

## Overexpression of Porcine *Kit* Gene in Transgenic Mice Does Not Affect Melanogenesis

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### Abstract

The *Kit* gene is proto-oncogene, encoded stem cell factor and involved in melanocyte development. Also, *Kit* is expressed in epidermis and proliferation of melanoblasts which occur to pigmentation in skin, eyes and other tissues. Here we established porcine *Kit* genes overexpressed transgenic mice. The role of *Kit* gene was regulation in melanogenesis remains unclear in porcine. Therefore, present study aims to ectopic expression porcine *Kit* gene in pigmentation of skin tissues. Our results showed that no effect on melanogenesis in ectopic *Kit* gene overexpressed transgenic mice. Further studies on *Kit* gene will provide insight into other specific roles in porcine and mice species.

**Keywords:** *Kit* gene; Transgenic mice; Skin; Melanogenesis

### Introduction

The proto-oncogene *Kit* is a tyrosine kinase receptor (as known as *c-Kit* and CD117), which codes for encoding stem cell factor (*SCF*), involved in differentiation, proliferation, chemotaxis, and apoptosis through *Kit* receptor activated by its ligand [1]. Also *Kit* gene has been reported in hematopoietic progenitor/stem cells, tissue mast cells, germ cells, interstitial cells of Cajal and melanocyte [2]. *SCF* is specific ligand of *Kit*; it is activated by *Kit* ligand and plays a key role in haematopoiesis, melanogenesis and spermatogenesis [3]. *Kit* has been observed in malignant tumors and melanoma [4,5]. *SCF/Kit* signaling is required for migration and survival of pigment precursors [6]. It is important for the migration, proliferation, and differentiation of melanoblasts [7]. Melanotic disorders are into hypermelanotic and hypomelanocytic, which are because of absence of melanocyte number or melanin deficiency and reduction. Initially melanogenesis is occur to melanoblast migration from the neural crest by myoblast development pathway key regulated genes such as paired-box 3 (*PAX3*), sex-determining region Y-box (*SOX10*), microphthalmia transcription factor (*MITF*), endothelin 3 (*EDN3*), endothelin receptor B (*EDNRB*) and *Kit* [8]. In mammals, melanin is synthesized through catalyzed by specific enzyme complexes such as phenylalanine (*PAH*), tyrosinase-related protein 1 (*TRYP1*) and tyrosinase-related protein 2 (*TRYP2*). Which is affecting the cellular differentiation processes [9]. Hair pigmentation is regulated by melanocyte differentiation at the embryonic stage. *Kit* is able to induce proliferation of melanoblasts in the epidermis by survival signal [10]. *SCF* and *Kit* are encoded map to the steel (*sl*) loci and the dominant white spotting (*w*) loci, which is a mutation occurs showing unpigmented hairs phenotype in mice [11–13]. *Kit* is almost exclusively expressed in the epidermis [14]. In previous study, mutated white spotting locus is affect various aspects such as defective pigmentation, spotting, ventral spot, diluted coat color, change the eyed color in mouse [15]. Furthermore, murine white-spotting locus mutation affect melanogenesis during development, and ectopic *Kitw42* minigene transgenic mice was shown that change the coat color phenotypes [16]. Also, *Kit* gene mutation is affected the coat colors and patterns in pig [17]. However, overexpressed pig *Kit* gene is unclear how they are affected on hair pigmentation. The aim of the study was to find out the effect on melanogenesis of skin tissues using ectopic porcine *Kit* overexpressed transgenic mice.

### Materials and Methods

#### Animals

All mice used in the study were maintained in the Korea Research Institute of Bioscience and Biology (KRIBB) animal facility under pathogen-free conditions in a temperature-controlled climate 63 at 22 ± 2°C and with a 12-h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee of KRIBB (approval number: KRIBB-AEC-17103) and were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health [18–20]. All animals had free access to food and water during the experiments.

#### Vector cloning

For the convenience of cloning, each of the keratin promoter and porcine *Kit* gene was synthesized (Cosmo Genetech, Korea) and inserted into the targeting vector. In short, two parts of synthesized keratin and *Kit* gene was ligated and NLNDA vector was digested by XhoI (New England Biolabs, USA) and NotI (New England Biolabs) to insert the keratin promoter and *Kit* gene. The final vector NLDT-Keratin promoter-*Kit* was confirmed by sequencing and linearized by XhoI and NotI used for pronuclear injection.

#### Generation of TG mouse

Ectopic swine *Kit* gene expression TG mice were generated linearized NLDT-keratin promoter-*Kit* plasmid DNA microinjection into mouse zygotes of C57/BL6 strain. We obtained total 87 F0

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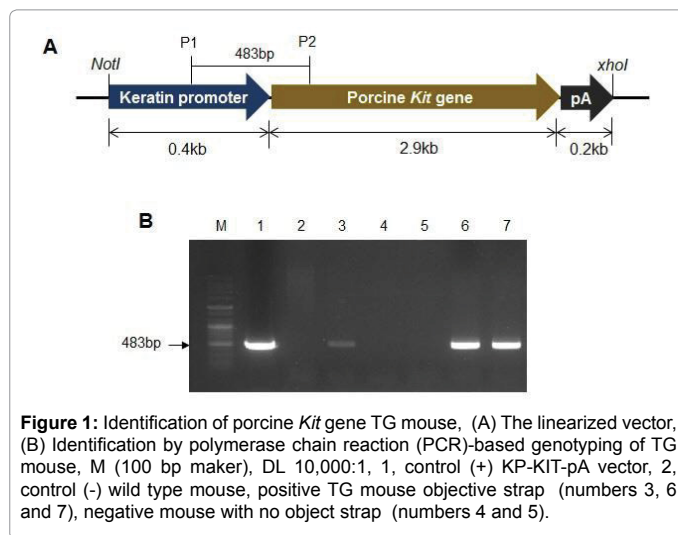
found mice, then genotyping was carried out by PCR analysis using mice tail genomic DNA. PCR primers were sequence as follows: P1, 5'-CTCTTAAGAGGCCGGGTGA-3' and P2, 5'-CCGCCTTCATTGGTGCATGTA-3' (primer location: Figure 1A). Genomic DNA PCR was performed for 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 75°C for 30 seconds. Transgenic lines were established by breeding ransgenic *Kit* F0 founder mice with wild-type mice to obtain F1 transgenic mice. Then confirm the construction of *Kit* F1 transgenic mice using PCR 86 analyses. For functional analyses, skin was excised from 4 month old wild type and transgenic mice.

### Reverse transcription PCR (RT-PCR) analysis

RNA was extracted from heart, liver, spleen, lung, kidney, skin and muscle of transgenic and wild type mice by using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA was treated with DNase I and reverse transcribed into cDNA using TOPscript cDNA Synthesis *Kit* (Enzymomics, Korea). cDNA was synthesis, 5 µg of total RNA incubated 55°C for 60 min and 95°C for 5min. Tissue specific expression of porcine *Kit* was examined by reverse ranscription PCR. The porcine *Kit* gene specific primers were as follows: porcine *Kit* gene forward 5' - TGAAGGCGGTTTAAGCAGT-3' and porcine *Kit* gene reverse 5' -CTTCGACAGCACGGACTTG-3'. RT-PCR was performed for 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Expression levels were normalized comparing to the amount of GAPDH mRNA in wild type and transgenic mice.

### Quantitative real-time PCR (qRT-PCR) analysis

RNA was extracted from skin tissue and cDNA synthesis, cDNA was used as a template for qRT-PCR amplification in combination with



**Figure 1:** Identification of porcine *Kit* gene TG mouse, (A) The linearized vector, (B) Identification by polymerase chain reaction (PCR)-based genotyping of TG mouse, M (100 bp maker), DL 10,000:1, 1, control (+) KP-KIT-pA vector, 2, control (-) wild type mouse, positive TG mouse objective strap (-) numbers 3, 6 and 7), negative mouse with no object strap (numbers 4 and 5).

melanocyte development specific primers designed (Table 1). qRT-PCR was duplicated with three samples, respectively, using the QuantiTect SYBR Green PCR *Kit* (Qiagen, Germany) and a Rotor-Gene Q thermal cycler (Qiagen, Germany). qRT-PCR was performed for 40 cycles of 95°C for 20 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. Expression levels were normalized comparing to the amount of GAPDH mRNA of WT-mice and data were analyzed using the  $\Delta\Delta C_t$  method.

### Western blotting analysis

Skin and muscle tissue protein lysates were prepared in RIPA buffer with cocktail of protease inhibitors, and then quantified using BSA Progein Assay Reagent (Bio-Rad, USA). Protein samples were boiled in 4x SDS buffer for 10 min, separated by SDS-PAGE on 8% gel running for 3 hrs and then semi-dry transferred to a PVDF membrane (Millipore, USA) at 200 mA for 2 hrs. After blocking with 5% skim milk in 0.1% TBST for 1 hr, the membranes were incubated with primary antibodies against anti-pig *c-Kit/CD117* (1:200 Lsbio, USA) or anti- $\beta$  actin (1:1000 Cell signaling, USA) overnight at -4°C. After 3 times washing in 0.1% TBST and then membranes were then treated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse 1:5,000 and anti-rabbit 1:5,000, Santa Cruz, USA) for 2 h. After 3 times washing in 0.1% TBST and immunodetection analysis was accomplished using ECL reagent (GE healthcare, USA). Luminescent densities were measured using LAS-3000 Luminescent Image Analyzer System (Fujifilm, Japan).

### Hematoxylin and eosin (H&E) staining

The skin tissue was isolated from WT and TG mice, male and aged 16weeks, and then fixed in 4% formaldehyde for overnight at 4°C, The fixed skin were embedded in paraffin, and then paraffin sections (4 µm) were deparaffinized in xylene and re-hydrated in serial diluted alcohol. The samples were washed in running tap water in 5 min and then, incubated in Mayer's hematoxylin solution for 1 min and washed in running tap water for 20 min. The samples were moved into a jar filled with eosin solution and incubated 1 min. After dehydration and clearing, the samples were visualized under microscope (Nikon, Japan).

### Statistical analysis

All experiments were repeated at least three times and statistical analysis were performed using student's t-test.  $P < 0.05$  was considered significant. The results are expressed as the mean  $\pm$  SEM of triplicate independent samples.

## Results

### Establishment of *Kit* transgenic mice

The transgene construct for skin tissue expression of porcine *Kit* gene in a transgenic mouse Structure of keratin promoter and *Kit*

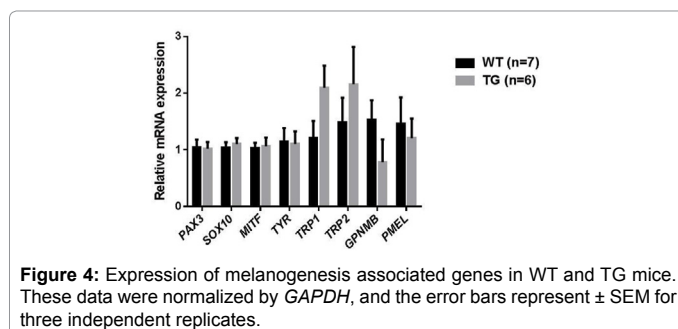
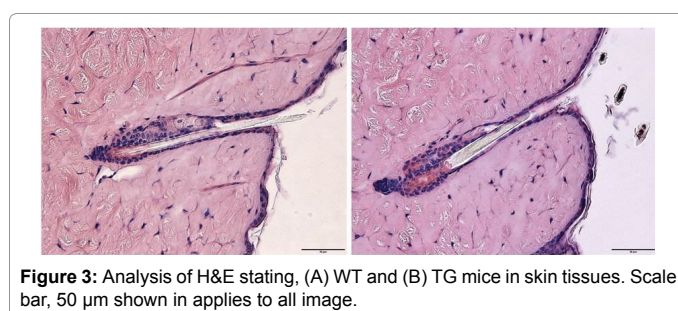
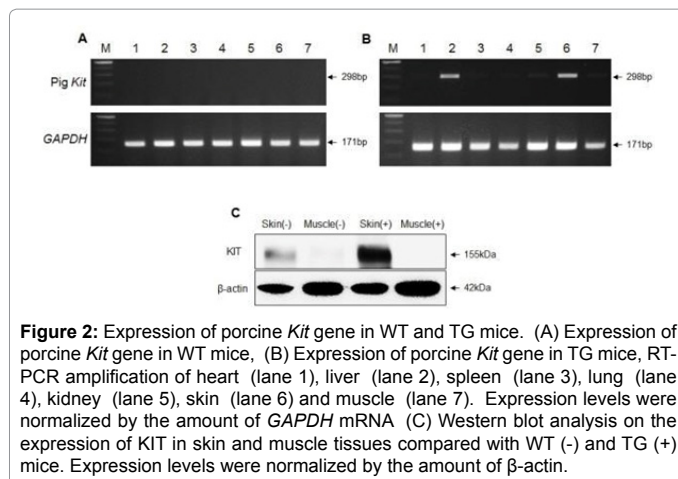
Genes	Forward sequence	Reverse sequence	Product size (bp)
<i>PAX3</i>	GGGAAGTGGAGGCATGTTTA	GTTTTCCTGCCAGCAATTA	151
<i>SOX10</i>	CCCACACTACCCGACCAG	GGCCATAATAGGGTCTCTGAGG	143
<i>MITF</i>	TACGACAACCTCTGGATCTCAC	CCTGGCGACTGATGAC	186
<i>TYR</i>	GTAAGTGGAGGTCGTCACC	ATCTGCTGAAGATGGGAGCC	202
<i>TRP1</i>	GTGCTTGGAGGTCCTGTAT	CCGCATCAGTAAAAGTGTGC	224
<i>TRP2</i>	CACTGGCAATGAGTCCTTTGC	TCCATTACACAGTGTGACCCG	213
<i>GPNUMB</i>	GGGCATACATCCCATCTCG	AGTGTGTTCCCAAAAGTTCCA	215
<i>PMEL</i>	TTCCATTGCCCTGCACCTCC	ATACACTGGCTGTCTCTCCC	115
<i>GAPDH</i>	TGAAGTCGGTGTGAACGG	CGTGAGTGGAGTCATACTGGAA	171

**Table 1:** Primer sequence for qRT-PCR.

gene CDS is schematically depicted in Figure 1A. Fertilized oocytes were microinjected with transgene construct in pro-nuclei; founder transgenic mice were identified by genomic DNA PCR. The results are shown in the 3 transgenic founder mice (3/7, #1, 6, and 7, Figure 1B). Among them, #3 *Kit* TG mice shown that low amplification band intensity compared with positive and other transgenic mice. The result may be caused by lower copy number and integration of the foreign porcine *Kit* gene.

### Characterization of porcine *Kit* transgenic mice

To investigate the expression of *Kit* gene in various tissue of transgenic mouse, we used porcine specific RT-PCR primers. These results showed that no expression in wild type mice, but porcine *Kit* gene was expressed in liver and skin tissues (Figure 2A). We constructed skin specific expression using keratin promoter, but additionally observed expression in liver tissue. Finally, we investigated porcine *KIT* protein expression by western blot. These result showed high protein expression in skin tissues of TG mice, but no protein was detected in muscle tissues (Figure 2B).



### Effect of skin melanogenesis in porcine *Kit* transgenic mice

To determine the effect of skin tissue melanocyte development of transgenic mice, H&E stained of skin tissue of transgenic mice and wild type. These results showed that no changes in the morphologies and phenotypes (Figure 3). Furthermore, melanogenesis associated gene expression of skin tissue in transgenic mice and wild type (Figure 4). Porcine *Kit* gene was not affected on melanocyte associated genes.

### Discussion

In this study, we established porcine *Kit* gene transgenic mice, and determined the porcine *Kit* gene involved the melanogenesis. Ectopic porcine *Kit* gene transgenic mice were scarified within 4 months of age, and then histological and gene expression analysis were performed on the genes associated in skin tissue. We hypothesized that *Kit* transgenic mice would exhibit a pigmentation of skin tissues. Because it is involved in white spotting colors in European pigs [8]. But we did not observe any obvious effect on the skin tissue alterations. Melanogenesis, the production of melanin pigment is shown responsible for melanin production and distribution [21].

Melanocytes are located in basal layer of hair follicles and skin epidermis [22]. *Kit* and *SCF* genes are involved in melanoblast proliferation and skin macules, which mutation can occur to melanoblast development migration (piebaldism) [8]. *Kit* and *Mitf* are needed for initial expansion of melanoblast, and also *Kit* plays in the development of pigment cells by *Mitf*, a crucial transcription factor. The elimination of expression of the *Mitf*-M isoform is caused by deafness and depigmentation in pigs [23]. Also, the duplication of the *Kit* gene is associated dominant white mutation pigs [10]. In addition, the molecular events of variable pig genes shown that different coat colors such as missense mutation of melanocortin 1 receptor (*MC1R*) [24], copy number variation and splice mutation of *Kit* [25], 6 bp deletion of tyrosinase related protein1 (*TRYP1*) [26], missense 176 mutation of endothelin receptor beta (*EDNRB*) [27,28], missense mutations of *Kit* ligand (*KITLG*) [29], and missense mutations of oculocutaneous albinism 2 (*OCA2*) [30].

### Conclusion

In conclusion, we established ectopic overexpression of porcine *Kit* gene in transgenic mice. Porcine *Kit* gene was only expressed in skin and liver tissue. However, it did not effect on melanocyte development and skin morphology. Further studies on *Kit* gene will provide insight into other specific roles in hair colors and liver tissue metabolism.

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### Conflict of Interests

The authors declare no competing interests.

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