferon stimulating gene factor
JAK	Janus kinase
JH	Janus kinase homology
KGF	Keratinocyte growth factor
LEF1	Lymphoid enhance factor one
LPP	Lichen plano pilaris
MPRK	Mitogen activated protein kinase
mRNA	Messenger ribonucleic acid
NLK	Nemo-liked kinase
NT	Neurotrophin
ORS	Outer root sheath
PASI	Psoriasis area severity index
PGE2	Prostaglandin-E two
RT-PCR	Real time polymerase chain reaction
SALT	Severity of alopecia tool
SH	Src homology
SHH	Sonic hedgehog
Smad	Small mother against decapentaplegic

STAT	Signal transducer and activator of transcription
TAD	Transcriptional activation domain
TAK	Transforming growth factor beta-activated kinase
TE	Telogen effluvium
TGFβ-1	Transforming growth factor beta one
Th	T helper
TLK-2	Tyrosine kinase two
TNF-α	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WNT	Wingless signal

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Recently hair loss disorders are highly concerning problem in generalized population. Many are caused by failure to entry the growth phase of hair follicle (anagen) and arrested in telogen phase including non-scaring alopecia.

Androgenic alopecia (AGA) is one of the non-scarring alopecia which characterized by pattern hair loss can be found in both male and female with high prevalence rate, at least 50% of men by age greater than 50 years old, causing effect on quality of life, stressfulness, low self-esteem. It is believed that hereditary and androgen-stimulated hair follicle result in replacement of terminal hair by miniaturization hair in affected area**.** Consequence in process of increasing in miniaturization hair is caused by progressive shortening of the duration of anagen and premature entry into catagen. In association with other factor such as stress, microinflammation, and microbial colonization of the follicular infundibulum.

Nowadays, Food and drug administration (FDA) has approved standard treatment for AGA which are oral finasteride and topical minoxidil, but the clinical success rate of treatment is still limited beside from their side effect. Oral finasteride, which prevent conversion of testosterone to dihydrotestosterone (DHT) by competitive inhibitor of type 2 5α-reductase, has side effect in decrease of libido and precaution use in pregnant women. Topical minoxidil, an adenosine-triphosphate-sensitive potassium channel opener which effect as vasodilating agent and has been reported to stimulated vascular endothelial growth factor (VEGF) in dermal papillar cell, has side effect on skin irritation, cause progressive hair loss in during early use, and may cause hair growth in unwanted area and patients have to use for life long.

Tofacitinib is a new oral disease modifying antirheumatic drug (DMARD) which mainly inhibit enzyme Janus kinase (JAK) JAK1 and JAK3 and, to a lesser extent, JAK2. It means that tofacitinib interfere JAK-STAT signaling pathway result in suppression of immune process. In human tofacitinib is approved

for treatment of rheumatoid arthritis. Recently many clinical trials are focus on improvement of several hair loss disorders by tofacitinib. Recent study has shown that despite of promoting hair growth by anti-inflammatory process, tofacitinib also provide growth phase of hair follicle by enhanced many molecules in onset of anagen but the whole mechanism of this drug for promoting hair growth is still unknown.

BMP4 and Noggin are both important molecule in hair cycle, by inhibition of BMP4 and stimulation of noggin result in onset of growth phase of hair follicle (anagen). Interestingly BMP4 and noggin also crosstalk to many molecules which can enhanced by tofacitinib.

In considering, we examine the role of topical tofacitinib on BMP4 and noggin for promoting hair growth in mice tissue. The result will help us understand new mechanism of this drug on promoting hair growth and benefit in new therapeutic method for treatment of non-scaring alopecia in future.

1.2 Objective

1.2.1 Primary objective

To compared the promoting effect of 2% topical tofacitinib to DMSO (negative control) on hair growth in mice tissue.

1.2.2 Secondary objective

To discovered effect of 2% topical tofacitinib on BMP4 and noggin for promoting hair growth in mice tissue.

1.3 Hypothesis

2% topical tofacitinib may promote hair growth greater than DMSO (negative control) and down-regulate expression of BMP4 and up-regulate expression of noggin in mice tissue result in stimulation of hair growth.

1.4 Keywords

BMP4
DMSO
JAK3 inhibitor
Non-scarring alopecia
Noggin
Hair growth
Tofacitinib

1.5 Operation definition

Fourteen Male C57BL/6 mice aged 8 weeks

1.6 Ethical consideration

Animal experiments were approved by the Animal Experiment Ethics Committee and conduct according to Ethical Principle and Guidelines for the Use of Animals for Scientific Purpose.

1.7 Limitation

1.7.1 This study does not perform noggin and BMP4 proteins concentration analysis. In order to confirm the impact of tofacitinib on our interested target molecules, researcher will have planned for quantitative measurement of noggin and BMP4 proteins by enzyme-linked immunosorbent assay (ELISA).

1.7.2 Duration of the study

1.8 Obstacles

In this study, researcher has observed that DMSO as vehicle for tofacitinib result in skin irritation and inflammation which observed from clinical presentation and histology. In order to solve the problem, researcher suggests another suitable vehicle for tofacitinib.

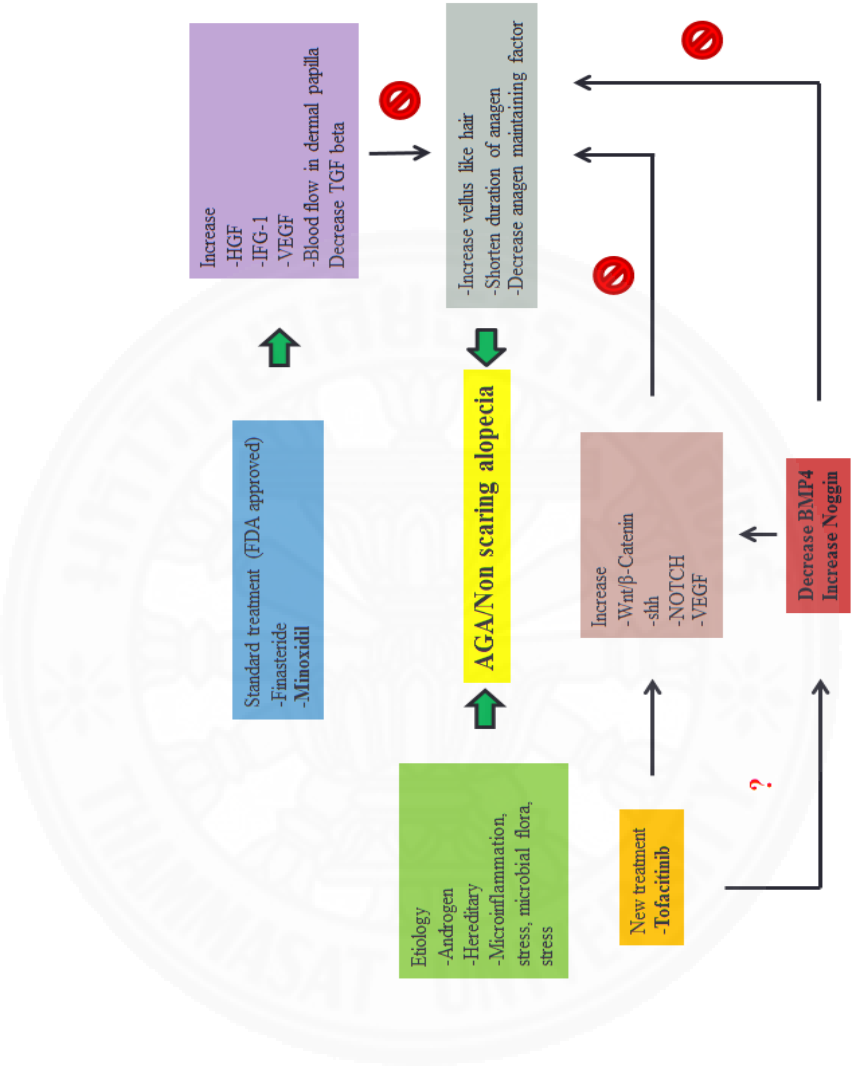


Figure 1.1 Conceptual of framework

Table 1.1 Time frame

Schedule of Events	**2016**	**2017**			**2018**
	Sep-Dec	**Jan-Apr**	**May - Aug**	**Sep-Dec**	**Jan-Apr**
1. Literature review	■				
2. Research proposal		■			
3. Experiment			■		
4. Data analysis			■		
5. Conclusion and report			■		
6. Publication				■	■

CHAPTER 2
REVIEW OF LITERATURE

Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway was first discovered twenty years ago to play an essential role inflammatory response [64]. JAK family, which regulate cytokine mediated leucocyte maturation and activation, cytokine production, and immunoglobulin production, consisted of JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2) are selectively associated with the cytoplasmic domains of various cytokine receptors such as IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-21, interferon erythropoietin, and growth hormone [34, 64, 88].

Tofacitinib (CP690,550) is a new oral disease modifying antirheumatic drug (DMARD). It is a targeted synthetic small molecule (molecular weight 312.4 Da; 504.5 for the citrate salt) [45], which mainly inhibit enzyme JAK1 and JAK3 and to a lesser effect on JAK2 [21]. It means that tofacitinib interfere JAK-STAT signaling pathway result in suppression of immune process and cytokine/chemokine production such as Interleukin-1, Interleukin-6, tumor necrosis factor, other cell surface receptors (CD20, CD80, and CD86) [45, 71]. Oral tofacitinib was approved by U.S. food and drug administration for treatment of rheumatoid arthritis since 6 November 2012 [71]. Oral tofacitinib has shown improvement of clinical outcome such as American college of rheumatology 20 score (ACR20), Health Assessment Questionnaire-Disability Index score (HAQ-DI) in treatment of rheumatoid arthritis in many clinical trials [16, 29, 121]. Oral tofacitinib, 5 and 10 mg twice daily, has shown higher in Psoriasis area severity index 75 (PASI75) in treatment of moderate to severe chronic plaque type psoriasis compare to placebo (39.9%, 59.2% and 6.2%, $p < 0.001$) [94]. In ulcerative colitis, oral tofacitinib result in improvement primary outcome of a clinical response at 8 weeks occurred in 78% of patients on 15 mg (highest dose used) compared with placebo ($p < 0.001$) [109]. In phase IIa randomized trial, 2% topical tofacitinib has shown significant reduction ($p < 0.001$) of eczema area severity index in treatment of atopic dermatitis for 2% topical tofacitinib (-81.7%) compare to vehicle (-29.9%) [10]. The study shown adverse event of 2% topical tofacitinib ointment in treatment of atopic dermatitis, the most common adverse event is upper

respiratory tract infection (5.8%), gastroenteritis (1.4%), application site pain (1.4%), application site pruritus (1.4%) with no death, severe or serious adverse event occurred after four weeks of treatment [10]. In this study they are also show pharmacokinetic of 2% topical tofacitinib ointment, the maximum plasma concentration is 2.72 ng/ml compare to 17.3 ng/ml of 5 mg oral tofacitinib. This information suggested that 2% topical tofacitinib ointment is the suitable concentration which provided good efficacy, acceptable safety and local tolerability profile after four weeks of treatment in atopic dermatitis [10]. The drug has been in other clinical trials in many settings such as vitiligo [23], ankylosing spondylitis [120].

Focus on stimulation of hair growth, Milène Kennedy Crispin *et al*. conducted single arm trial three month of using 5 mg oral tofacitinib citrate for alopecia areata (AA) with >50% scalp hair loss, alopecia totalis (AT), and alopecia universalis (AU). The result show 50% improvement of severity of alopecia tool (SALT) in 32% of patient ($p < 0.05$) [56]. Oral tofacitinib has been in other clinical trials that shown improvement of alopecia areata by its known anti-inflammatory effect [37, 51, 56]. In 2015, Sivan Harel *et al*. conducted the experiment in treatment of topical tofacitinib on C57BL/6 mice at age 8.5 weeks for 21 consecutive days resulted in stimulation of hair growth and suggested that local inhibition of the JAK-STAT pathway results in rapid onset of anagen [41]. They were demonstrated that inhibition of JAK-STAT pathway result in premature onset of anagen phase and leading hair growth in mice hair follicle, by inductivity of many molecules in hair cycle including Wnt/β-catenin, Sonic hedgehog (Shh) [41], the central pathway for anagen initiation [46, 102]. In this study they are show that JAK-STAT inhibition result in activation of hair follicle progenitor cells by present of the proliferating cell (Edu+), which clearly visible within the hair germ (P-cadherin+), in tofacitinib-treated skin [41]. In human organ culture model, tofacitinib treatment also resulted in increased elongation of hair shaft ($p = 0.023$), induced larger spheres of human dermal papilla, significantly greater number of hair follicle ($p = 0.00013$) compare to controls [41]. In addition, tofacitinib treatment also stimulated pathways involved in cell motility and migration such as Rho and integrin signaling resulted in telogen-anagen transition [41]. Furthermore they demonstrated targeting genes enriched in

inductive of dermal papilla. Tofacitinib suppressed proapoptotic genes such as Bcl20associated X protein (BAX), Bcl2-like11 (BCL2L11) and Caspase12 (CASP12) and up-regulated members of the transforming growth factor beta (TGF-β) pathway [41], the molecules that play a role for onset of anagen [30, 96, 104]. Lymphoid enhance factor one (LFF1) and members of NOTCH pathway, molecules that play a role for dermal-epidermal interaction [6, 62, 133], are also up-regulated in tofacitinib treatment [41].

In conclusion, their study shows that JAK inhibition via tofacitinib treatment increase the growth rate of anagen hair shafts in both mice tissue and human organ culture. Topical application of tofacitinib stimulating the activation and proliferation of hair follicle stem cell and enhances onset of anagen by inductivity of many molecules in hair follicle cycling.

Bone morphogenetic proteins (BMPs) has been first isolated from bone extract and played ability to heal bone defect in experimental animals [127]. BMP family is consisted of 100-140 amino acid [103] bind to bone morphogenetic protein receptor type I and type II (BMPR-I, BMPR-II) result in phosphorylation of the intracellular BMP-Smad (Bone morphogenetic protein-Small mother against decapentaplegic) and BMP-MAPK (Bone morphogenetic protein-Mitogen activated protein kinase) pathways [12]. BMPs are member of transforming growth factor beta (TGF-β) family proteins that function for development controlling, cell proliferation, differentiation and apoptosis in the skin and hair follicle [12]. Recently BMPs family is consisted more than 20 members, which vary in functions [12]. BMP2 and BMP4 have been reported to be involved in JAK-STAT pathway. Stimulating apoptosis in myeloma cells, BMP2 and BMP4 antagonized promoting activity of interleukin-6 via downregulation of STAT3 transcription factor [44].

Focus on postnatal hair follicle growth, BMP4 is produced by dermal papilla fibroblasts and secondary germ keratinocyte during telogen phase [12]. In 2001, Botchkarev *et al*. demonstrated that BMP4 plays important role in hair follicle cycling, the onset of anagen was initiated by inhibition of BMP4 [13]. The previous study has shown that inhibition of JAK-STAT pathway result in downregulation of BMP7, the molecule that function by same receptor as BMP4 in osteoblast precursor cells [25].

Another study has shown that application of topical tofacitinib upregulated expression of many molecule in hair cycle such as Wnt/β-catenin pathway [41]. BMP4 is also crosstalk with other important molecules in hair follicle such as Wnt/β-catenin pathway [66]**.** In initiation of anagen, β-catenin activity is activated by Wnt signal and suppressed by BMP4 [66]**.**

Thus, VEGF is one of the important molecule promoting hair growth [61, 132] , expression of BMP4 reduce the level of VEGF in mouse retina [130]. From this rationale we have hypothesis that topical tofacitinib may result in reduce expression of BMP4 result in upregulation of Wnt/β-catenin pathway and VEGF leading hair growth.

Noggin is 222 amino acid protein that bind to BMP4 receptor act as BMP4 antagonist [134] and function for initiated negative feedback of BMP4 [33]. Activation of hair growth phase in postnatal hair telogen hair follicle is associated with upregulation of noggin in follicular epithelium [12]. Noggin is also crosstalk with shh, epithelial shh acts to regulate dermal papilla maturation and maintain dermal papilla functions via noggin to drive hair follicle morphogenesis [126]. From this rationale we have hypothesis that topical tofacitinib may upregulate expression of noggin result in stimulation of Shh leading hair growth. Apart from Shh, noggin also crosstalk with VEGF, noggin treatment also decreased glial scar thickness, increased levels of VEGF protein in glia cell of mice brain [114], the previous study show that tofacitinib also upregulate expression of VEGF (unpublished data) so we have hypothesis that the upregulation of VEGF by tofacitinib may cause from upregulation of noggin.

Nowadays, hair loss disorders are highly concerning problem in generalized population. Many are cause by failure to entry the growth phase of hair follicle (anagen) and arrested in telogen phase including non-scaring alopecia examples androgenic alopecia (AGA), alopecia areata (AA) and telogen effluvium (TE).

TE, non-scaring hair loss, was discovered by Kligman in 1961. It is a most common form of diffuse hair loss which characterized shedding of telogen hair occurs around 3 months after triggering event and usually self-limited within 6 months after treated underlying condition. It caused by premature termination of

anagen and precipitates into catagen and later result in premature entry of telogen [73]. In 2000, Jain *et al*., investigated to probable cause of TE in 100 patients, the result show that precipitating causes of TE are fever (33%), psychological stress (30%) and systemic illness (23%) [52]. The most important treatment for TE is counseling the patients about nature of disease identify and remove precipitating causes. Currently, there are no FDA approved efficient catagen inhibitor or anagen inducers for treatment of TE. Despite topical minoxidil, drug of choice for treatment of TE by its known effect in prolong anagen [15], there is no study on JAK-STAT inhibitor on treatment of TE.

AA is a non-scaring, autoimmune hair loss result from damage of hair follicle by T-cell lymphocyte (CD4, CD8) [111], with a lifetime risk 1.7% effect equally on men and women [108]. By unknown etiology, the lesion is usually present with oval patch of alopecia and may extend to entire body (alopecia universalis). Despite no FDA-approved treatments for AA, there are many treatment methods such as intralesional corticosteroid, topical immunomodulatory, phototherapy but no definitive cure treatment has been established. The disease is often undergone spontaneous resolution to complete hair loss that may persist for life. In 2015, Ali Jabbari *et al.* show the reversal of alopecia areata by treatment of JAK1/2 inhibitor oral baricitinib in both *vivo* experiment mice model and in *vitro* human clinical trial. The study show a remarkable improvement in patient's AA at three months after baricitinib treatment, with complete and steady regrowth after nine months of treatment compare with control [50].

Androgenic alopecia (AGA) is one of the non-scarring alopecia which characterized by pattern hair loss can be found in both male and female with high prevalence rate, at least 50% of men by fourth decade of life [40, 86] and sixth decade of life in female [89]. AGA has believed to be resulted from genetically process [28] and androgen-stimulated [54] hair follicle result in replacement of terminal hair by miniaturization hair in affected area [95] and shortening of the duration of anagen and premature entry into catagen. Result from decreased expression of anagen maintaining factor such as insulin-like growth factor 1 (IFG-1), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), and increased expression of cytokine promoting apoptosis, such as transforming growth factor beta

1 (TGFβ-1), interleukin-1alpha (IL-1α), and tumor necrosis factor alpha (TNFα) [97]. In association with other factor such as stress, microinflammation [53], and microbial colonization of the follicular infundibulum [72].

Currently, FDA has approved two standard treatments for AGA which is oral finasteride and topical solution of minoxidil by aim to increase hair coverage, but clinical success rate is still limited. Oral finasteride, dose 1 mg/day, prevent conversion of testosterone to DHT by inhibit type 2 5α-reductase [55] has side effect in decrease of libido in men and precaution use in pregnant women. Topical solution of minoxidil, which are availiable in 2 and 5 percent of minoxidil, act as an adenosine-triphosphate-sensitive potassium channel opener which effect as vasodilating agent and has been reported to stimulated hepatocyte growth factor (HGF), IFG-1, VEGF in dermal papillar cell [65, 68, 92], inhibits of TGF beta induced apoptosis of hair matrix cells [92], dilates hair follicle arteries and increases blood flow in dermal papilla [92] has side effect on skin irritation, cause progressive hair loss in during early use, and may cause hair growth in unwanted area and patients have to use for life long, result in drop out of patients. Until now there is no reported in relationship between BMP4 and noggin to these two FDA approved standard treatments. Until now, there is no clinical study of JAK-3 inhibitor in treatment of AGA.

Recently clinical success rate treatment of non-scaring alopecia such as AGA by standard treatment is still limited with problem on adverse events leading to drop out of patients. Tofacitinib, a new DMARD, was proven to improvement of hair coverage by promote premature entry of anagen due to anti-inflammatory effect and regulate many molecules which control onset of anagen. However, the mechanism of tofacitinib is still not completely clear. In this research we conducted the experiment to examine the effect of tofacitinib on BMP4 and noggin, the important molecule in onset of anagen, we hope that this research will be the key to access understanding mechanism of tofacitinib in promoting hair growth.

CHAPTER 3
HAIR

Hair is a unique continuously regenerating organ structure found in all mammals. In human, it has multiple important functions including protection skin from external harmful environment, sensory function. Scalp hair prevents sunlight, physical damage and keeps warm to the skin on head and neck area also cosmeceutical function which required for social communication. If the diseases occurred to scalp hair, surely it will effect to the protective function also self-confidence in human.

3.1 Hair follicle morphogenesis

Initiation stage of hair follicle development resulted from an interaction between two adjacent layers, epidermis and underlying dermis. It's begin from the signal from the dermal mesenchyme resulted in the focal thickening in the basal layer of epidermis. This induction process occurred from Wnt (wingless) and β-catenin signaling cascade from dermal mesenchyme. Despite from epidermal thickening, dermal cell become thickening and develops into follicular dermal papilla due to expression of many growth factors and transcription factors. Later, the down-growth of hair placode from epidermis into dermis was occurred caused the formation of many layers of epithelium lining by epithelial signaling of Shh (sonic hedgehog). Finally, with the interaction between epidermis and dermis, hair follicles are fully develop by the formation of inner root sheath, hair shaft, sebaceous glands, arrector pili muscle develop upward from dermal papilla [14].

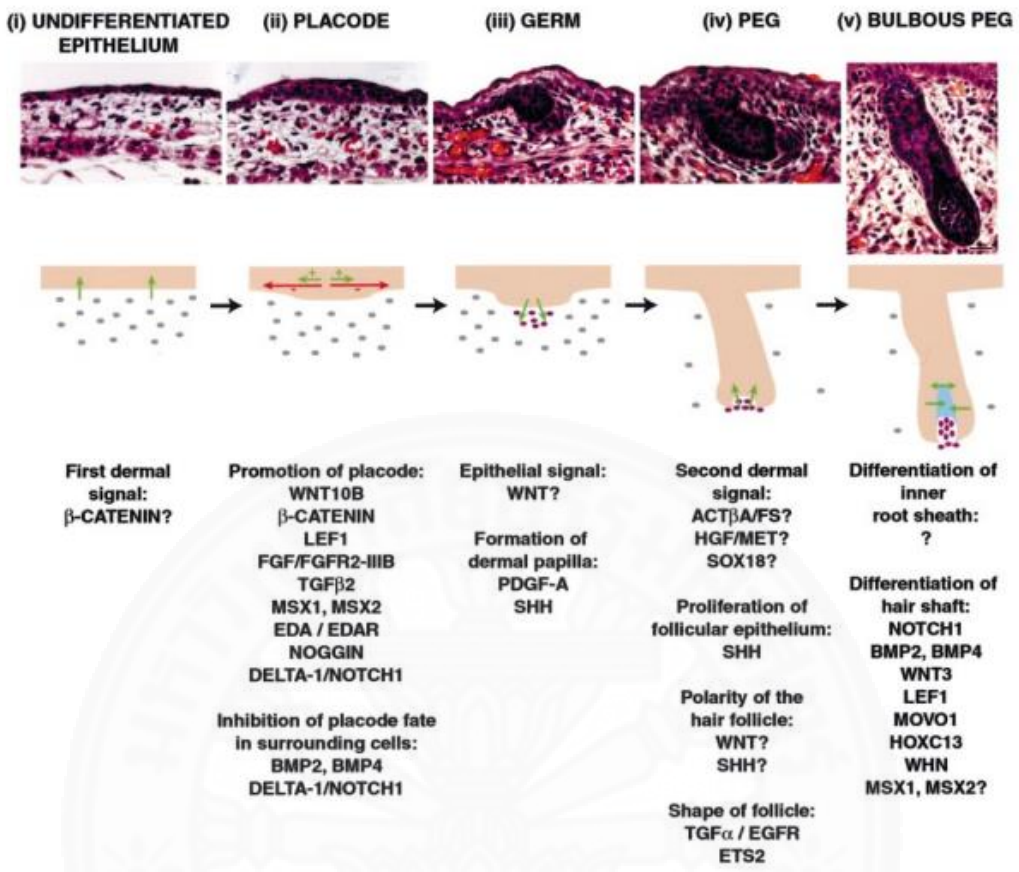


Figure 3.1 The event of hair follicle morphogenesis [78]

3.2 Hair cycle

Postnatal hair follicle has a regenerative property which represent in cycle and continuously within a lifetime. Hair follicle cycling means the changing in morphology, length and histology of hair shaft and follicle. In human hair follicles cycling are change in mosaic pattern while mouse which follicle cycling is begins wavily from anterior to posterior portion. Mainly hair cycles are dividing into three different stages. Begin with the growth phase (anagen), regression phase (catagen) and resting phase (telogen) then growth again. In each stage has unique morphology, characteristic and the duration of individual phase [117].

3.2.1 Anagen

Anagen has been described as a growth phase of hair follicle involved in active regrowth of hair shaft. The anagen has been divided into six substage [18]. Duration of anagen in human scalp is about 2-6 years compared to mice which duration of 1-3 weeks after postnatal with wavy pattern [59]. The length of hair shaft is determined by the time a follicle stay in anagen phase. During early anagen, the hair stem cells in dermal papilla proliferate and grow deep down into dermis until reach the end point and reverse into cylindrical shape making growth in upward direction which produced a seven layer of keratin lineages including IRS cuticle, Huxley's layer, Henle's layer and hair shaft with the rate of matrix cell cycle at 23 hours [117]. Hair bulge, the place for permanent stem cell reservoir of hair follicle, is the source of making up the outer root sheath [22].

3.2.2 Catagen

After end of anagen phase, hair follicles growth stops and enters the catagen phase. In humans, catagen last about 2-3 weeks [60]. In mice model, catagen phase are first present on 17-21 days after postnatal [99]. The initiation of catagen begin when mitosis activity in proliferating cells are terminated in combination of the inhibition of many growth factors [22]. Many molecules are upregulated during catagen such as brain derived neurotrophic factor (BDNF), transforming growth factor beta-I (TGF-β1), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) [117]. Catagen has been divided into eight substages from late anagen to early telogen [83, 118]. First sign of catagen is characterized by the withdrawal of papilla cell fibroblast from basement membrane follow by the shrinkage of the dermal papilla which resulted from loss of extracellular matrix substance [26]. The cessation and apoptosis of bulbar epithelial cell change the morphology at base of hair shaft into "club hair". Many studies have shown that the absence of fibroblast growth factor (FGF) 5 during late anagen result in entry of catagen phase. Many other molecules which downregulated during catagen phase are insulin-like growth factor I receptor (IGF-IR), keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) [117].

3.2.3 Telogen

At the end of catagen phase, hair follicle regressed and placed just only upper dermis. With extreme shrinkage of dermal papilla and poor extracellular matrix, hair follicle enters telogen phase. In mice first telogen phase is very short last about 1-2 days. Otherwise, in second telogen phase of mice is last approximately more than 2 weeks [4]. However, human telogen phase last about 2-3 months. In this stage, hair follicle are rest and prepare for enter another anagen phase [36].

3.2.4 Telogen – Anagen transition

In order to transition from telogen to anagen the interaction between epithelial and mesenchymal are occurred. Wingless (WNT) signal, sonic hedgehog (SHH) and noggin from dermal papilla are important biochemical factors in telogen – anagen transition [36]. In contrast, bone morphogenetic proteins (BMPs) signal from subcutaneous fat needs to be withdrawn in order to entry of new anagen phase [100].

3.2.5 The stem cell of follicle

Stem cell defines as a cell that have self-renews property and this ability retain over lifetime of the animal. Hair follicle stem cells are found at convex extension of distal part of outer root sheath (ORS) called “hair bulge” [91]. In anagen phase, stem cell at hair bulge express many markers such as SOX9 in order to initiate a new growth phase [87].

3.3 Alopecias

Recently hair loss disorders (alopecias) are highly concerning problem in generalized population and cause of distress for patients. Typically, it’s mainly categorized into two differential groups which is scarring, non-scarring alopecia and in addition with hair shaft disorders. Scarring alopecia or cicatricial alopecia caused by the permanent follicular damage followed by the replacement of scar in follicular unit. Scarring alopecia which mainly involved in lymphocytic infiltration included discoid lupus erythematosus (DLE), frontal fibrosing alopecia (FFA), lichen planopilaris (LPP), central centrifugal alopecia (CCCA), and pseudopelade of Brocq. Scarring alopecia which mainly involved in neutrophilic infiltration is folliculitis decalvans, tufted folliculitis, and dissecting cellulitis [106]. On the other hand, non-

scarring alopecia resulted from many etiologies from different diseases and can result in good prognosis of hair regrowth. Common features of non-scarring alopecia included AGA, TE, AA, tinea capitis, tricholillomania. Nowadays many therapeutic options and ongoing researches are focus on stimulation of hair growth in non-scarring alopecia. This chapter will be review mainly focus on non-scarring alopecia.

3.3.1 Telogen effluvium

Kligman was the first who described telogen effluvium, the most common diffuse hair loss, in 1961. The disease is characterized by diffuse hair loss during telogen phase after few months after triggering event and it usually self-limiting after 6 months. Physicians need to find relevant history and exclude endocrine disease, nutritional deficiency and connective tissue disease [73]. Main etiologies of telogen effluvium are fever (33%), psychological stress (30%) and systemic illness (23%) [52].

The goal of treatment in telogen effluvium is counseling patient to get rid of triggering factors such as stress, underlying infection status and adequate nutritional intake. Currently there is no FDA approved standard treatment of telogen effluvium. Some experts used minoxidil as a treatment option for telogen effluvium due to its anagen maintenance effect [15]. Some studies suggest keeping serum ferritin above 40ng/dl or 70ng/dl otherwise there is still debated [73].

3.3.2 Alopecia areata

Alopecia areata (AA) is a non-scarring hair loss disorder characterized by well circumscribed patches of hair loss. The etiologies of AA is believed to be result from autoimmune mediated damage to hair follicle and stimulation of inflammatory cascade due to disruption of immune privileged of anagen hair. Histopathological of AA show infiltration of cytotoxic T lymphocyte (CD 8^+) and $CD4^+$ T lymphocyte together with increase of antigen presenting cell (Langerhans cell) at bulb region of anagen hair follicle. This inflammation induced arrest of anagen phase and disrupt hair shaft formation resulted in weakening and breaking of hair shaft as called “exclamation mark hair”. When the disease is more progress its end up with the entry of catagen and miniaturized hair. The number of hair follicle in AA is still the same, so the hairs are able to regrowth when the

inflammation was stopped [119]. Currently, intralesional corticosteroid has promised as a standard treatment of AA in combination with topical corticosteroid and topical minoxidil (2% or 5%). Interestingly, many studies show successful treatment of AA by the janus kinase/signal transducers and activator of transcription pathway (JAK-STAT) inhibitor [119].

3.3.3 Androgenetic alopecia

Androgenetic alopecia (AGA) is a common type of non-scarring pattern alopecia involved in polygenetic condition. The incident of AGA is 30% in male with age of 30 years, 50% in male with age of 50 years, and 80% in male with age of 70 years [70]**.** Clinical of AGA presented with progressive miniaturization of hair result in vellus transformation of terminal hair. The alteration of hair cycle leads to decrease duration of anagen phase and increase duration of telogen phase [95, 98]. Hamilton et al. first described the important role of androgens as the origin of AGA in 1951 [40]. Pathogenesis of AGA is involved in multifactorial mechanism, androgen interplay effect in many human skin functions such as sebaceous gland growth, hair growth and wound healing. It's the important hormone that stimulates or inhibits hair growth depending on the site of body. In scalp hair follicle, androgen inhibits hair growth, stimulates miniaturization of hair follicle and decrease duration of anagen. The previous study demonstrated that androgen stimulate release of TGF-β, a catagen inducer, from dermal papilla cells (DPCs) [47]. In addition to androgen, pathogenesis of AGA is also involved in genetics. The gene susceptibility for AGA was identified on chromosome 20p11 without relationship to the androgen pathway [105]. Later on many studies found many AGA susceptibility gene including 7p21.1 which located in histone deacetylase 9 (HDAC9), 17q21.31 [70]. The pathogenesis of AGA is also involved in Wnt/β-catenin pathway. Some studies demonstrated that androgen and its receptor interact with β-catenin resulted in inhibition of Wnt singaling in regulation of hair growth in DPCs [57, 122]. P63 protein, the molecule which play a role in hair follicle development, $CD34^{+}$ stem cell, and prostaglandin D2 were discovered as a candidate factors for the pathogenesis of AGA [32, 79, 116]. The latest factor involved in pathogenesis of AGA is oxidative stress, its alter DPCs function, proliferation, migration and induce TGF-β signaling [9].

Currently, the treatment of AGA depend on effectiveness, cost, and practically among each patients. The goals for treatment in AGA are prolong anagen phase, prevent miniaturization and reverse it. Nowadays there are only two F.D.A. approved treatment of AGA: minoxidil which is potassium channel opener and finasteride, a 5α-reductase inhibitor, with good effectiveness but require lifelong daily use and provide many side effects. In addition to medical therapy, surgical procedure including hair transplantation provide clinical outcome.

Minoxidil was firstly developed as an anti-hypertensive drug but later on the many patients who use this medication also develop hypertrichosis [113]. Over 20 years 2% and 5% topical application of minoxidil was approved by U.S. F.D.A. in treatment of AGA which effect in increased the diameter of hair fiber, prolonging anagen phase and shortening duration of telogen phase. Several studies demonstrated that minoxidil has effect on proliferation of epidermal keratinocytes, hair follicle keratinocyte and fibroblast in human skin [77]. Minoxidil also stimulate synthesis of prostaglandin E2 (PGE2), the molecule which found during anagen phase. Another mechanism that minoxidil stimulate hair growth by increased cutaneous blood flow was approved by the evidence from Lachgar et al. that minoxidil can stimulate production of vascular endothelial growth factor (VEGF) [65]. The previous studies have reported other effect of minoxidil in prolong duration of anagen phase by promote many cell growth factors including IGF-1, HFG and inhibit, a catagen inducer, TGF-β [92]. Despite good clinical outcome in treatment of AGA, minoxidil also has many adverse effects such as irritant and allergic contact dermatitis on the scalp, increase hair loss during early use of the drug and patient need to apply this drug for lifelong otherwise the hair will starting to shed again [107].

Finasteride is a competitive inhibitor of type II 5α-reductase, enzyme that converts testosterone to dihydrotestosterone (DHT). Due to lowering of DHT, it is result in prolong anagen phase, inhibits and reverse of miniaturization. Nowadays, 1mg of finasteride is the only dosage approved by FDA in treatment of AGA [39]. A systemetic review in efficacy and safety of finasteride in treatment of AGA in short term (<12 months) and long term (>24 months) has shown that the proportion of patients result in improvement of scalp hair regrowth greater in finasteride group than placebo with short term relative risk 1.81 and long term relative

risk 1.71 with > 95% confidence interval [76]. The common side effect of finasteride is sexual adverse effect at range between 2.1% - 3.8%. Erectile dysfunction is the most common followed by loss of libido and ejaculatory dysfunction. Side effect of finasteride has no evidence of dose dependency and the duration of therapy in treatment of AGA. But the side effect stop when patient discontinued treatment. In addition, the patients who receive finasteride need to examine serum prostatic specific antigen (PSA) as a baseline in order to mislead in diagnosis of prostatic cancer and patients need to continue this drug for lifelong in order to maintain good efficacy in treatment of AGA [85].

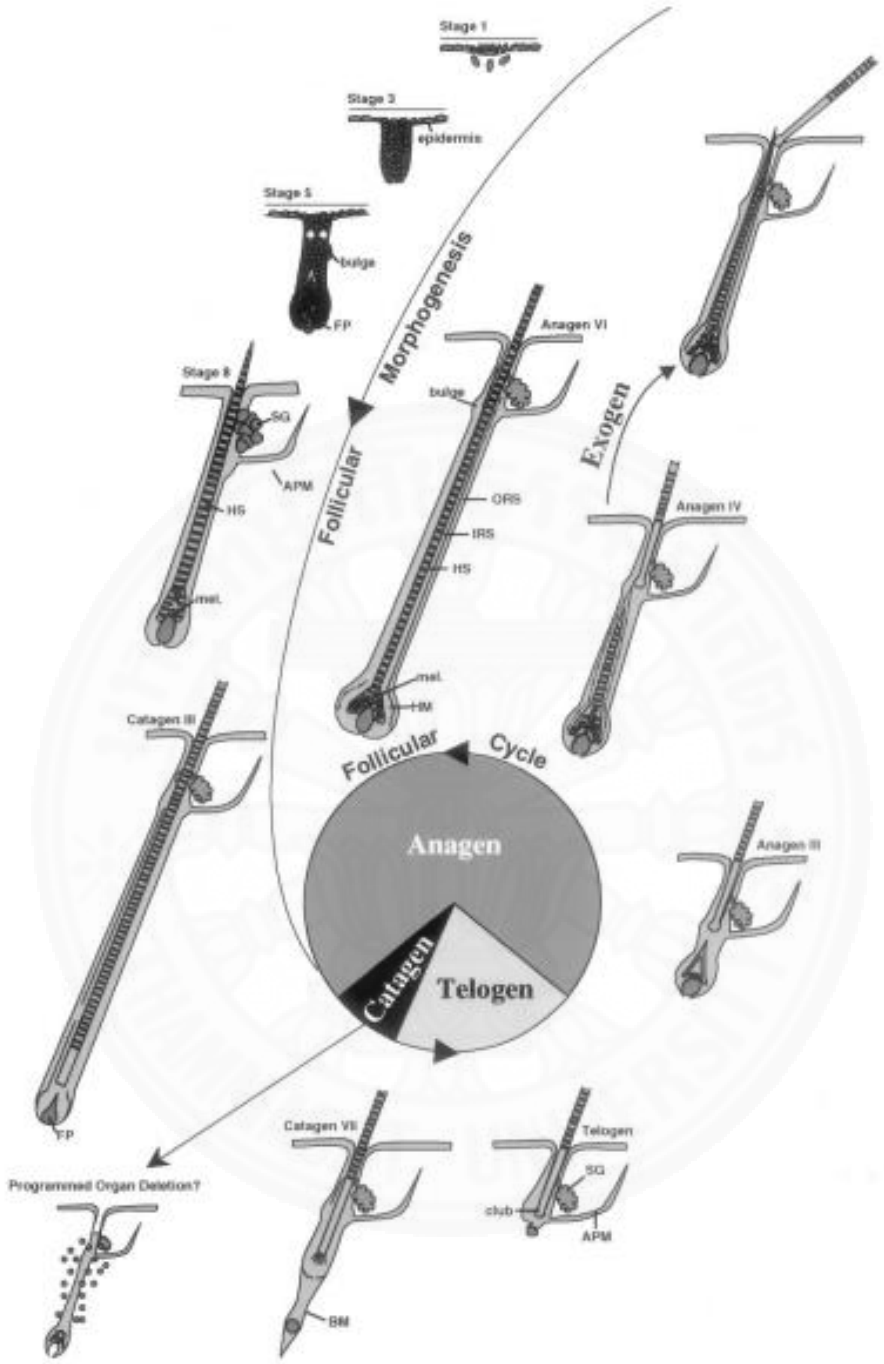


Figure 3.2 Hair cycle [117]

Fibroblast growth factor (FGF)		
FGFR1	Papilla	
FGFR2	Matrix	
FGFR3	Precuticle cells of bulb	
FGFR4	IRS, ORS bulb periphery	
FGF1	Follicular epithelial cell	
FGF2	Follicular epithelial cell	Blocks follicle morphogenesis
FGF5 (short form)	ORS	Terminates anagen
FGF5 (long form)	Macrophage-like round cells in dermis	Blocks short form
FGF7 (KGF)	Papilla	Induces anagen; cytoprotective to chemotherapy
Sonic hedgehog (SHH)		
SHH	Anagen bulb, IRS	Initiates anagen
PATCH	Bulb and surrounding mesenchyme	Mutation leads to basal cell carcinoma
Transforming growth factor-β (TGF-β)		
TGF-β-RI	ORS: late anagen/catagen	Signal transducing receptor for TGF-β isoforms; plays a role in catagen development
TGF-βRII	ORS: late anagen/catagen	
TGF-β1	All expressed in developing follicle; in mature follicle in IRS, ORS, and CTS	TGF-β1 plays a role in catagen induction and blocks anagen induction in vivo ar anagen growth in vitro; TGF-β1 and TGF-β2 stimulates ORS cell proliferation and opposes TGF-2 stimulus
TGF-β2		
TGF-β3		
BMP2	Anagen bulb prekeratogenous zone	Suppresses proliferative activity and supports differentiation
BMP4	Lower follicle mesenchyne	Suppresses hair growth
BMP6	Epithelium	Supports hair follicle development and growth
Noggin	Follicular mesenchyne	Suppresses activity of BMP4 allowing for hair growth
WNT		
WNT-3	Prekeratogenous zone	Hair shaft structure
β-Catenin	Keratogenous zone ORS	Follicular morphogenesis
Lef-1	Peripapillary matrix Epithelium and papilla cells	
Dishevelled-2	ORS; precursor cells and hair shift cortex and cuticle	
Insulin-like growth factor (IGF)		
IGF-I	Upper bulb	Essential for follicle growth in vitro
	ORS Anagen FP (but not catagen or telogen) CTS	
IGF-I receptors	Basal cell of the ORS, sebaceous gland, upper hair matrix keratinocytes	
IGFBP-3	FP	Thought to play regulatory role on IGF expression
IGFBP-5	FP, CTS	
IGFBP-4	Papilla epithelial matrix margin, CTS	
Epidermal growth factors (EGF)		
EGF		Follicle morphogenesis stimulates cell growth in the ORS but inhibits it in the matrix
TGF-α		
EGF-R	In anagen ORS and matrix; in catagen on all undifferentiated cells of epithelial strand and secondary hair germ	
Hepatocyte growth factor (HGF)		
HGF	Papilla	Mediates E-M interactions, stimulates follicle growth in vitro
HGF receptor, *c-met*	Follicular bulb epithelium	Upregulation shows accelerated hair follicle morphogenesis and retarded entry to catagen

Figure 3.3 Molecular factor of hair follicle growth [117]

Term	Definition
Bulb	Prominent, onion-shaped thickening on the proximal end of the HF; consists of relatively undifferentiated matrix cells, HF melanocytes and of cells from the proximal ORS
Bulge	Convex extension of the distal part of the ORS, near the epidermis, location of epithelial follicle stem cells and point of insertion of the *m.* arrector pili.
Club hair	Resting hair shaft with a hollow brush of keratinized keratinocytes on the proximal end, tightly attached to the cortical cells of the hair cortex
Connective tissue sheath (CTS)	Part of the dermal connective tissue, tightly attached to the outer side of HF, composed of fibroblasts (and macrophages) and connective tissue
Dermal papilla (DP) syn: follicular papilla (FP)	Mesodermal part of the HF, which consists of closely packed mesenchymal cells; framed by the bulb matrix during anagen
Epithelial strand (ES)	Column of epithelial cells between the germ capsule and the compact DP; laterally demarcated by the thickened glassy membrane
Secondary germ capsule syn: secondary hair germ	Bag-like structure of glycogen-free cells (germ cells) of distal ORS, surrounding the club hair
Hair shaft	The hair *per se*, composed of trichocytes (= terminally differentiated HF keratinocytes), divided into hair cuticle, cortex and medulla
Hair shaft medulla	Central part of the hair shaft, composed of large, loosely connected keratinized cells with large intercellular air spaces
Hair shaft cortex	The mass of the hair shaft, composed of keratinized cells, longitudinally packed with keratin filaments (and melanin granules in pigmented hair shafts)
Hair canal	Passage way between epidermal surface and the most distal part of the IRS, demarcated by surrounding ORS.
Hyaline membrane syn: vitreous membrane, glassy membrane	Outermost noncellular part of the HF; composed of basal lamina and two layers of orthogonally arranged collagen fibers; separates ORS from CTS
Infundibulum	Most distal part of the HF in the dermis extending from sebaceous duct to the epidermal surface (including hair canal and distal ORS)
Isthmus	Middle portion of the HF extending from the sebaceous duct to the insertion of the m. arrector pili (bulge region)
Inner root sheath	Multilayered structure composed of terminally differentiated HF keratinocytes surrounded by the ORS; consists of Henle's layer, Huxley's layer and cuticle; surrounds the hair shaft up to the hair canal(IRS)
Outer root sheath (ORS)	Outermost sheath of HF keratinocytes, which merges distally into the basal layer of the epidermis and proximally into the hair bulb
Papillary stock syn: papillary stalk	Fibroblasts that link the proximal pole of the DP and the perifollicular CTS
Sebaceous gland	Glandular structure close to the insertion of the *m. arrector pili* with holocrine function; composed of lipid-filled sebocytes with a foamy appearance

Figure 3.4 Term frequently used in hair research [83]

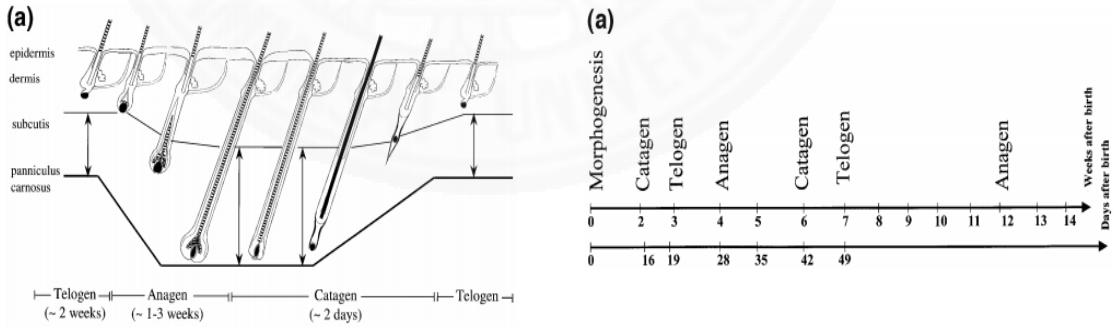


Figure 3.5 Mice hair cycle [83]

CHAPTER 4
BMP AND NOGGIN

Bone morphogenetic proteins (BMPs) are multi-functional growth factors that belong to the transforming growth factor (TGF-β) family. BMPs have roles in embryonic development, cell proliferation, differentiation and cellular functions in postnatal human and animal. The study in BMPs family was started in 1960s, when it was first identified. Nowadays, the BMPs family contains more than 20 molecules with similar in structure and their specific to BMP receptors. Many studies have demonstrated that BMP signaling pathway plays important roles in heart, neural, cartilage and bone development. In addition to bone formation, BMP family also plays crucial roles of epidermal homeostasis, hair follicle growth and melanogenesis in human postnatal skin [12, 19]. Noggin is polypeptide act as a potent antagonist of BMP signaling which binds to BMP receptor type I, II then inactivates BMP2, 4 and 7 signaling pathway. Expression of noggin required for induction of growth phase in hair cycle [2, 19]. This chapter will focus on BMP and noggin in aspect of hair follicle biology.

4.1 Structure of BMP and their receptors

BMP is a molecule consists of approximately 100-140 amino acids in each monomer. In synthesis of BMP is required a subtilisin-like protease for release a carboxyl-terminal group. Each monomer of BMP is linked with disulfide bond which later release into extracellular matrix and interact with their receptors. BMPs have been categorized into many subgroups due to their amino acid sequence but share same cysteine residue and form a rigid structure [17, 103]. BMP bind to a membrane receptor, intracellular serine/threonine kinase, called bone morphogenetic protein receptor (BMPR) type I and II results in phosphorylation and transmission of intracellular signal. The control of BMP signaling are regulated in many levels include (i) at cell surface level by regulated the binding of BMP to BMPR, (ii) at

cytoplasm level by regulate transduction pathway of BMP and (iii) at nucleus level by regulate transcription of target gene [12, 74, 81].

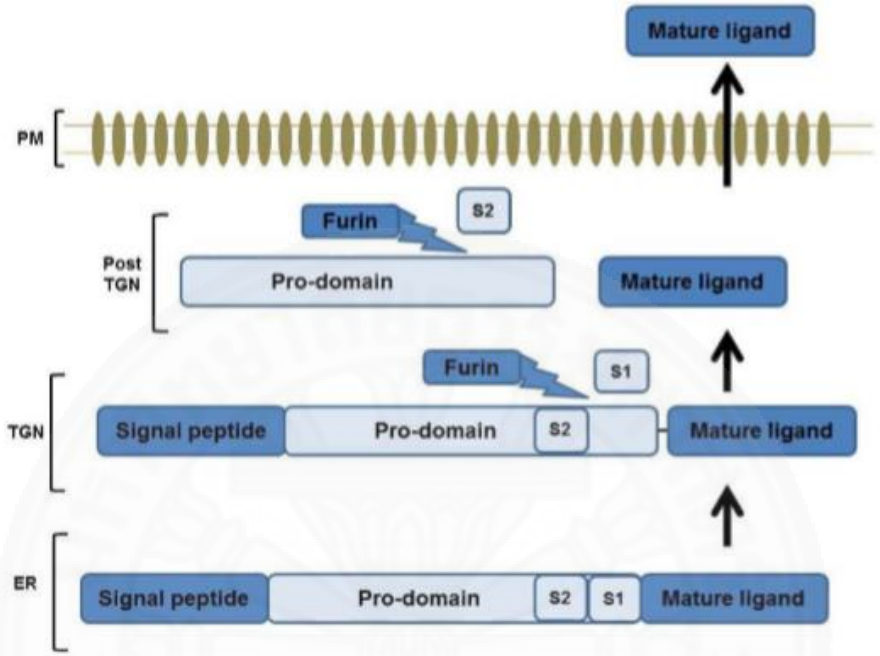


Figure 4.1 Model of BMP synthesis [2].

BMP subfamily	Designation (generic name)	Structure	% identity (human/mouse)
BMP2/4	BMP-2 (BMP-2A) BMP-4 (BMP-2B)	114 aa 116 aa	100 98
BMP-3	BMP-3 (Osteogenin) BMP-3B (GDF-10)	110 aa 110 aa	98 99
BMP-7/OP-1	BMP-5 BMP-6 (Vegetal related-1) BMP-7 (Osteogenic protein-1) BMP-8 (Osteogenic protein-2) BMP-8B (Osteogenic protein-3)	138 aa 139 aa 139 aa 139 aa 139 aa	96 96 98 94 100
BMP-9/ BMP-10	BMP-9 (GDF-2) BMP-10	110 aa 108 aa	95 100
BMP-11/GDF-8	BMP-11 (GDF-11) GDF-8	109 aa 109 aa	100 100
BMP-12/BMP-13/BMP-14	BMP-12 (GDF-7) BMP-13 (GDF-6) BMP-14 (GDF-5)	104 aa 120 aa 120 aa	98 99 98
BMP-15	BMP-15	125 aa	71

Figure 4.2 Molecular structures of BMP family members [12].

4.2 Intracellular BMP signaling cascade

BMP signaling cascade is involved in two major transduction pathways (i) canonical or BMP-Smad pathway (ii) noncanonical or BMP-mitogen-activated protein kinase (MAPK) pathway.

4.2.1 BMP-Smad pathway

After binding of BMP to BMPR and form complex molecule, BMPR II phosphorylates glycine/serine domain of BMPR I which results in phosphorylation of Smad proteins. Smad1, 5 and 8 or receptor activated Smad (R-Smad) were first phosphorylated by BMPR I kinase and subsequence forming complex with Smad4 called common partner Smad (Co-Smad). These complexes (Co-Smad) then translocate into nucleus and regulate transcription of BMP responsive genes. On the other hand, Smad6 and 7 (inhibitory Smad or I-Smad) inhibit the phosphorylation of Smad1, 5 and 8 which regulate in negative feedback of BMP-Smad pathway [12, 43].

4.2.2 BMP-MAPK pathway

BMP-MAPK has been discovered after BMP-Smad pathway. The activation of BMPR complex also has an interaction with another intracellular protein called XIAP which activate TGF-β activated kinase (TAK) 1 protein. TAK1 was considered as a MAPK kinase family protein which activity stimulated by TGF-β1 and BMP4. Subsequence from activation of TAK1, nemo-like kinase (NLK) was activated and inhibited phosphorylation of Lef-1 transcription factor which result in downregulate Wnt/β-catenin pathway. BMP-MAPK and BMP-Smad pathway has shown to be linked with each other via Smad6 protein [48, 131].

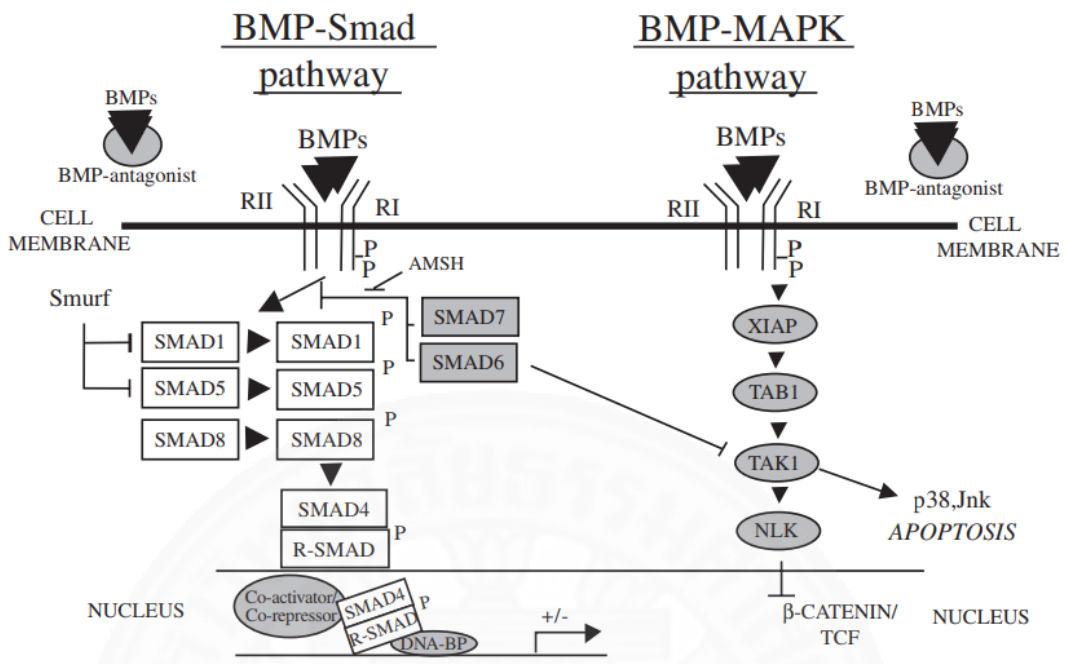


Figure 4.3 Intracellular BMP signaling cascade [12].

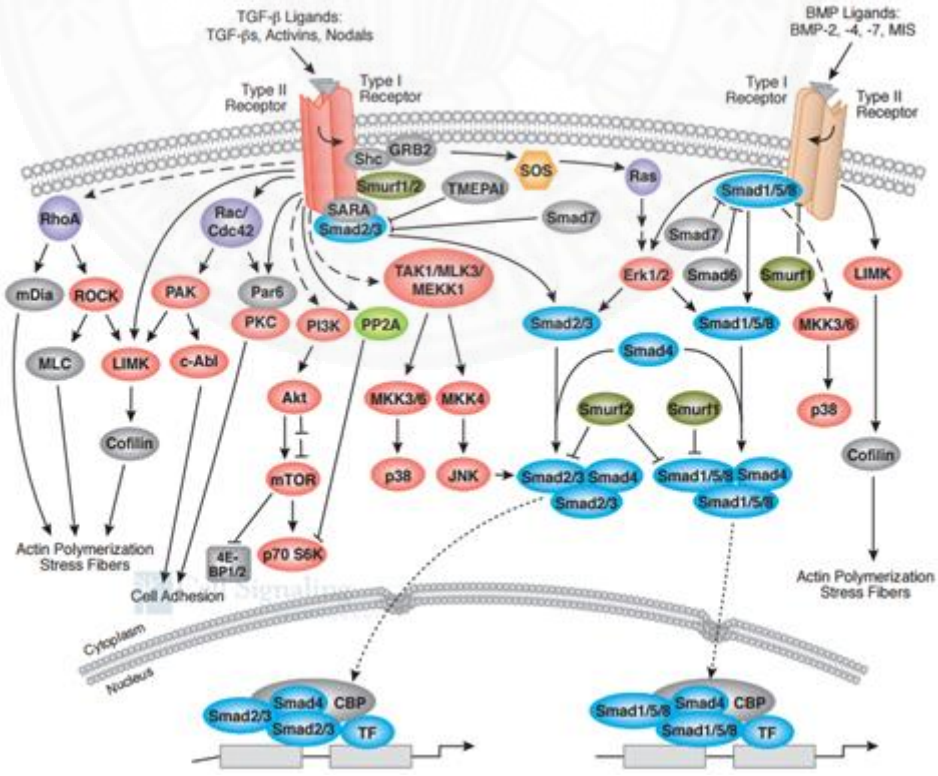


Figure 4.4 Example of Intracellular BMP4 signaling cascade via smad protein.

4.3 Noggin

Noggin is complex of 222 amino-acid that binds and neutralizes with very high affinity (10-15 times higher) to BMP2 and BMP4 than its receptor and act as an antagonized molecule of BMP. It also binds to BMP4 receptors and abolishes BMP4 activity by blocks the binding sites of cell surface receptor. Noggin also binds with lower affinity to BMP7 [134].

4.4 BMP and Noggin in postnatal hair follicle growth

As described in previous chapter, postnatal hair follicle was show in cyclic activity the growth phase (anagen), regression (catagen) and resting (telogen). The hair cycle results from regulation of many stimulatory and inhibitory molecules. In initiation of a new anagen phase in postnatal hair is characterized by a construction of new hair fiber from hair bulb. Many studies have demonstrated the important of Wnt/β-catenin, Shh and also BMP pathway as a major pathway involved in onset of the growth phase [31]. BMP4, BMPR I and noggin have known to dynamically change in their expression during the experiment in C57BL/6 mice. BMP4, produced by dermal papilla fibroblast and secondary germ keratinocyte during telogen phase, has been shown to preventing the onset of anagen development [13]. In contrast to BMP4, administration of noggin results in stimulation of hair growth in postnatal mice hair follicle [13]. The study of Zimmerman et al. [134] supported the idea that noggin which has higher affinity to BMP4 10-15 times prevent BMP4 interact with BMPR-I receptor during telogen and induce onset of anagen. BMP4 has been shown to be an inhibitory factor for onset of anagen during telogen-anagen transition. Wilson et al. demonstrated that administration of BMP4 results in blockage of anagen development and keratinocyte proliferation in secondary hair follicle [125]. Noggin has been demonstrated crosstalk to Shh signaling. Shh, the essential molecule for anagen initiation and hair follicle morphogenesis [27], has been shown to be upregulated in hair follicle after administration with noggin [12]. Botchkarev et al. suggest that administration of noggin results in neutralization of BMP4 effect and stimulate Shh during the telogen-anagen transition subsequence in hair growth [12].

In postnatal hair follicle, BMP4 is crosstalk with other molecules in hair cycle. The activity of β-catenin is activated by Wnt signal and suppressed by BMP signal. BMP4 has been shown to inhibit regeneration of hair population by maintain hair follicle unresponsive to Wnt signal [66].

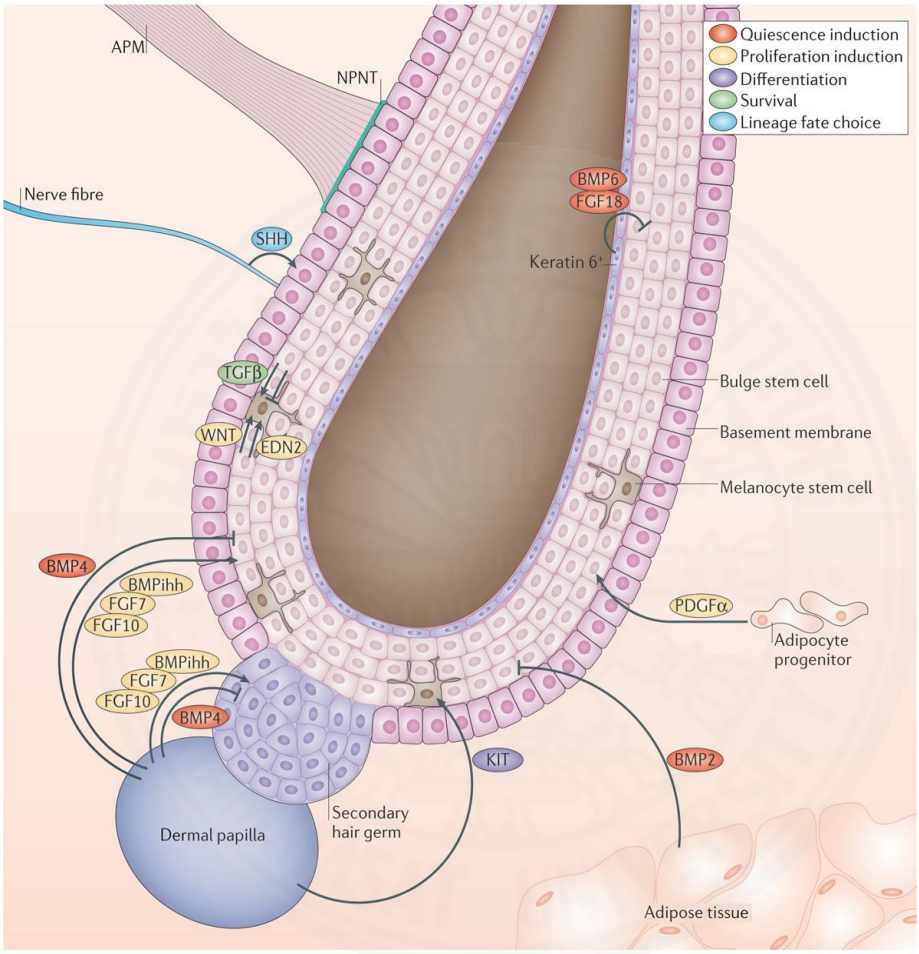


Figure 4.5 BMP4 and BMP4 inhibitor (noggin) in skin and hair follicle tissue.

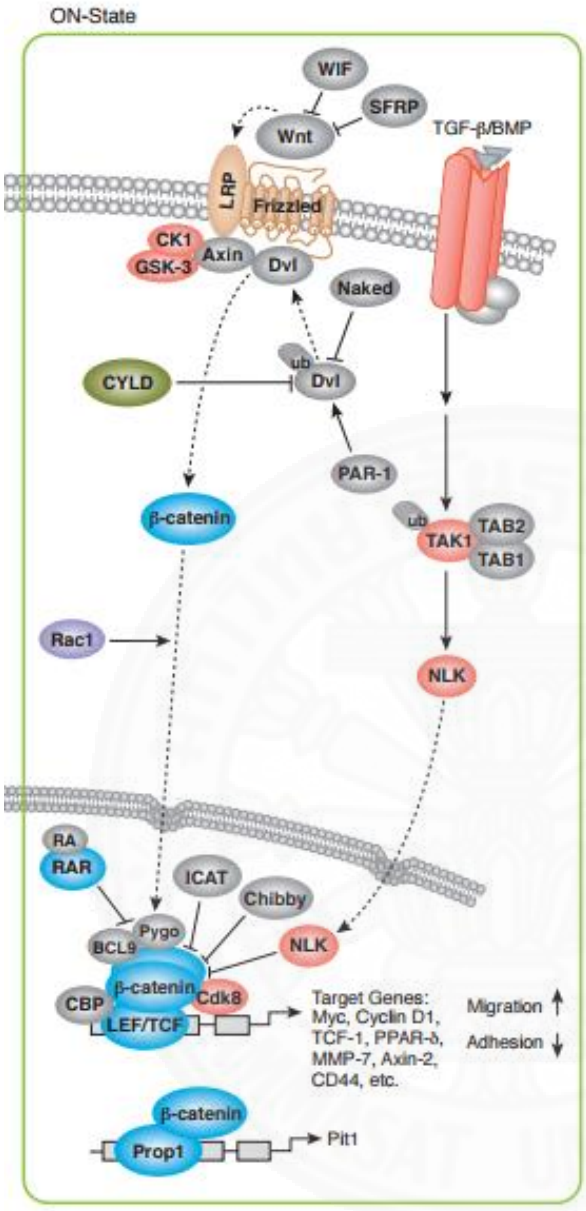


Figure 4.6 Crosstalk between BMP4 and Wnt signaling pathway.

CHAPTER 5
JAK-STAT

Interferons (IFNs) have been first described as a member of cytokine for 50 years ago. Later on, many other four helix cytokines and there receptors were discovered, opened new era of biomolecular science. The studies of how cytokines induce gene expression result in discovered of JAK-STAT signaling pathway. Totally, 4 JAKs and 7 STATs provide responsible in intracellular transduction pathway of more than 50 members of cytokine. This chapter will briefly review on JAK-STAT signaling pathway [112].

5.1 The JAKs

JAKs (Janus kinase) families, member of tyrosine kinases, are consisting of JAK1, JAK2, JAK3, and Tyk (tyrosine kinase) 2. They have responsible for many cell functions, except JAK3, which specific response to leukocyte function. Molecular weight of JAKs range size from 120 to 140 kDa which consist of 7 JAK homology (JH) domains. Activation of JAK results from phosphorylation process. FERM (four point one, ezrin, radixin, moesin), a terminal of JAK molecule, play a role in association with cytokine receptor [112].

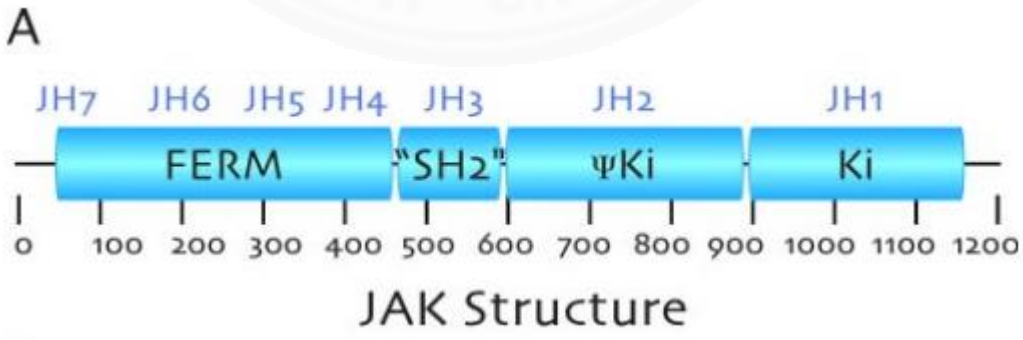


Figure 5.1 JAK structure [112].

5.1.1 Tyk2

Tyk2 associates with the function of IFN-I, IL-6, IL-10 and IL-12/23 cytokine family [58, 124]. Malfunction of Tyk2 can cause allergic and lethal impair antimicrobial response due to inability of response to IFN-I, IL-6, IL-10 and IL12/23 cytokine in human [124]. In contrast to mice model, Tyk2 deficiency dose not result in lethal effect as in human [124].

5.1.2 JAK1

JAK1 has function associated with type I (IFNα/β), type II (IFN-γ), IL-2 and IL-6. IFNAR (IFN-α/β receptor) 1 and 2 are responsible with Tyk2 and JAK1 in order for phosphorylation process. Deficiency of JAK1 in mice model has end up with perinatal death due to immunological dysfunction [112].

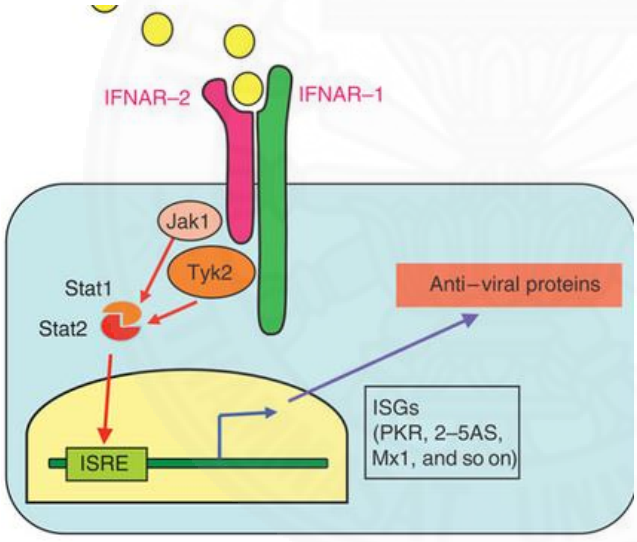


Figure 5.2 Tyk2 and JAK1 signaling pathway [49]

5.1.3 JAK2

JAK2 has function associated with the single chain receptor include GH-R (growth hormone receptor), Epo-R (erythropoietin receptor), IL-3 cytokine families and IFN 2, 3, 5 receptors. Deficiency of JAK2 in mice model results in severe anemia. In human, JAK2 mutation can cause myeloproliferative disorder [112].

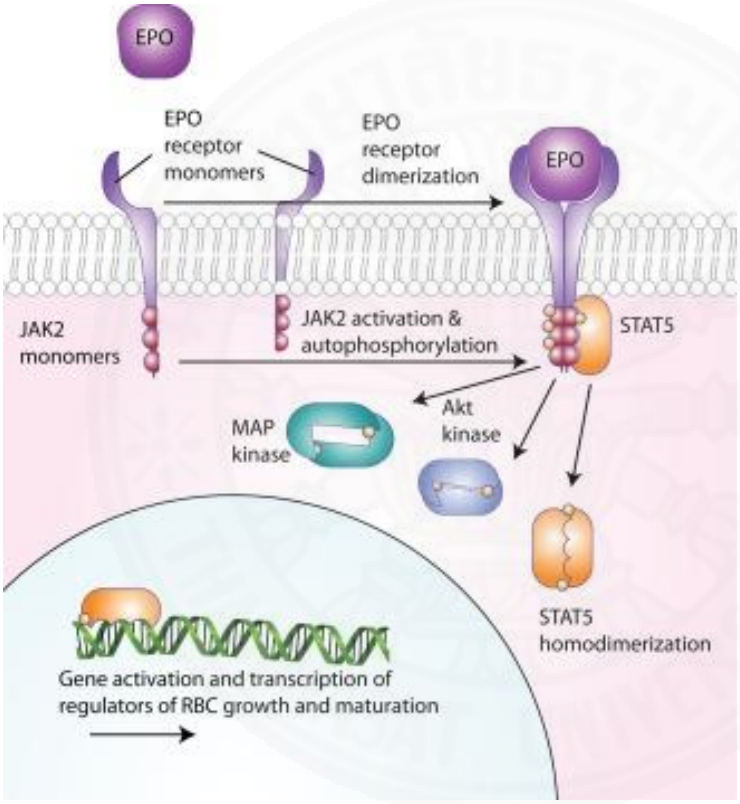


Figure 5.3 JAK2 and Epo (erythropoietin) signaling pathway [110].

5.1.4 JAK3

JAK3, only leukocyte specific molecule, has function associated with IL-2 receptor-γ-chain. JAK3 has involved as a component of many cytokines such as IL-4, IL-7, IL-9, IL-15 and IL-21. In human, mutation of JAK3 revealed severe combined immunodeficiency disease but less severe in mice model. Recently, JAK3 was exhibit as a pharmacological target for immunomodulatory agent such as tofacitinib.

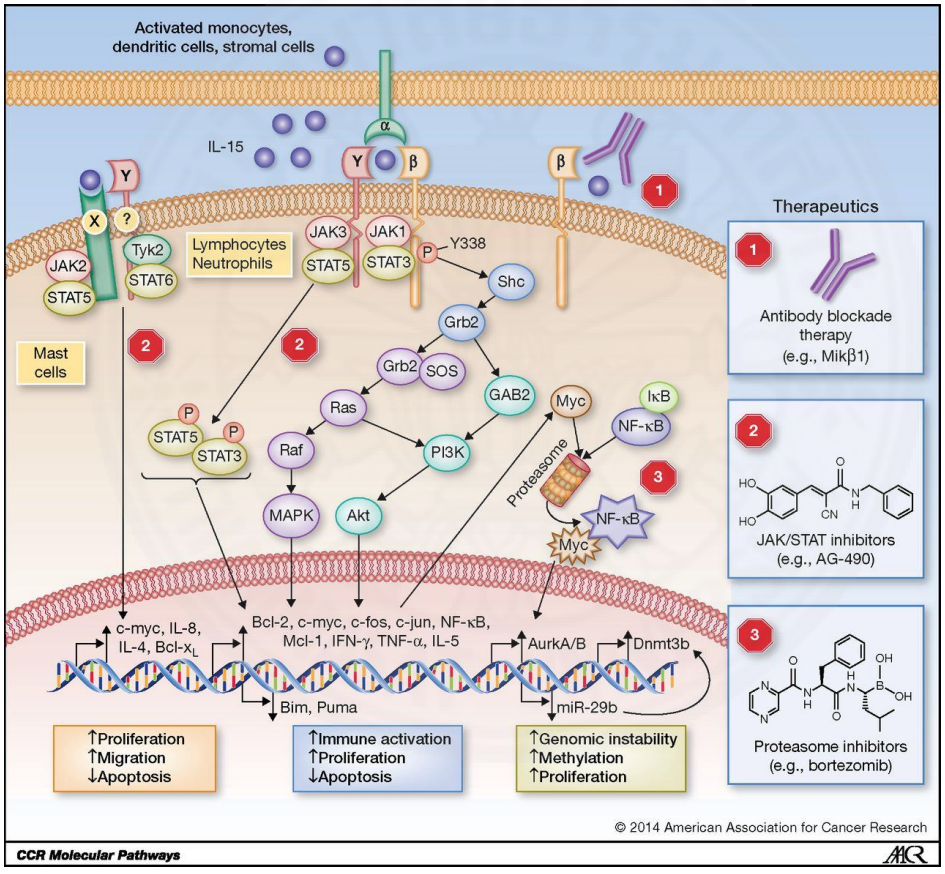


Figure 5.4 Example of IL-15 signaling via JAK3 [80].

5.2 The STATs

Recently, there are 7 members of STAT families which range from 750-900 amino acids includes STAT 1, 2, 3, 4, 5a, 5b and 6. In resting state, STATs stay in inactive stage as a protein in cytoplasm. After the activation of JAK, its result in phosphorylation of associated tyrosine kinase receptor and then recruit specific STAT by attach at SH-2 domain of STAT. Activated STAT translocate into nucleus and bind to GAS enhancer family (a palindrome, TTTCCNGGAAA). Structure of STAT molecule is consisted on many domains such as the amino terminal (NH2), coiled-coil, DNA-binding (DBD), SH2 and transcriptional activation domain (TAD). NH2 domain plays a role in DNA binding site to GAS in nucleus result in transcription activation and protein synthesis.

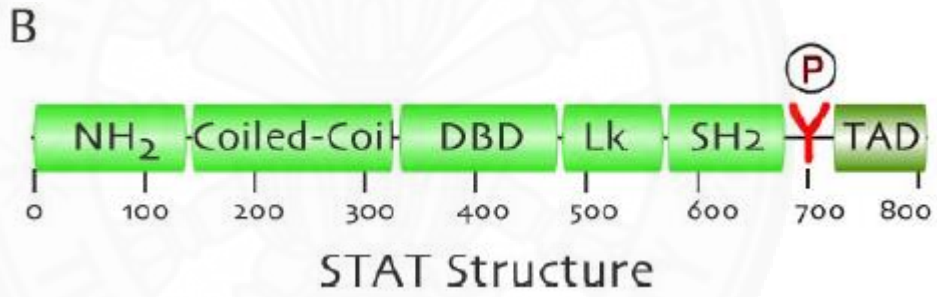


Figure 5.5 STAT structure [112].

5.2.1 STAT1

STAT1 is a component of ISGF-3 bind to ISRE in nucleus during stimulation of IFN-α. Later on, STAT1 has been shown to be involved in response to type-I and type-II IFNs. IFN-I and IL-6 families are responsible in activation of STAT1 signaling. Mutation of STAT1 causes a risk to bacterial and viral infections. STAT1 demonstrated function to promote inflammation and inhibition proliferation, in contrast to STAT3 [112].

5.2.2 STAT2

STAT2, the largest STAT, is a component of ISGF-3 which play a role in responsible to type-I IFNs autocrine loop [112].

5.2.3 STAT3

STAT3 is IL-6 dependent transcription factor responsible in acute phase gene expression**.** STAT3 play a role in transduction signal of IL-6, IL-10, IL-21, IL-27 and G-CSF (granulocyte colony stimulating factor), leptin. Many studies demonstrate that STAT3 associate to cancer growth and anti-inflammatory process [112].

5.2.4 STAT4

STAT4 is responsible to IL-12 during CD4+ and NK cell activation**.** Later on, STAT4 has been shown to play an important role in IL-23 dependent Th17 expansion [112].

5.2.5 STAT5

STAT5 consists of STAT5a and STAT5b which responsible to IL-3, IL-5, GM-CSF, IL-2, IL-7, IL-9, IL-15, GH (growth hormone), erythropoietin, prolactin. While there are many responsible molecules to STAT5, it's mainly function in erythropoiesis and lymphopoiesis [112].

5.2.6 STAT6

STAT6 is a transduction signal of IL-4 and IL-13. It has function responsible for IL-4/IL-13 in turning naiive CD4+ lymphocyte to T helper 2 (Th2), mast cell activation and promote B-cell function [112].

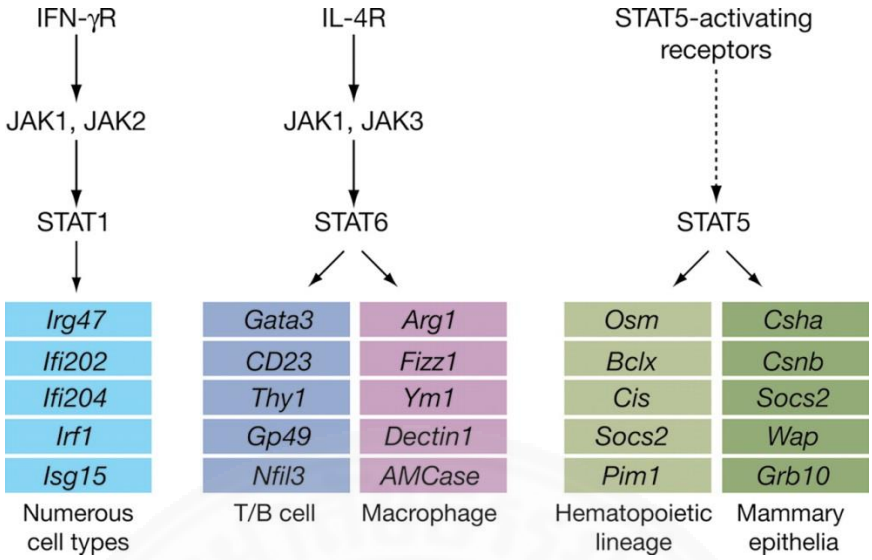


Figure 5.6 Representative example of gene expression induced by STAT signaling in different tissue [84].

CHAPTER 6
JAK INHIBITOR IN DERMATOLOGY

Currently, there are two generation of JAK inhibitor, the first generation of JAK inhibitor consists of tofacitinib, ruxolitinib, baricitinib, and oclacitinib. FDA has approved only tofacitinib for treatment of rheumatoid arthritis since 2012. Baricitinib is currently in clinical trial in treatment of rheumatoid arthritis and psoriasis but not yet FDA approved [24].

Inhibition of JAK-STAT pathway is the hot issue in treatment of many skin diseases. Despite US FDA approved as a drugs for treatment of rheumatoid arthritis and myeloproliferative disorders. This chapter will review clinical study of JAK-STAT inhibitor, a new drug class; in treatment of skin diseases include psoriasis, hair loss disorder, atopic dermatitis, and vitiligo.

6.1 JAK inhibitor and Atopic Dermatitis

Atopic dermatitis is the inflammatory skin disease which pathogenesis related to T-helper cell type II (Th2) response. The disease results from increase production of IL-4, IL-5 and IL-13 from T-cell stimulation. As we all know, signaling cascade of these cytokines are function via JAK-STAT signaling pathway. Previous studies have shown that tofacitinib and oclatinib can inhibit Th2 differentiation in animal model [8, 34, 35]. In mice, topical applications of JAK inhibitor (JTE-053) demonstrate improvement of skin barrier by reduce IL-4 and IL-13 signaling [5]. Support by Levy, LL *et al*. study which demonstrated that the patients, who has recalcitrant to conventional therapy, take oral tofacitinib 5 mg daily or twice daily result in 66.6% improve in severity scoring index of atopic dermatitis with 69.9% decrease in pruritus score [67]. Another randomized double-blind placebo control phase 2 study has demonstrate that 2% topical tofacitinib ointment show reduction of eczema area and severity index score up to 81.7% on week four of treatment [123].

6.2 JAK inhibitor and Non-scaring alopecia

Alopecia areata (AA) is a one of non-scaring alopecia which pathogenesis involved in dysfunction of CD8+ T cells and its cytokines included IFN-γ and IL-15. As we all know, signaling cascade of IFN-γ and IL-15 are function via JAK-STAT pathway. Xing *et al.*, demonstrate the successful treatment of alopecia areata in mice model from both systemic and topical treatment of tofacitinib and ruxolitinib [129]. Focus on stimulation of hair growth, Milène Kennedy Crispin *et al*. conducted single arm trial three month of using 5 mg oral tofacitinib citrate for alopecia areata (AA) with >50% scalp hair loss, alopecia totalis (AT), and alopecia universalis (AU). The result show 50% improvement of severity of alopecia tool (SALT) in 32% of patient ($p < 0.05$) [56]. Oral tofacitinib has been in other clinical trials that shown improvement of alopecia areata by its known anti-inflammatory effect [37, 51, 56]. In 2015, Sivan Harel *et al*. conducted the experiment in treatment of topical tofacitinib on C57BL/6 mice at age 8.5 weeks for 21 consecutive days resulted in stimulation of hair growth and suggested that local inhibition of the JAK-STAT pathway results in rapid onset of anagen [41]. They were demonstrated that inhibition of JAK-STAT pathway result in premature onset of anagen phase and leading hair growth in mice hair follicle, by inductivity of many molecules in hair cycle including Wnt/β-catenin, Sonic hedgehog (Shh) [41], the central pathway for anagen initiation [46, 102]. In this study they are show that JAK-STAT inhibition result in activation of hair follicle progenitor cells by present of the proliferating cell (Edu+), which clearly visible within the hair germ (P-cadherin+), in tofacitinib-treated skin [41]. In human organ culture model, tofacitinib treatment also resulted in increased elongation of hair shaft ($p = 0.023$), induced larger spheres of human dermal papilla, significantly greater number of hair follicle ($p = 0.00013$) compare to controls [41]. In addition, tofacitinib treatment also stimulated pathways involved in cell motility and migration such as Rho and integrin signaling resulted in telogen-anagen transition [41]. Furthermore they demonstrated targeting genes enriched in inductive of dermal papilla. Tofacitinib suppressed proapoptotic genes such as Bcl20associated X protein (BAX), Bcl2-like11 (BCL2L11) and Caspase12 (CASP12) and up-regulated members of the transforming growth factor beta (TGF-β) pathway [41], the molecules that play a role for onset of anagen [30, 96, 104]. Lymphoid enhance factor one (LFF1) and members of NOTCH

pathway, molecules that play a role for dermal-epidermal interaction [6, 62, 133], are also up-regulated in tofacitinib treatment [41].

6.3 JAK inhibitor and Psoriasis

The pathogenesis of psoriasis is involved in multifactorial and multiple pathway of cell and cytokine response. IL-12 and IL-23 are important cytokine mediators of this disease. Currently, many studies and clinical trial are focus on inhibition of JAK-STAT pathway in treatment of psoriasis. As we all know, these two cytokines are function via JAK-STAT pathway. Previous study has shown that inhibition of JAK-STAT pathway result in reduction of IL-23 which upstream blockage production of IL-17 cytokine in psoriasis [129]. Tofacitinib, 5mg twice daily and 10mg twice daily, has shown improvement of psoriasis area severity index of 39.5% and 63.6% respectively. In this study also show that 10mg twice daily of oral tofacitinib does not inferior to 50mg subcutaneous twice weekly of etanercept in treatment of psoriasis [7]. Focus on topical treatment of psoriasis, 1% and 1.5% of baricitinib cream applied twice daily result in decrease in sized of psoriasis plaque after 4 weeks of treatment [101].

6.4 JAK inhibitor and Vitiligo

The central pathogenesis of vitiligo is involved in destruction of melanocyte due to CD8+ producing IFN-γ. As we all know, IFN-γ functions via JAK-STAT pathway. The previous study has demonstrated that topical application of tofacitinib show repigmentation of acro-facial vitiligo after 5 month of treatment [23]. On the other hand, oral ruxolitinib 20mg daily also show improvement of facial vitiligo after 20 weeks of treatment [42]. However, discontinuation of the drug results in recurrent of depigmentation. Currently there is an ongoing study on topical 1.5% ruxolitinib twice daily in treatment of vitiligo [24].

6.5 Safety data and adverse effect of JAK-STAT inhibitor

Mainly, safety data of tofacitinib was received from FDA approved treatment for rheumatoid arthritis and psoriasis. On the other hand, safety data of roxulitinib was received from FDA approved treatment for myelofibrosis and polycythemia vera. Infection urinary tract infection and recurrent varicella zoster are the most common side effect observed in oral roxulitinib and tofacitinib. Mild dyslipidemia is also can observed in patient who takes oral roxulitinib and tofacitinib without increase risk of stroke or myocardial infarction. Because of JAK2 has function in hematopoiesis, thrombocytopenia is commonly observed in ruxolitinib, mainly JAK2 inhibitor, than tofacitinib, mild JAK2 inhibitor. Many studies comfirm unrelationship between normal dosages (<20mg/day) of tofacitinib which used to treat inflammatory disease such as rheumatoid arthritis and lymphoproliferative disease [24, 93].

Drug	Inhibits	FDA-approved indications	FDA-approved dosage
Tofacitinib	JAK1/3 > 2	Rheumatoid arthritis	5 mg, twice daily 11 mg ER, once daily
Ruxolitinib	JAK1/2	Myelofibrosis	5-25 mg, twice daily
		Polycythemia vera	5-25 mg, twice daily
Baricitinib	JAK1/2	None	None
Oclacitinib	JAK1	Canine atopic dermatitis	N/a

ER, Extended release; *FDA*, Food and Drug Administration; *JAK*, Janus kinase; *N/a*, not applicable.

Figure 6.1 First generation of JAK inhibitor [24].

Disease	Evidence for oral therapy	Evidence for topical therapy
Alopecia areata	OCT-tofacitinib[15] OCT-ruxolitinib[17] CS-tofacitinib[18,19] CR-tofacitinib[20-25] CR-ruxolitinib[26-29] CR-baricitinib[30]	CR-ruxolitinib[16]
Atopic dermatitis	CS-tofacitinib[31]	RCT-tofacitinib[32]
Chronic actinic dermatitis	CR-tofacitinib[33]	
Chronic mucocutaneous candidiasis	CR-ruxolitinib[29,34]	
Cutaneous T-cell lymphoma	Other[5]	
Dermatomyositis	CR-tofacitinib[35,36] CR-ruxolitinib[37]	
Erythema multiforme	CR-tofacitinib[38]	
Graft-versus-host disease (cutaneous)	CS-ruxolitinib[39]	
Hypereosinophilic syndrome	CS-tofacitinib[40]	
Lupus erythematosus	CR-tofacitinib[41,42] CR-ruxolitinib[43]	
Mastocytosis and mast cell disease	CR-ruxolitinib[44]	
STING vasculopathy	CR-tofacitinib[42] CR-ruxolitinib[45]	
Palmoplantar pustulosis	CR-tofacitinib[46]	
Polyarteritis nodosa	CR-tofacitinib[47]	
Psoriasis	RCT-tofacitinib[48,49] RCT-baricitinib[11] Others*	RCT-tofacitinib[50,51] CS-ruxolitinib[52]
Vitiligo	CR-tofacitinib[53] CR-ruxolitinib[27]	CS-ruxolitinib[54]

Other designates in vitro data on human tumor cells.
CR, Case reports (<5 patients/study); *CS*, case series (≥5 patients/study); *JAK*, Janus kinase; *OCT*, open-label clinical trial; *RCT*, randomized-controlled trial; *STING*, stimulator of interferon genes.
*Multiple earlier studies not included.

Figure 6.2 Summary of JAK-inhibitor in treatment of dermatologic conditions [24].

CHAPTER 7
RESEARCH METHODOLOGY

7.1 Material

7.1.1 Animals

Eight weeks C57BL/6 male mice purchased from National Laboratory Animal Center. Randomly assigned to tofacitinib-treated group (N=7) and vehicle control group (N=7) kept under hygienic conventional standard and provide standard environment, food, water. Animal experiment was approved by Thammasat University's Animal Ethical Committee and conducted according to Ethical Principles and Guidelines for the Use of Animals for Scientific Purpose.

Sample size is A 14 C57BL/6**,** eight-week-old male, mice are selected for this experiment.

Program G*Power 3.1.7 (54):

Effect size f = 0.8

α error probability = 0.05

Power (1-β error probability) = 0.80

Number of groups = 2

Total sample size = 14

Inclusion criteria:

Male C57BL/6 mice aged 8 weeks

Exclusion criteria:

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

-Death during the experiment.

7.1.2 Drug

Tofacitinib purchased from Abmole® Bioscience (Houston, U.S.A.), and dissolved in dimethyl sulfoxide (DMSO) into 2% concentration, will be used in compare to the solution vehicle (DMSO alone) as negative control.

7.2 Research Design

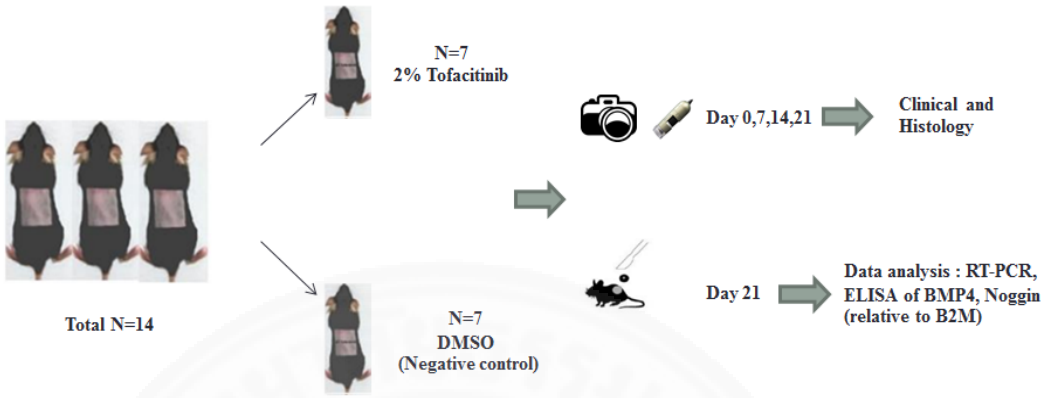


Figure 7.1 Outline of experiment

Experiment was performed an investigation with eight week-old mice which are in telogen phase. Fourteen wild type male C57BL/6 mice were divided into two groups (each group consisted of 7 mice). Mice were shaved on the dorsal back area (size 3x3 cm, position below scapular) and treatment compared between application of 2% topical tofacitinib (n=7), and topical DMSO (vehicle control group), (n=7) for 21 consecutive days. Tissue from the dorsal back after treatment will be collected by punch biopsy 6 mm in diameter after day 21. A digital image of the coated area will be weekly recorded until three weeks. The ratio of hair regrowth will be done using Dino-Lite microscope (AM7013MZT (R4) Series) at 40x magnification.

7.3 Outcome measurement

7.3.1 Hair growth evaluation

Digital photograph will be taken weekly by using Sony® DSC-RX100M3 (20.1 megapixel) for evaluation of hair growth. The percentage in area of hair regrowth was analyzed by Adobe Photoshop CS6 software (Adobe System Incorporated, U.S.A.). The mean length of hair re-growth will be measure by using

Dino-Lite microscope (AM7013MZT (R4) Series, AnMo® Electronic Corporation, Taiwan) at 65x magnification.

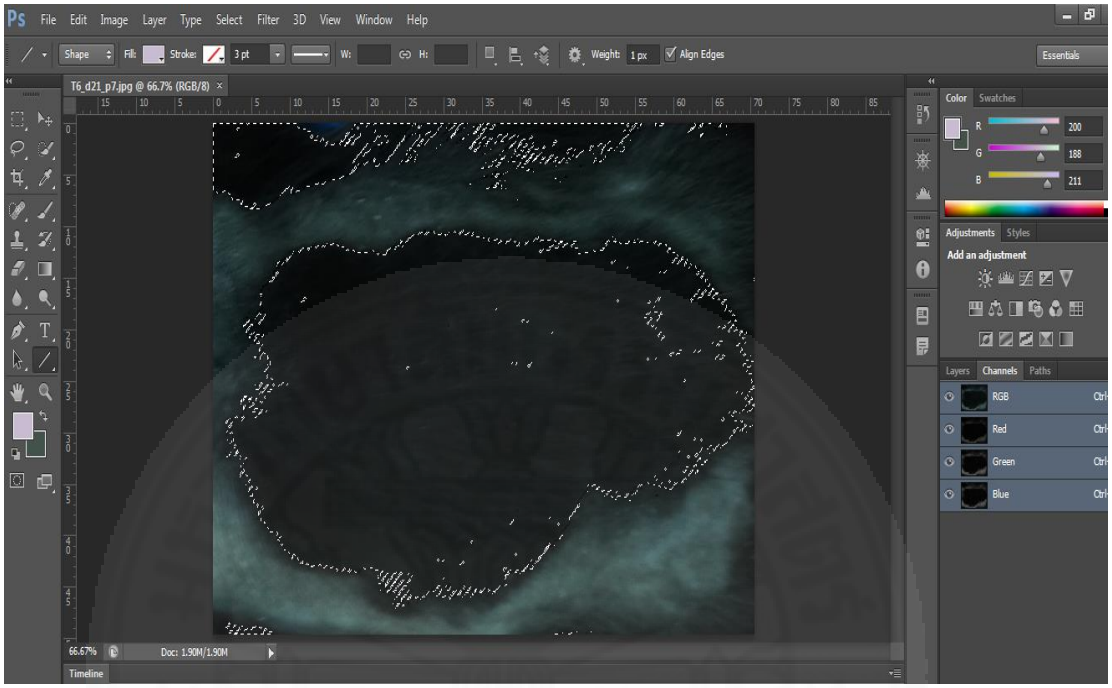


Figure 7.2 Example in evaluation of area percentage in hair regrowth using Adobe Photoshop CS6 software (Adobe® System Incorporated, U.S.A.)

7.3.2 Histopathological evaluation

At day 21 of experiment, tissue for dorsal back will be collected by punch biopsy (6 mm in diameter) after anesthesia with isoflurane. Tissue samples were fixed in 10% buffered formalin for 24 hours and embedded in 3-µm paraffin wax sections and stained with hematoxylin and eosin (H&E) compared between 2% topical tofacitinib and DMSO (negative control) groups.

Ratio of anagen hairs were evaluated in percentage compare to other hair stage. Interfollicular epidermis thickness and length of hair infundibulum were evaluated under x4 and x10 objective at a Leica® DM2000 microscope. Tissue histology was taking at least 5 photographs per samples and evaluated by veterinary pathologist.

7.3.3 Quantitative real-time polymerase chain reaction (RT-PCR)

A sample of mice hair follicle were collected by punch biopsy (6mm) and stored at -80 C. In RNA isolation, we followed the protocol of RNeasy® Mini Kit (QIAGEN, Hilden, Germany). RT-PCR analysis was performed on a BIO-RAD Real-Time PCR system (BIO-RAD®, California, U.S.A.). As listed.

7.3.3.1 After tissue samples were removed from the -80 refrigerator and brought into the porcelain mortar and pestle. Liquid nitrogen was directly put into each sample. After freezing, tissue samples were mashed into small pieces and transfer to micro-centrifuge tube.

7.3.3.2 Disrupt the tissue and homogenize the lysate in 600µL buffer RLT.

7.3.3.3 Pipet the lysate and placed into a 1.5 ml collection tube then centrifuge for 2 min at full speed.

7.3.3.4 Transfer supernatant into new micro-centrifuge tube and add 600µL of 70% ethanol to the clear lysate and mix immediately by pipetting.

7.3.3.5 Transfer 700 µL of the sample to an RNeasy spin column in a 2ml collection tube then centrifuge at 10,000 rpm for 15 seconds. Discard the flow-through

7.3.3.6 Add 700µL buffer RW1 to the RNeasy spin column then centrifuges at 10,000 rpm for 15 seconds. Discard the flow-through.

7.3.3.7 Add 500µL buffer RPE to the RNeasy spin column then centrifuges at 10,000 rpm for 15 seconds. Discard the flow-through.

7.3.3.8 Add 500µL buffer RPE to the RNeasy spin column then centrifuges at 10,000 rpm for 15 seconds.

7.3.3.9 Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30µL RNase-free water directly to the spin column membrane then centrifuge at 10,000 rpm for 60 seconds to elute the RNA.

7.3.3.10 Primers and Probes for mice BMP4 (qMmuCEP0054665) and noggin (qMmuCEP0058332) were purchased from Bio-Rad® (California, U.S.A.). Data were calculated relatively to expression of Beta-2-microglobulin (B2M) (qMmuCIP0042770) as internal control gene by using standard exponential curve.

One step RT-PCR was performed by using the ImProm-II® Reverse Transcription system. Using the component of mixture as listed.

-2x Probe RT-PCR master mix 10µL

-QN Probe RT mix 0.2µL

-Primer mice BMP4 (Mm6813) FAM 1 1µL or Primer mice Noggin (Mm135266) HEX 1 1µL

-Primer mice B2M (Mm163) Cy-5 1 1µL

-Isolated RNA

-RNase free water

7.3.3.11 RT-PCR was performed by using C1000 thermal cycler BIO-RAD CFX96 Real-time system (BIO-RAD, Hercules, California, U.S.A.). Data were calculated relatively to expression of reference gene Beta-2-microglobulin (B2M).

7.4 Data analysis

Results were recorded as mean ±SD. The independent sample t test was performed to analyze differences between data obtained from each groups. For non-parametric data, Mann Whtiney U test was performed to analyze the data. A *P*-value of less than 0.05 was considered statistically significant. All statistical analyses will be performed out using IBM SPSS® version 22.

CHAPTER 8
RESULTS

8.1 Hair growth

On day 0, 7, 14, and 21, after inhaled with isoflurane, photographs of experimental area in each group (tofacitinib-treated and DMSO-treated) have taken by 20.1 mega pixel digital camera (DSC-RX100M3, Sony Corporation, Japan). The percentage in area of hair regrowth was analyzed by Adobe Photoshop CS6 software (Adobe System Incorporated, U.S.A.). Hair growth rate was analyzed under 65x Dino-Lite microscope model AM7013MZT (R4) (AnMo Electronics Corporation, Taiwan).

8.1.1 Tofacitinib treatment induce higher percentage area of hair regrowth than DMSO-treated group

At the starting point of the experiment, there is insignificantly different in mean percentage of remnant hair after shaved (tofacitinib-treated 10.47%, DMSO-treated 9.56%, P=0.794) which assume that the study reveal approximately same start point for hair regrowth evaluation between two groups. Independent sample t test was used to analyze the data. On day 7 of the experiment, tofacitinib-treated mice demonstrated initiation of hair growth area while DMSO-treated did not (tofacitinib-treated 18.11%, DMSO-treated 9.64%, P=0.019). Furthermore, on day 14 of experiment, tofacitinib-treated group revealed significant acceleration of hair regrowth while DMSO-treated group showed lesser area of hair regrowth (tofacitinib-treated 52.36%, DMSO-treated 12.88%, P=0.003). At the end of experiment on day 21, tofacitinib-treated mice showed almost full area of hair regrowth on dorsal back while DMSO-treated, as vehicle control, result in less than half area of hair regrowth (tofacitinib-treated 90.11%, DMSO-treated 37.82%, P=0.001). Data represent in Table 8.1 and Figure 8.1.

Table 8.1 Mean percentage area of hair regrowth compare between tofacitinib and DMSO-treated group. Data analyzed by independent sample t-test.

	Tofacitinib (n=7)	DMSO (n=7)	p-value
Day 0	10.47%	9.56%	0.794
Day 7	18.11%	9.64%	0.019
Day 14	52.36%	12.88%	0.003
Day 21	90.11%	37.82%	0.001

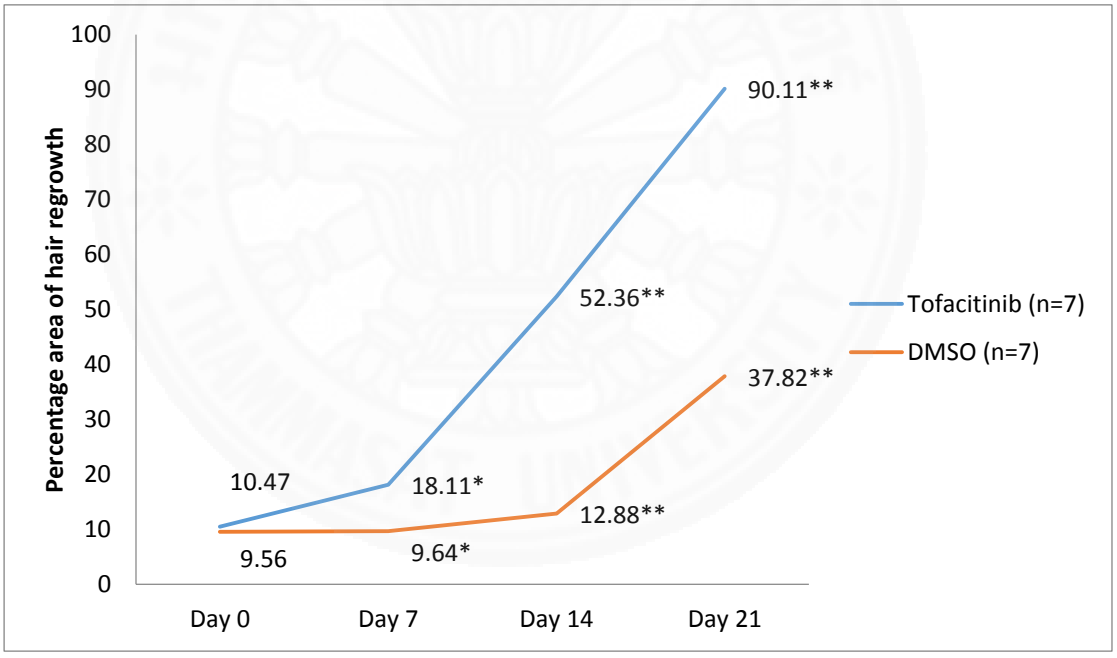


Figure 8.1 Represent percentage in area of hair regrowth comparison between tofacitinib- and DMSO-treated groups,* $P < 0.05$, ** $P < 0.01$.

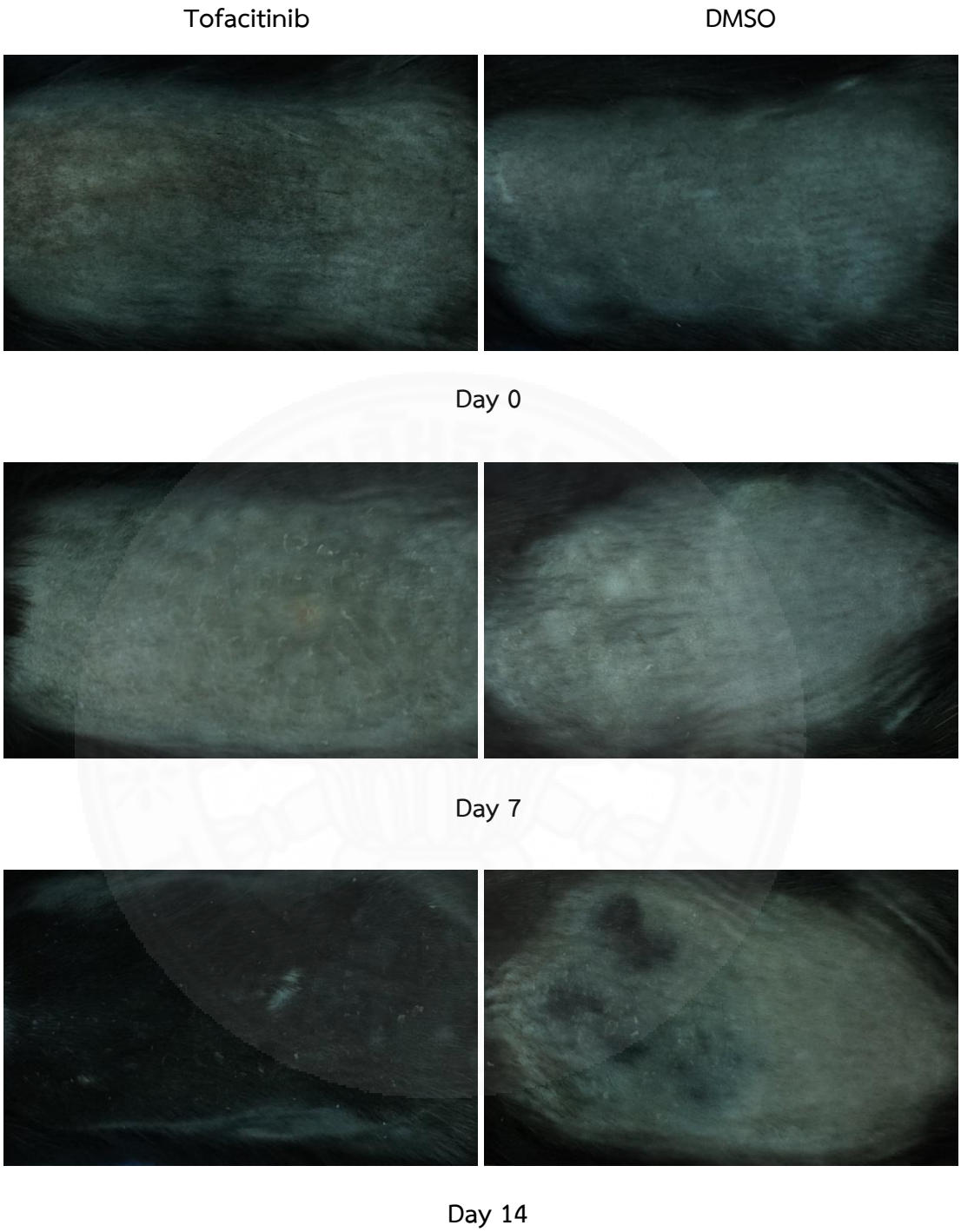
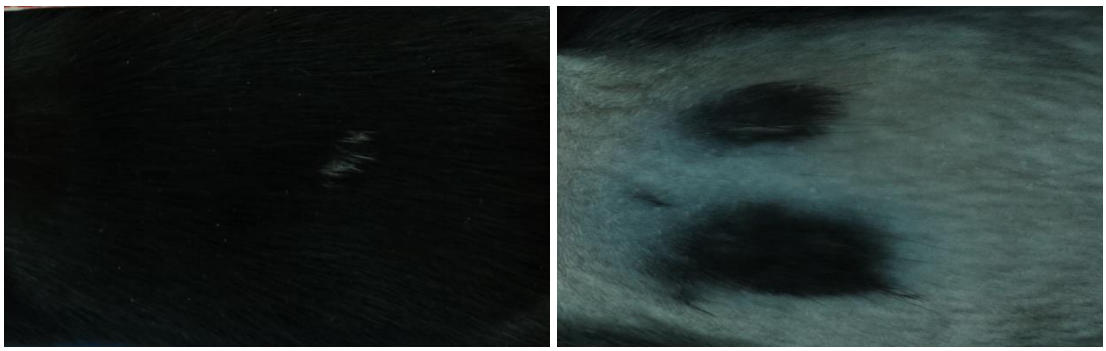


Figure 8.2 Pictures of hair regrowth compare between tofacitinib- and DMSO-treated mice.



Day 21

Figure 8.2 Pictures of hair regrowth compare between tofacitinib- and DMSO-treated mice

8.1.2 Tofacitinib accelerate hair growth rate greater than DMSO

The study evaluated hair regrowth by using Dinolite microscope at x65 magnification. Hair regrowth rate was measured in unit of millimeter during day 0, 7 14 of experiment, compare between tofacitinib-treated and DMSO-treated mice. Due to marked hair regrowth in tofacitinib-treated group on day 21 of experiment, measurement of hair length from microscopic evaluation cannot be performed. At the starting point of the experiment, there is insignificantly different in mean of hair length (tofacitinib-treated 0.18614 mm, DMSO-treated 0.18486 mm, P-value=0.88) which assume that the study reveal approximately same start point for hair length evaluation between two groups. Independent sample t test was used to analyze the data. On day 7 of the experiment, tofacitinib-treated mice showed significant higher in hair length compare to vehicle control group (tofacitinib-treated 0.32257 mm, DMSO-treated 0.26714 mm, P-value=0.001). Interestingly, on day 14 of experiment, topical tofacitinib-treated mice exhibit obviously higher in hair length compare to DMSO-treated mice (tofacitinib-treated 0.93286 mm, DMSO-treated 0.37714 mm, P-value=0.001), as shown in Table 8.2 and Figure 8.3.

Table 8.2 Mean length of hair (mm) compare between tofacitinib and DMSO-treated group. Data analyzed by independent sample t-test.

	Tofacitinib (n=7)	DMSO (n=7)	p-value
Day 0	0.18614	0.18486	0.88
Day 7	0.32257	0.26714	0.001
Day 14	0.93286	0.37714	0.001

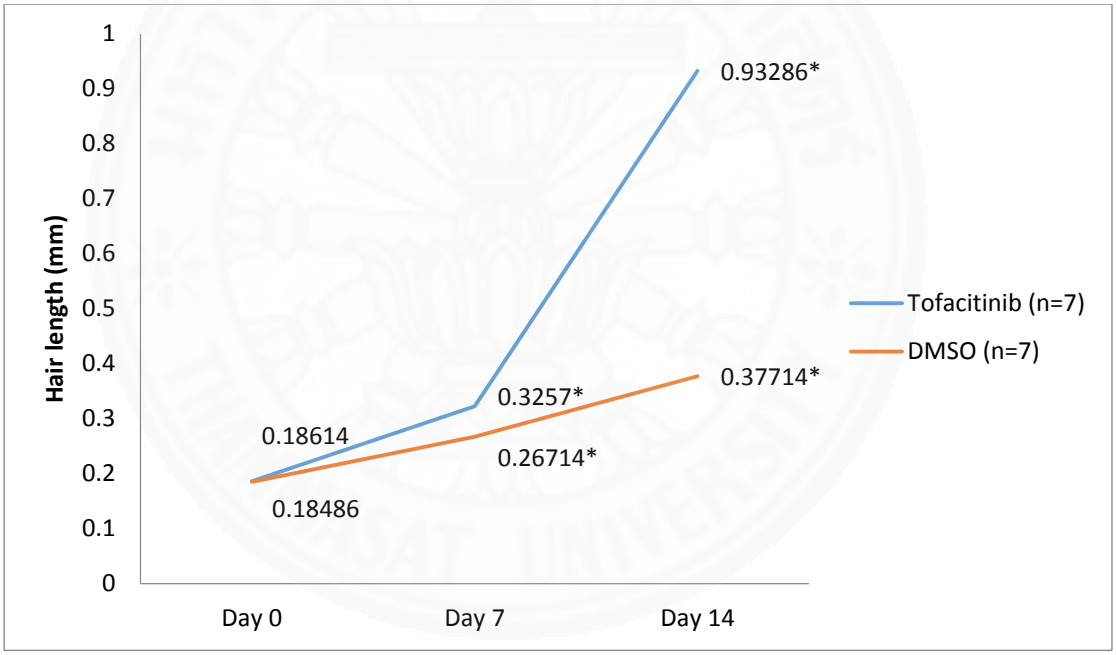


Figure 8.3 Represent percentage in area of hair regrowth comparison between tofacitinib- and DMSO-treated groups, *$P < 0.01$

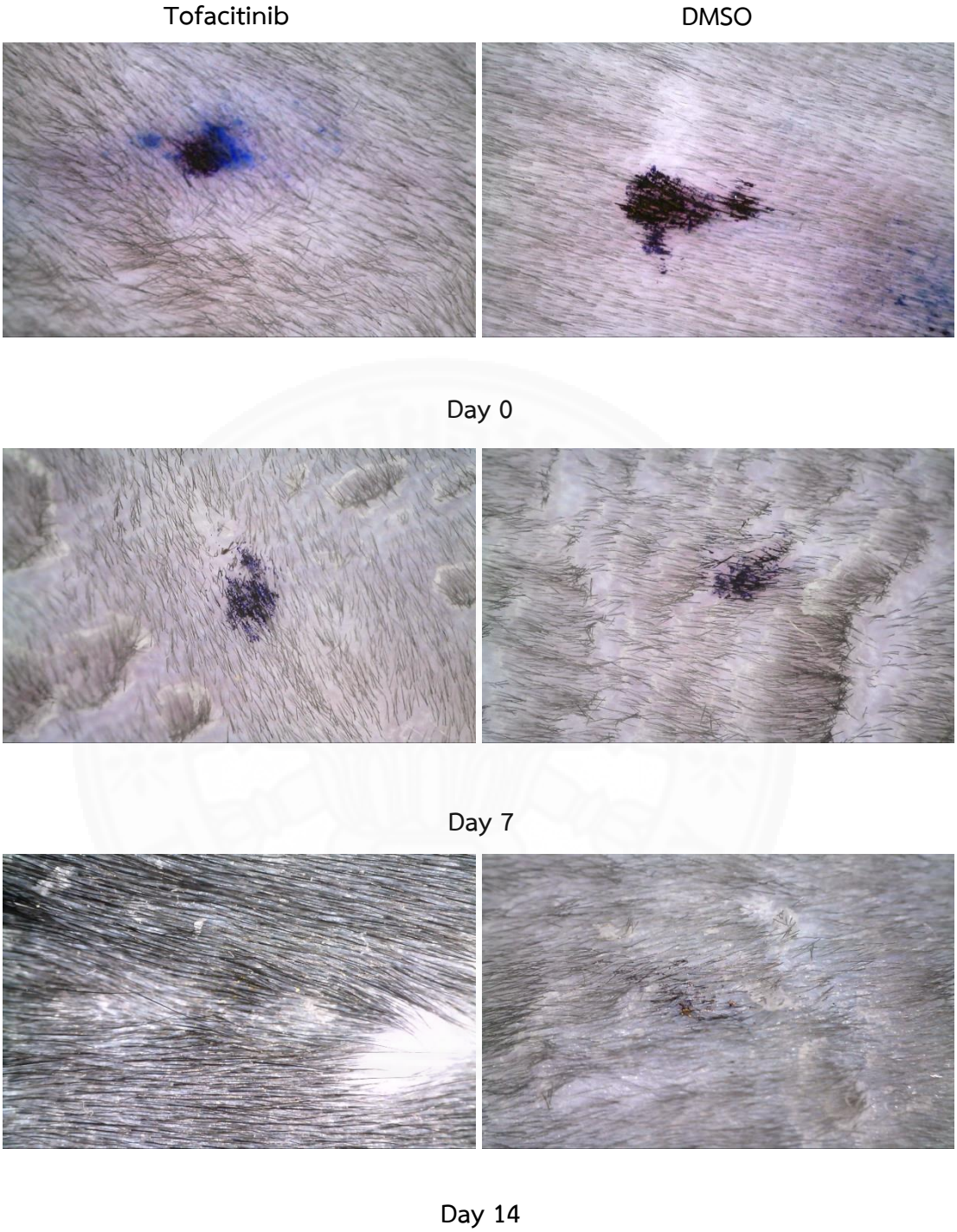
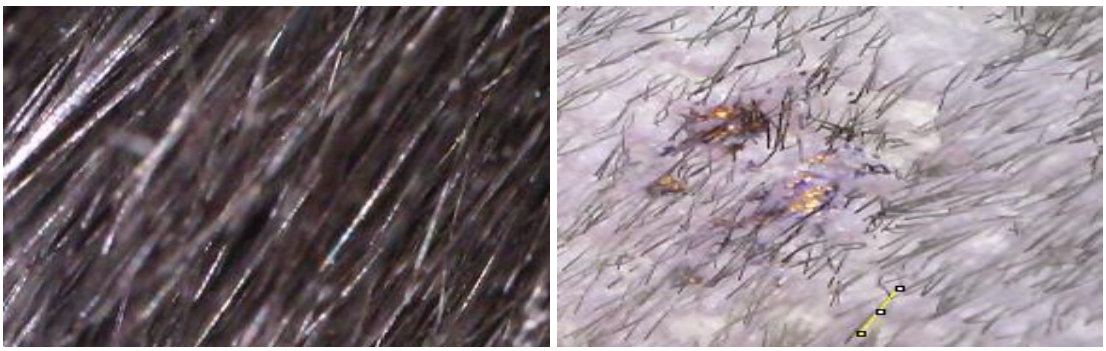


Figure 8.4 Dino-lite digital microscopic pictures of hair regrowth compare between tofacitinib- and DMSO-treated mice at x65 magnification.



Day 21

Figure 8.4 Dino-lite digital microscopic pictures of hair regrowth compare between tofacitinib- and DMSO-treated mice at x65 magnification

8.2 Histopathology

At the end of experiment tissue samples were took from mice by 6mm punch biopsy and fixed in paraffin section. After stained with hematolysin and eosin, histopathological sections were evaluated under Leica® DM2000 4x and 10x microscope. Tissue histology was taking at least 5 photographs per sample for analysis.

8.2.1 DMSO-treated group

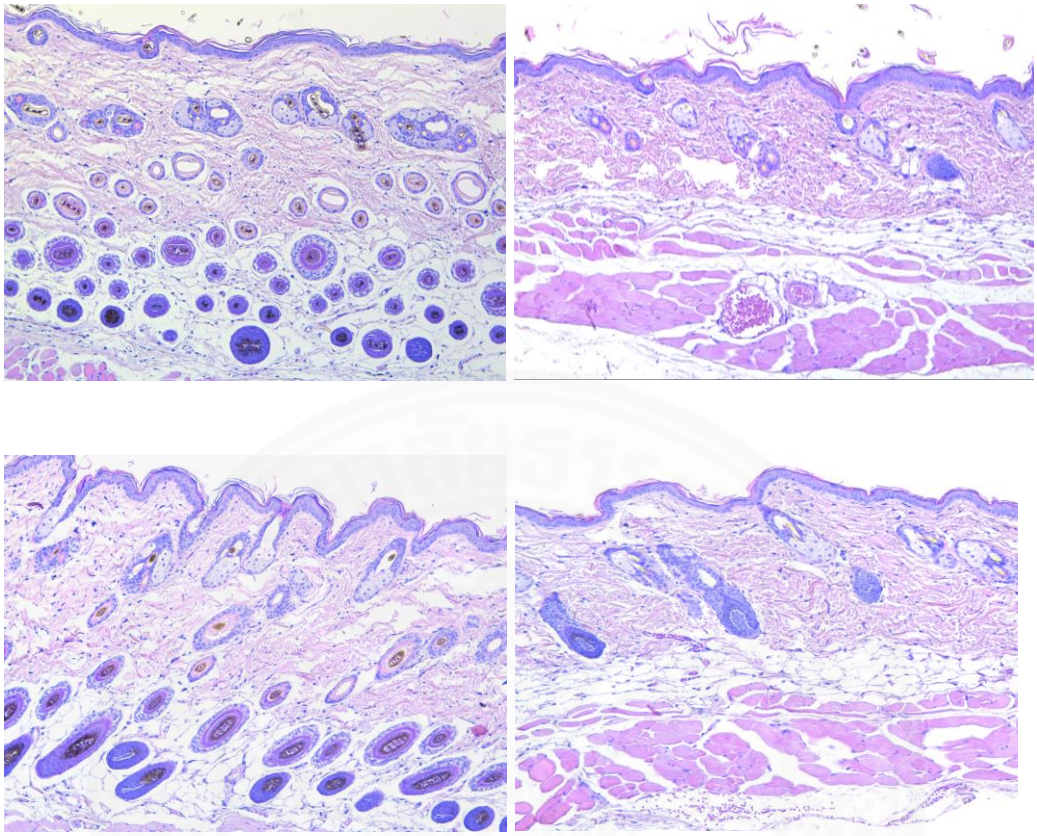


Figure 8.5 Histology of DMSO-treated mice on day 21, observed from microscope under x10 magnification.

Microscopic findings of DMSO-treated group

- Well epithelialization with keratinization
- Moderate organization of granulation, abundant collagen bundles, mild interstitial edema and moderate mononuclear cells infiltration
- New capillaries in dermis and hypodermis
- Increased interfollicular epidermis (IFE) thickness

8.2.2 Tofacitinib-treated group

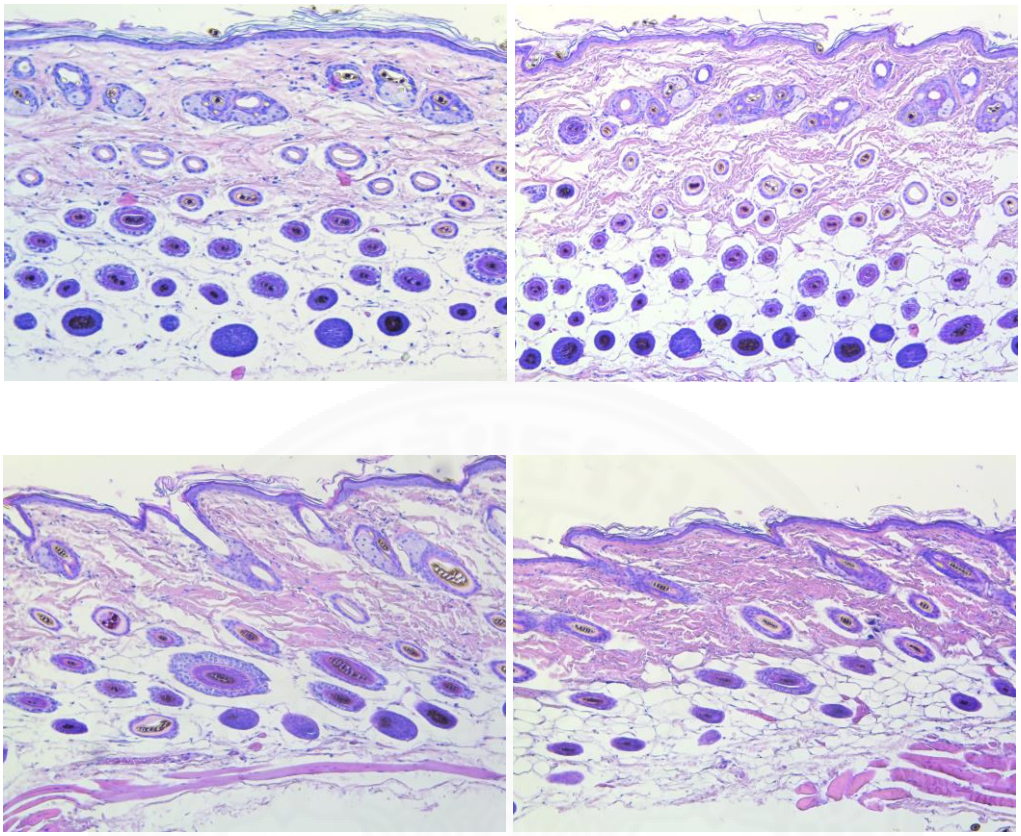


Figure 8.6 Histology of tofacitinib-treated mice on day 21, observed from microscope under x10 magnification.

Microscopic findings of DMSO-treated group

- Moderate epithelialization with keratinization
- Moderate organization of granulation, abundant collagen bundles, moderate interstitial edema and mild mononuclear cell infiltrations in dermis
- New capillaries in hypodermis

8.2.3 Tofacitinib treatment induce higher number of hair follicles

After the end of experiment (day 21), topical tofacitinib treated group represent higher number of hair follicle compare to vehicle control group. The evaluation of hair follicle number was performed per unit area of 6mm in each sample. In tofacitinib-treated mice demonstrate significantly higher in mean number of hair follicle (31.4, n=7) than DMSO-treated group (21.2, n=7)

Table 8.3 Independent t-test analysis demonstrated mean number of hair follicle per 6mm on day 21 of experiment compare between tofacitinib and DMSO-treated mice.

	Tofacitinib (n=7)	**DMSO (n=7)**	**P-value**
Mean number of hair follicle	31.40 (6.0962, 56.7038)	21.20 (4.9535, 37.4464)	0.02

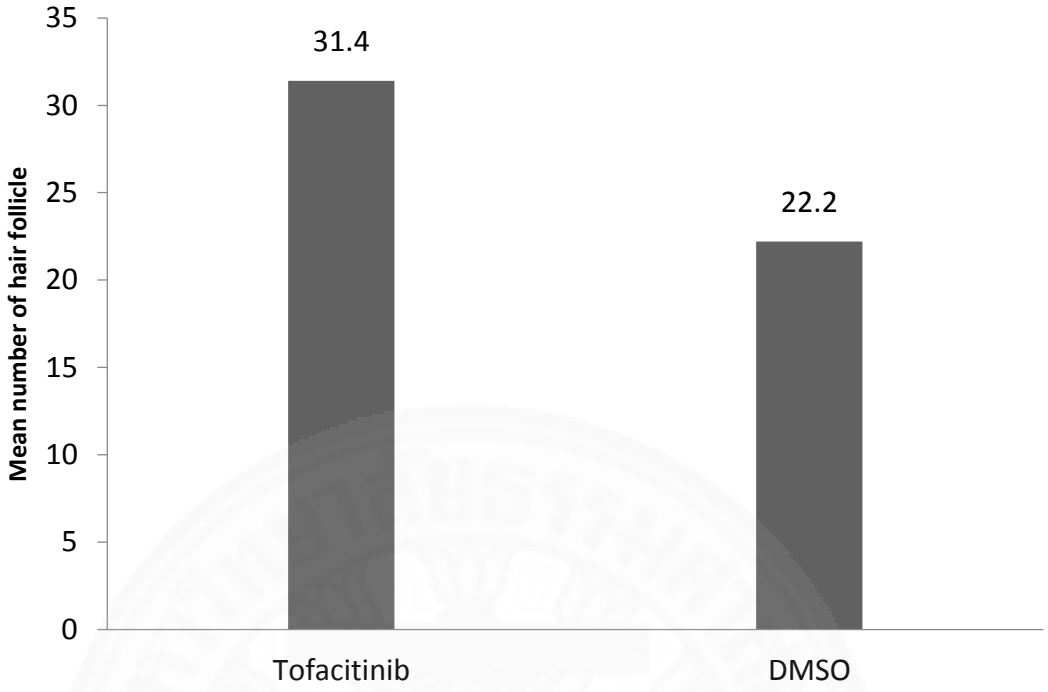


Figure 8.7 Represent of mean number of hair follicle per 6mm at the end of experiment (day 21) between tofacitinib-treated group and DMSO-treated group.

8.2.4 Tofacitinib treatment induce increase anagen hair

After the end of experiment (day 21), topical tofacitinib-treated mice showed higher ratio of anagen hair than DMSO-treated mice. In topical tofacitinib-treated group mostly found anagen hair (n=72, 84.7%), catagen (n=4, 10.6%) and telogen (n=4, 4.7%) whereas late anagen were in deep dermis and hypodermis follow by early anagen in superficial dermis. In contrast to DMSO-treated mice, found lower percentage of anagen hair (n=18, 62%) follow by telogen hair (n=11, 38%) with no catagen respectively, as shown in Figure 8.8.

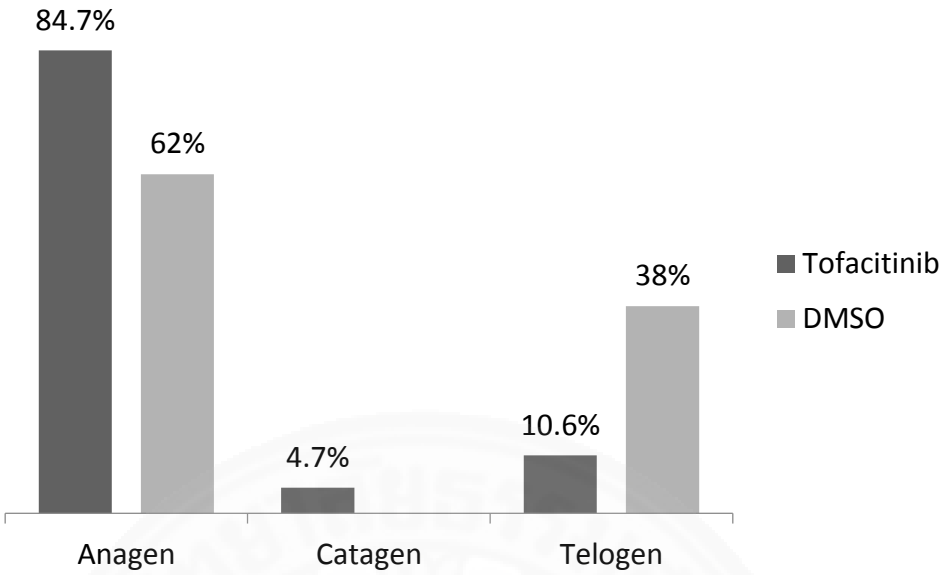


Figure 8.8 Represent in percentage in each stage of hair, comparison between tofacitinib and vehicle control. Tofacitinib treatment induce higher ratio of anagen hair.

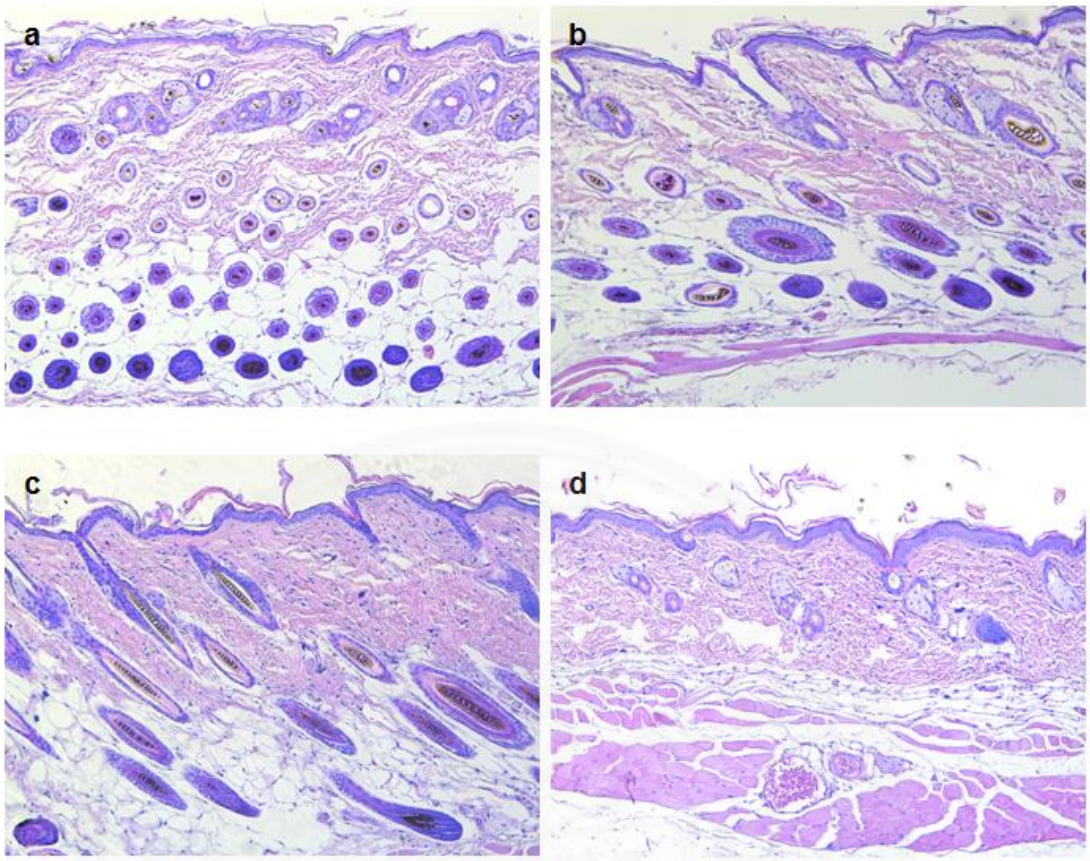


Figure 8.9 Tofacitinib treatment induce higher ratio of anagen hair. (a, b) Images of H&E staining from tofacitinib-treated mice in different section under 10 x magnifications. (c, d) Images of H&E staining from DMSO-treated mice in different section under x10 magnification.

8.2.5 Tofacitinib treatment induce increase length of hair infundibulum but not interfollicular epidermis thickness

After the end of experiment (day 21), length of hair infundibulum was increased in topical tofacitinib-treated groups (fig 8.10) and was relatively increased in anagen compared to other hair stages (fig 8.11a). Interfollicular epidermis (IFE) thickness was relatively increased in topical DMSO treatment (fig 8.11b).

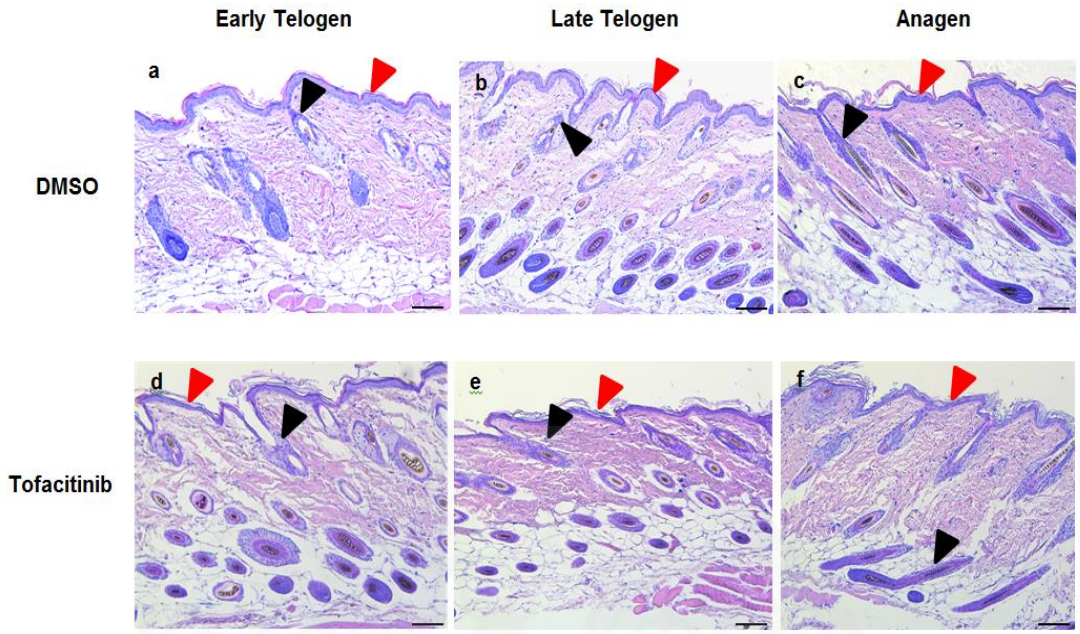


Figure 8.10 Thickness of IFE (red arrow) and Infundibulum length (black arrow) on H&E staining under x10 microscope. Scale bar: 100 µm.

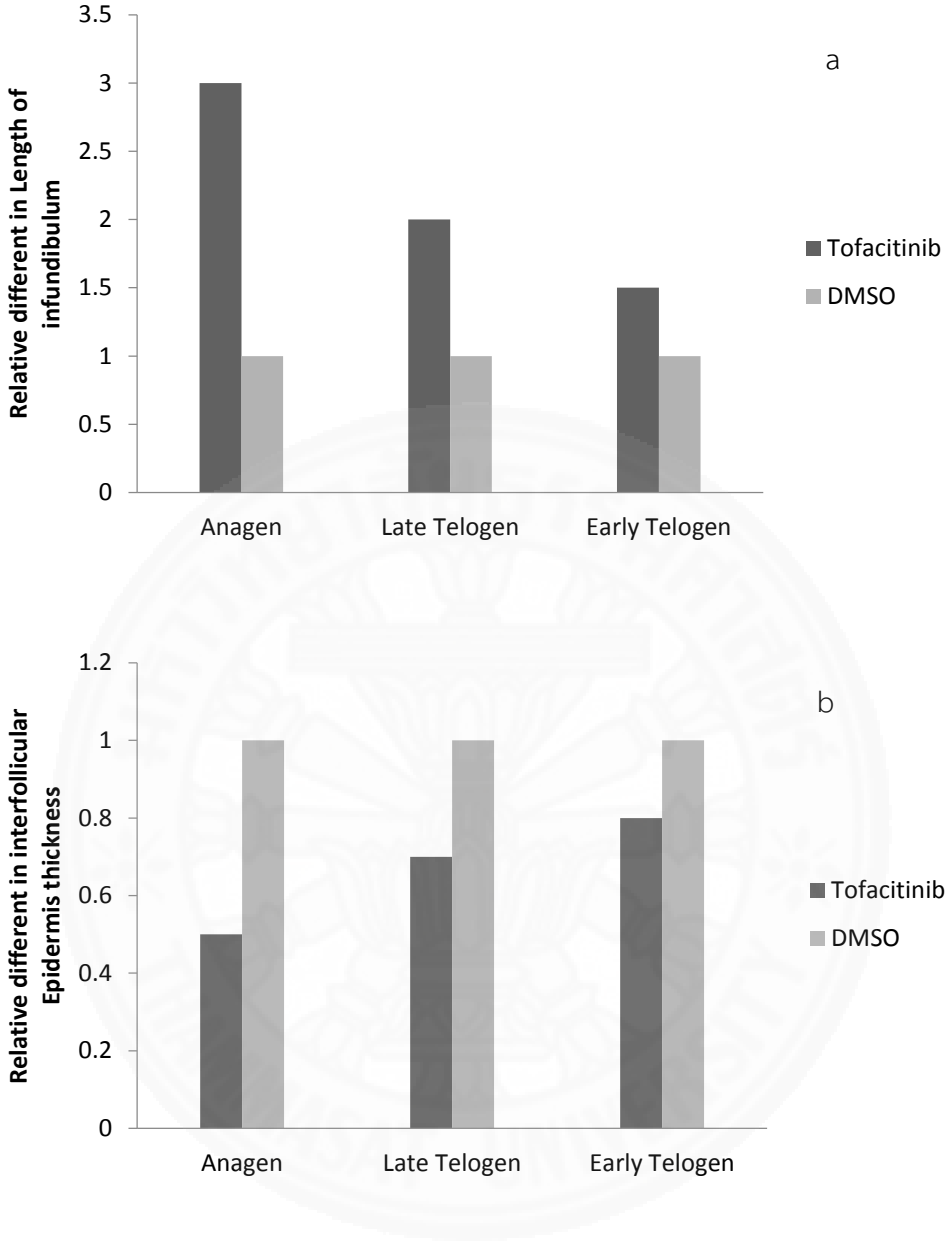


Figure 8.11 (a) Relative different in length of indundibulum and (b) relative different of interfollicular epidermis thickness between tofacitinib- (n=7) and DMSO- (n=7) treated group. Observed from H&E staining histology section under x10 microscope.

8.2.6 Tofacitinib treatment induce increase in proliferation of vascular structure and decrease of inflammatory cell infiltration

At the end of experiment, Topical tofacitinib-treated group also resulted in lesser inflammatory cell infiltration mononuclear cell were mainly observed (fig 8.12a) and greater proliferation of vascular structure than DMSO-treated group (fig 8.12b).

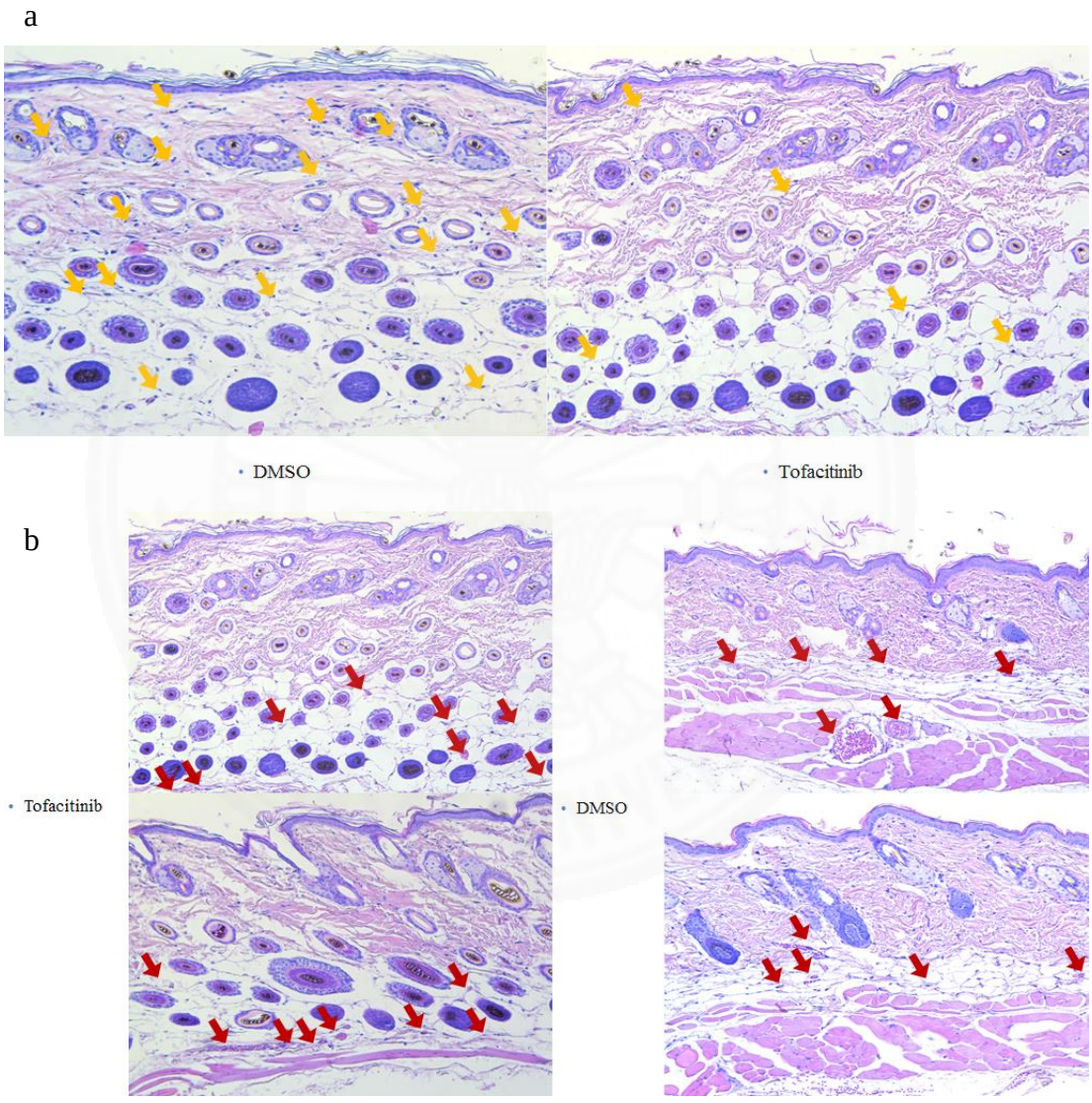


Figure 8.12 (a) Inflammatory cell infiltration (yellow arrow) (b) vascular structure (red arrow) between tofacitinib- (n=7) and DMSO- (n=7) treated group. Observed from H&E staining histology section under x10 microscope.

Table 8.4 Comparison of histology between DMSO- (n=7) and tofacitinib-treated (n=7) groups.

Histologic Criteria	DMSO (7)	Tofacitinib (7)
Angiogenesis	Moderated found newly formed capillaries	Mostly found newly formed capillaries
Inflammatory cells	Moderated found mononuclear inflammatory cells infiltration in dermis	Mild inflammatory cells infiltration in dermis but found in perivascular
The thickness of the inter follicular epidermis (IFE)	Found an increase interfollicular epidermis thickness (mild to moderate)	Not found an increased interfollicular epidermis thickness

8.3 BMP4 and Noggin analysis

8.3.1 BMP4

In order to analysis of cell growth factor mRNA expression, this study performs RT-PCR procedure. In DMSO-treated group (n=5), the study has loss two appropriate samples for RT-PCR analysis due to failure to express internal control molecule (B2M) of the sample.

For independent sample between tofacitinib-treated and DMSO-treated mice, this study used non-parametric Mann-Whtiney U method to analyze statistical data. Interestingly, against to the study hypothesis, mRNA expression of BMP4 was significantly higher in tofacitinib-treated group (8.29, n=7) than DMSO-treated group (3.60, n=6), P=0.042, (fig 8.13).

Table 8.5 Represent the mean of BMP4 mRNA expression evaluated from RT-PCR. Data obtained from Mann-Whitney U test. *P < 0.05*

Sample	n	Mean	P-value
Tofacitinib-treated	7	8.29	0.042
DMSO-treated	5	3.60	

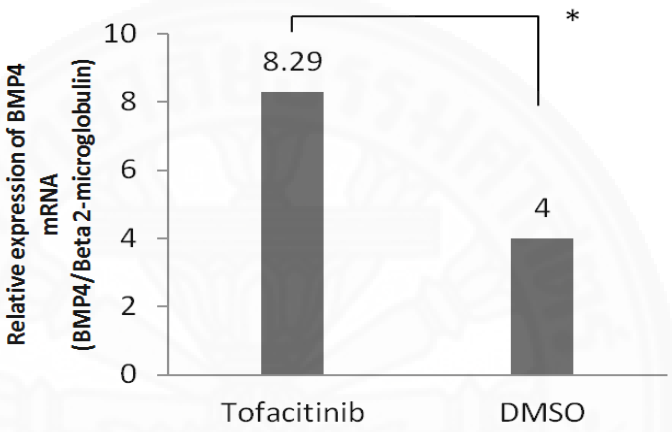


Figure 8.13 Result form qRT-PCR data show relative BMP4 mRNA expression. **P < 0.05*

8.3.2 Noggin

In tofacitinib-treated group, the study has loss one appropriate sample for noggin mRNA analysis due to the low level of mRNA. On the other hand, In DMSO- treated group (n=5), the study has loss two appropriate samples for RT-PCR analysis due to failure to express internal control molecule (B2M) of the sample

Correspond to the hypothesis, non-parametric Mann-Whtiney U method shows significantly upregulation of noggin mRNA in tofacitinib-treated group (mean=8, n=6) greater than DMSO-treated group (mean=3.6, n=5), *P*=0.028, (fig 8.14).

Table 8.6 Represent the mean of Noggin mRNA expression evaluated from RT-PCR. Data obtained from Mann-Whitney U test. *P < 0.05*

Sample	n	Mean	P-value
Tofacitinib-treated	6	8.00	0.028
DMSO-treated	5	3.60	

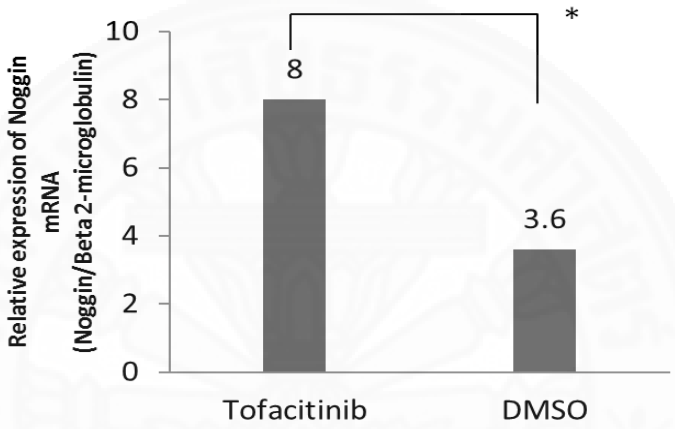


Figure 8.14 Result form qRT-PCR data show relative BMP4 mRNA expression, **P < 0.05*

CHAPTER 9
DISSCUSSION

Hair loss disorders are still highly concerning problem among worldwide population. Many are cause by failure to entry the anagen phase and arrested in telogen phase including non-scarring alopecia. Androgenic alopecia (AGA) is one of the non-scarring alopecia which characterized by pattern of miniaturization hair loss can be found with high prevalence rate since fourth decade of life causing effect on quality of life, stressfulness, low self-esteem [40, 86]. It has believed to be result from genetically process [28], androgen stimulated [54], shortening of duration of anagen and premature entry into catagen by decrease anagen maintaining factors [97]. Currently, U.S. FDA has approved two standard treatments for AGA which is oral finasteride and topical solution of minoxidil but clinical success rate is still limited with problems on adverse event leading to drop out of patients. 2% topical tofacitinib was used in the study due to widely used of this concentration in many clinical trials of dermatological studies. This concentration has been shown efficacy in treatment of many dermatological diseases such as atopic dermatitis and psoriasis without serious adverse event [11, 93] . This study conducted the experiment to examine the efficacy of topical tofacitinib for promoting hair growth in mice in terms of clinical presentation and histopathology. Furthermore, this study has demonstrated impact of the drug on BMP4 and noggin, the important molecules involved in onset of growth phase in hair cycle. This study also aimed to suggest a new possible mechanism of tofacitinib in treatment of hair loss disorder.

The experiment was performed in mice tissue aged 8 weeks second hair cycle occurred [99]. Our experimental method, shaved C57BL/6 wild type male mice, could be represent model of non-scaring alopecia including alopecia areata which results from prolonging of telogen phase [3, 90] and androgenetic alopecia which results from inability to reenter the anagen phase [41, 90]. The author has hypothesized that if topical tofacitinib treatment resulted in premature onset of anagen it might solve the problem of many diseases in non-scaring alopecia. All mice tissues have been in early phase of telogen when the experiment was started [82]. After

treatment with topical tofacitinib and DMSO once daily for 21 consecutive days, topical tofacitinb-treated mice show higher in ratio of hair regrowth which almost full area of the dorsal back while DMSO-treated group observed just partial hair regrowth area. Interestingly, the rate of hair regrowth observed from Dino-Lite microscope was acceralated in tofacitinib-treated mice greater than vehicle control group. Moreover, the histopathological data show that topical tofacitinib-treated group has shown significantly increase in number of hair follicle compare to vehicle control group. According to previous studied, normally mice in this period of time at the end of experiment, without any intervention, will represent hair in late telogen phase but not anagen [82]. Interestingly, the result has shown that topical tofacitinib-treated mice represent higher percentage of anagen hair while telogen is still commonly observed in DMSO-treated group indicated that topical tofacitinib induced premature onset of anagen in mice model. Topical tofacitinib treatment also show increase in length of infundibulum greater than DMSO treatment which marked observe in anagen hair indicated the entry of growth phase in hair cycle. Correspond with previous study, this study also observed the proliferation of vascular structure, without telangiectasia observed from clinical apperance, in topical tofacinib-treated group which is another evidence supporting that topical tofacitinib induce expression of VEGF [75]. After treated with topical DMSO, dorsal skin of mice samples have shown erythematous scaly patches which indicated the irritative skin side effect of DMSO. Correspond to histopathological result, DMSO-treated group also show the increase of inflammatory cell infiltration and interfollicular epidermis thickness, revealed a process of skin inflammation which correspond to Wright, E.T. *et al*. [128] studied. Interestingly, 2% topical solution of tofacitinib, which dissolved in DMSO as vehicle-treated mice showed normal skin presentation during entire of the experiment with lesser inflammatory cell infiltration and without increase of interfollicular epidermal thickness. According to our observation, this study suggest another evidence that topical application of JAK-STAT inhibitor can reduce inflammation process, eventhrough counter inflammatory effect of DMSO, in mice skin model which may benefit in treatment of inflammatory hair loss disorder include alopecia areata and may reduce hair follicle micro-inflammation which played a role in pathogenesis of androgenetic alopecia [72].

Many previous studies have reported that tofacitinib can promote hair growth which involved in multifactorial mechanism. Tofacitinib was believed to promote hair regrowth in patient with alopecia areata by inhibition of an interferon (IFN) and cytotoxic T lymphocyte (CTL) function [51, 129]. Futhermore, tofacitinib was show to upregulated many molecule that initiate onset of anagen including LEF1, a key regulators of the wingless (Wnt) signaling pathway, and sonic hedgehog (Shh) [41] which is known to be the central pathway for anagen initiation [20, 126]. Sivan Harel *et al*. also show that topical tofacitinib resulted in activation of hair follicle pregenitor cells by presence of Edu^{+} (proliferating) cells within the hair germ (P-$cadherin^{+}$) compartment [41]. Moreover they investigate the relationship of JAK-STAT pathway and hair cycle. Immunofluorescence study has shown that activated (phosphorylated) Stat3 is highly express in dermal papilla during catagen and telogen but repress in anagen phase [41]. Sivan Harel *et al*. next investigate genes which up-regulated by tofacitinib treatment and the result shown that TGF-β pathway, BMP pathway, and NOTCH pathway were upregulated by tofacitinib.

According to previous study, topical tofacitinib-treated mice resulted in stimulation of hair growth, induction of anagen phase, and promote angiogenesis [75]. In finding a new possible mechanism by which topical tofacitinib regulated molcules involved in onset of anagen, we analyzed the expression of two growth factors. Noggin which is known to be stimulating factor [13] and BMP4 which is known to be inhibitory factor [12] of anagen entry. To our surprise, these two molecular factors were both significantly upregulated by topical tofacitinib. Our experiment demonstrated that topical application of tofacitinib show significantly upregulated expression of noggin, the molecule which initiate onset of anagen [12], compared to DMSO in mice model. Noggin is crosstalk to many molecules which upregulated from tofacitinib treatment. Epithelial Shh maintain dermal papilla function via noggin to drive hair follicle morphogenesis [126], here we suggest that topical tofacitinib might upregulate expression of noggin and resulted in upregulate expression of Shh. Furthermore, previous study has described that noggin treatment improved ischemic brain tissue by increase level of VEGF in mice gila cell [114]. Correspond to our previous study [75], we suggest that topical tofacitinib treatment might prior induced

noggin expression which subsequence into induction of VEGF and result in proliferation of vascular structure in mice skin and hair follicle.

On the other hand, the onset of anagen occur from downregulation and inhibitory effect of BMP4 [13]. BMP4 is also crosstalk with other important molecules in hair follicle including Wnt/β-catenin pathway [66]**.** In initiation of anagen, β-catenin activity is activated by Wnt signal and suppressed by BMP4 [66]**.** Correspond to Sivan Harel *et al*. studies which demonstrated upregulation of BMP6, which is known to function in hair follicle growth and development, by tofacitinib. This study also found that topical application of tofacitinib also results in significantly upregulated expression of BMP4 which is known to function as inhibitory molecule in onset of anagen. To explain how tofacitnib results in onset of anagen and roburst of hair regrowth in this condition which BMP4 was upregulated. Zimmerman, L.B. *et al* explained that noggin is a potent inhibitor of BMP4 and act as an antagonized molecule with high affinity to BMP2/BMP4 and low affinity to BMP7 [134]. On the other hand, to explain how BMP6 still support hair follicle growth in this condition which noggin was upregulated. Song K *et al*. demonstrated the experiment which shown that BMP6 was resistance to noggin inhibition and noggin was fail to play a role as negative feedback regulator of BMP6 [115]. This study suggest that topical tofacitinib upregulate expression of noggin might overided and antgonized the effect of BMP4 but not BMP6 subsequence in hair growth.

CHAPTER 10
CONCLUSIONS AND RECOMMENDATIONS

10.1 Conclusion

Inhibition of JAK-STAT pathway by tofacitinib has promise a new hope in treatment of many diseases. Many ongoing clinical trials are showing efficacy of this drug in treatment of atopic dermatitis [10], vitiligo [23, 69], psoriasis [1, 38, 63], alopecia areata [56, 129]. Topical tofacitinib promote entry of anagen phase, hair regrowth compared to minoxidil, DMSO as vehicle control seen from clinical and histopathology results [75]. This study try to investigate new possible molecular effect of tofacitinib for promoting hair growth and more evidence which indicated the growth phase in clinical and histopathology. For the first time, we demonstrate that tofacitinib might initiate onset of anagen by stimulate expression of noggin which overide effect of BMP4 (fig 10.1). On the other hand, upregulation of noggin might subsequence in upregulation of Shh and increase level of VEGF which promote anagen entry (fig 10.1). This study could be another step to explain the whole molecular mechanism of tofacitinib for promoting hair growth.

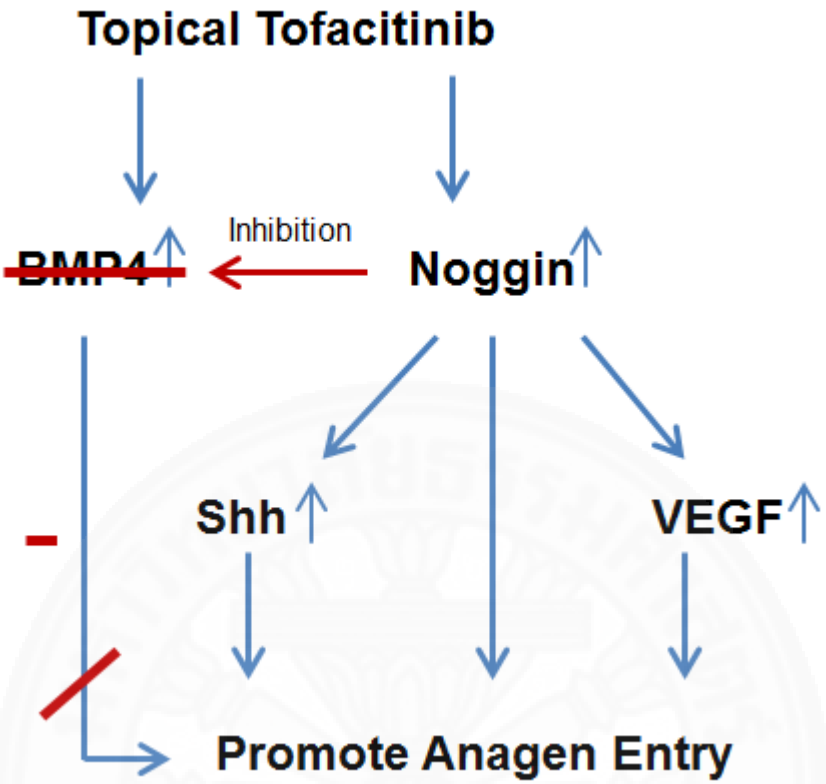


Figure 10.1 Possible mechanism of topical tofacitinib on Noggin, BMP4 and other related molecules for promoting anagen entry.

10.2 Recommendation

10.2.1 This study does not perform noggin and BMP4 proteins concentration analysis. In order to confirm the impact of tofacitinib on interested target molecules, the author suggested quantitative measurement of noggin and BMP4 proteins by enzyme-linked immunosorbent assay (ELISA).

10.2.2 This study has performed just short-term treatment duration of topical tofacitinib. Long-term efficacy and safety of topical tofacitinib for treatment of hair loss disorder need to be provided.

10.2.3 Due to irritative side effect of DMSO, vehicle agent in this study, other solvents may include in further studies to reduce irritative skin side effect.

10.2.4 The author suggest complete panal of analysis all molecules involved in regulation of hair cycle.

10.2.5 The author suggest dose dependent experiment by using other dose of topical tofacitinib for example 1% or 3% of topical tofacitinib in evaluation of clinical outcome, molecular effect and adverse effect in mice.

10.2.6 The author suggest time dependent experiment by using sequencetial biopsy of mice tissue on day 7, 14 and 21 for evaluation of histopathology and trend of molecular effect on BMP4 and noggin.

10.2.7 The author suggest more intervention of futher study by inject inhibitor of BMP4 and noggin on mice to evaluate whether tofacitinib can still result in up-regulation of our interested molecules in condition which mice were contain with BMP4 and noggin inhibitor.

10.2.8 The author suggest further clinical trial on human.

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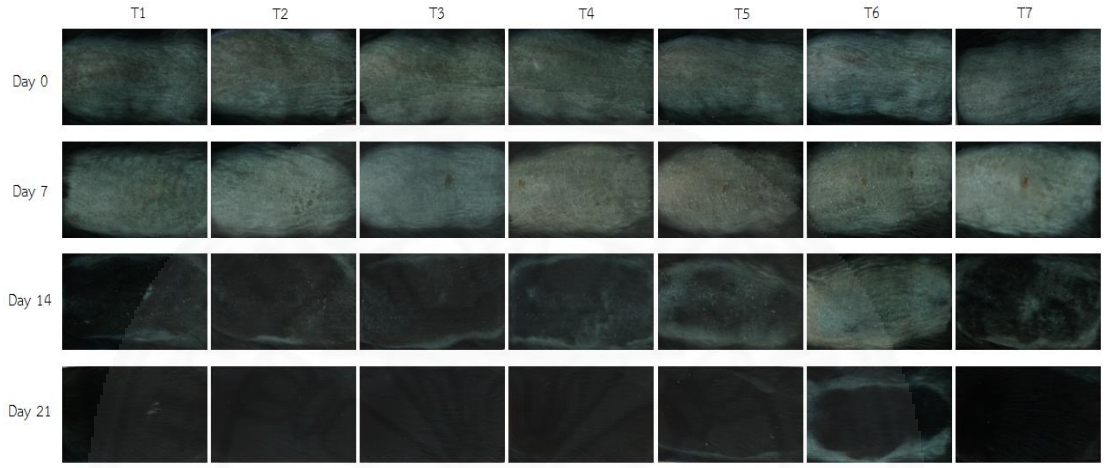
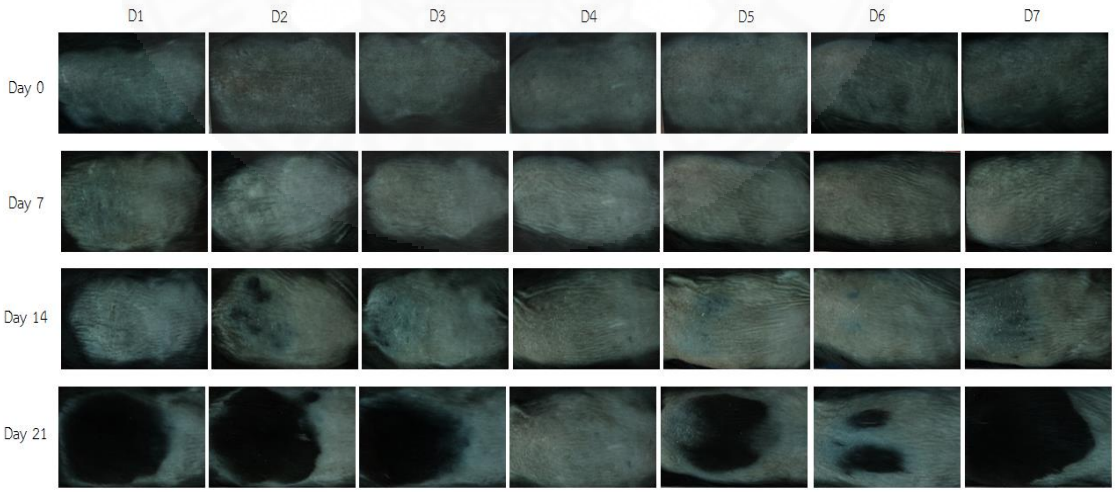
APPENDICES

APPENDIX A
CLINICAL PRESENTATION OF MICE

Tofacitinib-treated mice

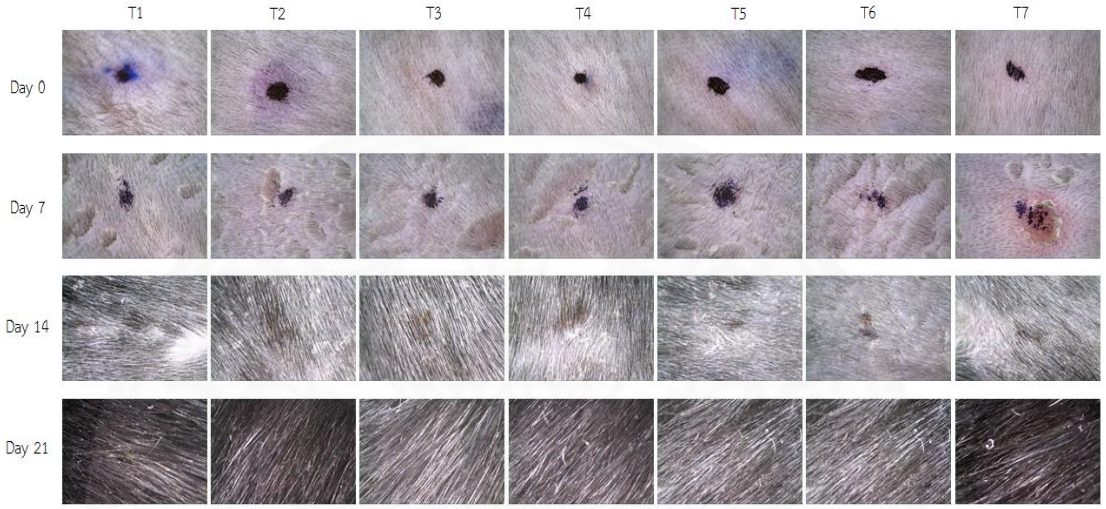


DMSO-treated mice

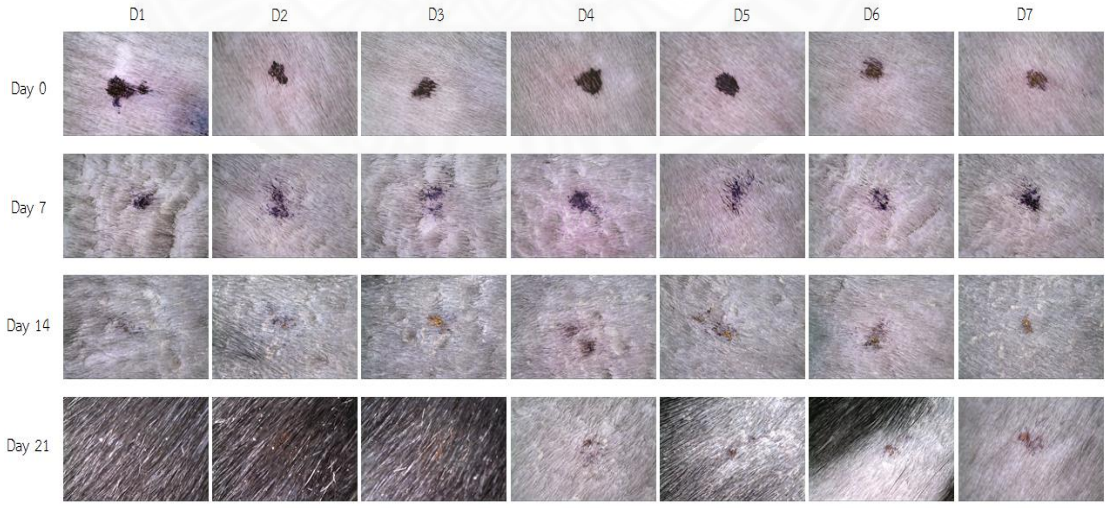


APPENDIX B
CLINICAL PRESENTATION OF MICE

Tofacitinib-treated mice



DMSO-treated mice



APPENDIX C

RAW DATA OF RT-PCR ANALYSIS

Target	Sample	Mean Cq	Mean Efficiency Corrected Cq	Relative Normalized Expression
B2M	D01	20.38	20.38	
B2M	D02	21.66	21.66	
B2M	D03	22.46	22.46	
B2M	D04	23.16	23.16	
B2M	D05	23.55	23.55	
B2M	D06	26.77	26.77	
B2M	D07	22.56	22.56	
B2M	T01	25.05	25.05	
B2M	T02	24.33	24.33	
B2M	T03	22.10	22.10	
B2M	T04	22.19	22.19	
B2M	T05	26.23	26.23	
B2M	T06	26.03	26.03	
B2M	T07	25.10	25.10	
BMP4	D01	33.11	33.11	0.47938
BMP4	D02	35.10	35.10	0.29250
BMP4	D03	36.04	36.04	0.26444
BMP4	D04			
BMP4	D05	36.31	36.31	0.46586
BMP4	D06			
BMP4	D07	35.43	35.43	0.43223
BMP4	T01	36.71	36.71	1.00000
BMP4	T02	35.59	35.59	1.31863

BMP4	T03	34.24	34.24	0.71399
BMP4	T04	34.97	34.97	0.45854
BMP4	T05	38.46	38.46	0.67354
BMP4	T06	39.28	39.28	0.33364
BMP4	T07	37.03	37.03	0.82832
NOG	D01	34.34	34.34	0.98904
NOG	D02	37.10	37.10	0.35407
NOG	D03	37.37	37.37	0.51035
NOG	D04	39.49	39.49	0.19026
NOG	D05	36.91	36.91	1.49109
NOG	D06			
NOG	D07	37.42	37.42	0.52861
NOG	T01	38.99	38.99	1.00000
NOG	T02	38.46	38.46	0.87579
NOG	T03	36.59	36.59	0.68042
NOG	T04	37.66	37.66	0.34513
NOG	T05	39.63	39.63	1.45425
NOG	T06	39.53	39.53	1.35863
NOG	T07	39.94	39.94	0.53366

BIOGRAPHY

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