

使用組織特異性條件式基因剔除小鼠研究 *Cdk12* 在基底和柱狀  
上皮細胞的角色

Study on roles of *Cdk12* in basal and luminal type epithelial cells by  
tissue specific conditional knockout mice

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碩士論文

Department of Life Sciences and Institute of Genome Sciences

National Yang-Ming University

Master Thesis

中華民國一〇四年八月

August, 2015

## Acknowledgement

還是逃不了要面對這一頁的感謝，越是想要好好寫的東西越是沒辦法輕易動筆。從決定要五年一貫成為預研生到現在趕著把論文印出來，總覺得這兩年的時間都是在忙碌中度過的。接下來可以預見的是匆匆地離校和入伍，我總是沒辦法好好說聲再見。

大一寒假沒想太多就被紀潔邀請進入范明基老師實驗室，一眨眼就是四年半，真的很感謝這些日子以來范老師的指導和照顧，從親自帶我做實驗和討論進度到口試前每天再忙也會抽出時間幫我批改論文，真的真的很謝謝您。也格外感謝虹如學姊，在我小時候擺爛不想做實驗的時候嚴厲地教訓我了一頓，讓我終於能把心思放在實驗上面。忙碌的日子裡，晚上跟你聊天總是最好的放鬆。也感謝其他范家的學長姊、學弟妹們：阮博士絃嘉、管理員韋志、小紅帽、林永、恩絨、克緯、雨蕙、瑜賸、子瑄、元添、子淮、雅惠、資敏、曼萱、子寧、思佑、Audrey、Yvonne、建樺。感謝共同指導的陳俊銘老師，不論是帶我看玻片、討論進度還是在三一八期間鼓勵我去看社會運動，感謝您的一切指導。感謝陳家和游家的學長姐：Fish、采靈、文馨、傑翔、致祐、子淯在實驗上的協助。感謝曾經參與我進度報告和口試的委員們：翁芬華、阮祺昌老師。以及榮總皮膚部的張雲亭主任、陳志強、朱思穎醫師、劉力瑛小姐，有了您們給予的意見和協助這篇論文才能順利完成。

謝謝周成功、娟娟、JY、小范、照雄、文亞、孫以瀚各位老師，感謝您們在課堂上教導我怎麼接近科學，以及課堂外的鼓勵。感謝圖資七樓的好朋友們：學甫、為婕、琬淇、家玉、婉瑜、安安、俐潔、添鈞、易翰、冠偉、安琪。感謝超cover 的球友們：思瑢、正妹、冠陞、小包、泓霖、冠智、正妹妹、老極、怡萱。感謝一起討論、辯論、讚嘆科學的科研讀書會成員們：大信、忠佑、花花、阿繆、皮卡、CY、Jumbo、澤翔、家綱、昀芸、查德、Jack。感謝宗儒、凌亞、浩瑋，讓我在科學以外還能碰碰法律。

最忙碌的這兩年也是我開始擔任世界魔術方塊協會代表的日子，很開心沒有因為唸書而放棄了自己最愛的嗜好。謝謝郭大和 Tyson 的推薦讓我加入 WCA 這個大家庭。也感謝這些日子以來一起征戰南北的方塊好手們：理查、短 Q、鬼花、L、aljohn、SC、五尾、阿良、壯壯、大貓、Zp4、丹丹、土城、小燒、亭安、中國代表小明、小圓、小強、香港代表 Kim、Philip、WCA 理事會 Ron、Ilkyoo、Natán、Sébastien、cubecomp 網站負責人 Luis。還有我最要好的日本朋友 Saji。

感謝爸媽老弟馬沙等家人給予的體諒和支持。

感謝紀潔，有妳的陪伴，這些日子每一天都很精彩。

羅鴻 08/11/2015

## 中文摘要

週期蛋白依賴性激酶 12，*CDK12*，其參與在基因體穩定、同源重組、基因轉錄、功能性剪接、轉譯、胚胎幹細胞的多能性以及神經軸突生長。近期的研究指出，在臨床的人類卵巢癌和乳癌樣品中有 *CDK12* 失去功能的突變發生。因為擁有較高的同合突變比率，因此 *CDK12* 被推斷為一可能的腫瘤抑制基因。為了瞭解 *CDK12* 功能性的喪失是否會造成癌症的發生，我使用了 *K5-CreER<sup>T</sup>* 和 *K8-CreER<sup>T</sup>* 這兩種可調控且具有組織專一性 Cre 重組酶的小鼠，在基底和柱狀上皮細胞來進行 *Cdk12* 在成鼠的條件性剔除。

但是，經過了超過 20 個月的觀察，沒有明顯看到腫瘤的生成，可能是因為單一基因的突變不足以促進腫瘤發生。而在 20 周大之後，觀察到有強烈的脫毛表現型發生的 K5 啟動 *Cdk12* 條件性剔除的小鼠的皮膚上。切片的結果發現，一種被稱為角化囊腫的角質化毛囊，出現在 *K5-CreER<sup>T</sup>; Cdk12<sup>del/fx</sup>* 小鼠的皮膚中。因此 *Cdk12* 是否在維持毛髮上有個重要的角色就成了個有趣的問題。透過免疫染色，發現在 *K5-CreER<sup>T</sup>; Cdk12<sup>del/fx</sup>* 小鼠中幹細胞的標記 CD34 消失，並且 K6 標記上升。AE13 和 AE15 兩個毛囊標記的染色結果顯示幹細胞往毛囊細胞的分化在失去 *Cdk12* 的情況下沒有受到影響。同時，最終分化的表皮細胞標記 involucrin 染色被觀測到在角化囊腫和毛囊中出現，說明幹細胞往表皮細胞做分化。我進一步去測試究竟在基底層的上皮細胞剔除 *Cdk12* 會不會造成毛髮再生的缺陷，而透過拔毛來引發的毛髮再生在 *K5-CreER<sup>T</sup>; Cdk12<sup>fx/fx</sup>* 小鼠中明顯出現延遲的現象。經過流式細胞儀的分析，當 *Cdk12* 基因剔除小鼠經過重複拔毛之後，位在毛囊腫脹區的幹細胞數量減少，但位在毛胚區的幹細胞並沒有觀察到不同。在表皮細胞中出現了較高的細胞增殖訊號，但在毛囊則是沒有差異。*Cdk12* 過去已知和基因體的穩定性有關，因此使用 γH2AX 來測定 DNA 的損傷，並且發現不論是在毛囊還是表皮都有明顯增加 DNA 雙股斷裂的發生率。相反地，當在柱狀上皮細胞剔除 *Cdk12* 的時候，沒有相似的表現型在 *K8-CreER<sup>T+/0</sup>; Cdk12<sup>del/f</sup>* 小鼠中發生。

總結來說，在基底層上皮細胞剔除 *Cdk12* 會造成角化囊腫的發生，並且失去毛囊幹細胞和產生不正確的分化途徑。分子機制則可能是透過增加基因體的不穩定性所造成。

# Abstract

Cyclin dependent kinase 12, *CDK12*, is involved in genomic stability, homologous recombination, transcription, alternative splicing, translation, self-renewal and axonal elongation. Recent studies showed that dysfunctional *CDK12* mutations were found in clinical samples of human ovarian and breast cancers. A high ratio of homozygous mutations of *CDK12* was observed in ovarian cancer, which suggests *CDK12* is a potential tumor suppressor gene. To study whether *CDK12* deficiency causes cancer formation, two inducible tissue-specific Cre mouse lines, *K5-CreER<sup>T</sup>* and *K8-CreER<sup>T</sup>*, were used to knockout *Cdk12* in basal and luminar epithelial cells in adult mice.

However, over 20-month investigation, no significant tumor progression was found, which may be due to that single gene mutation is not sufficient to promote tumor formation. Strong phenotype was observed on skin with hair loss in *K5*-driven *Cdk12* conditional knockout mice after 20 weeks old. Results from sections showed that there are horn cysts, cornified hair follicles, present in *K5-CreER<sup>T</sup>; Cdk12<sup>del/fx</sup>* mice. Whether *Cdk12* has important roles on maintenance of hair became an interesting question. Results from immunostaining showed that hair follicle stem cell marker CD34 was absent and K6 was increased in *K5-CreER<sup>T</sup>; Cdk12<sup>del/fx</sup>* mice. Staining pattern of AE13 and AE15 showed that stem-to-hair follicular cell differentiation did occur upon the loss of *Cdk12*. On other hand, terminal differentiated epidermis marker, involucrin, was observed in horn cyst and bulge of hair follicle, indicating differentiation of stem cells into epidermis-like cells. I further tested whether depletion of *Cdk12* in basal type epithelial cells would cause hair regeneration defects, and depilation-induced hair regeneration was significantly delayed in *K5-CreER<sup>T</sup>; Cdk12<sup>fx/fx</sup>* mice. Flow cytometry results showed that bulge stem cell populations were decreased after repetitive depilation, but no difference was found in hair germ stem cell populations. Increase of cell proliferation was found in epidermis, but not in hair follicles. Since *Cdk12* is correlated with genomic stability, DNA damage was detected by  $\gamma$ H2AX and significant increase of DNA double-strand break (DSB) was found in both hair follicles and epidermis. In contrast, no similar phenotypes were found if *Cdk12* depletion occurred in luminar epithelial cells in *K8-CreER<sup>T+0</sup>; Cdk12<sup>del/fx</sup>* mice.

Taken together, loss of *Cdk12* in basal type epithelial cells results in formation of

horn cysts, loss of bulge stem cells and performance of incorrect differentiation process. The molecular mechanism of this defects is possibly through an increase of genomic instability.



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# 1. Introduction

## 1.1 Cdk12

*CDK12* is a protein kinase, previously known as *CRKRS*. Previous studies in our laboratory showed that *Cdk12* influences alternative splicing of an E1a reporter gene by forming complex with cyclin L1 and L2 (Chen et al., 2006). In other studies, *Cdk12/cyclin K* complex were reported to regulate transcription of DNA damage response (DDR) genes, like *BRCA1*, *ATR*, *FANCI* and *FANCD2* which involve in homologous recombination repair, thus maintaining genomic stability (Bösken et al., 2014; Bajrami et al., 2013; Blazek et al., 2011; Ekumi et al., 2015; Joshi et al., 2014). *Cdk12* also facilitates expression of long-exon genes by phosphorylation of the C-terminal domain of RNA polymerase II in yeast (Bartkowiak et al., 2010). Our laboratory analyzed the function of *Cdk12* both *in vitro* and *in vivo*. *Cdk12* and *Cdk13* regulate axon elongation though downstream effector *Cdk5* in P19 neuronal differentiation model (Chen et al., 2014). Embryonic lethality was observed in *Cdk12* deficiency mice (Juan et al., unpublished data). Depletion of *Cdk12* in central nervous system (CNS) results brain atrophy and thinner cerebral cortex by radial migration defects and apoptosis of neural progenitor pool (Chen et al., unpublished data).

### 1.1.1 Cdk12 in cancers

Recent studies showed that dysfunctional *CDK12* mutations were related to clinical tumor samples. Nine significant *CDK12* mutations were found in 316 ovarian cancer samples, and 7 mutations are located in kinase domain, which indicate that *CDK12* lose its kinase activity in these tumor samples (Cancer Genome Atlas Research, 2011). In another ovarian cancer screening report, *CDK12* was found to have 12 significant mutations in 214 human ovarian carcinomas by ABSOLUTE analysis, and 7 of them were homozygous mutations. The high ratio of *CDK12* homozygous mutations suggests that *CDK12* might be a potential tumor-suppressor gene in human ovarian carcinomas (Carter et al., 2012). In breast cancer, a fusion transcript with *LASPI* and out-of-frame *CDK12* was found in a rare but malignant breast cancer, breast micropapillary carcinomas (Natrajan et al., 2014).

In addition to clinical cancer samples, *CDK12* expression level was also found to

be related to sensitivity of anti-cancer drugs like cisplatin and oraparib in ovarian cancer cell lines (Bajrami et al., 2013). In MCF7 cells, a cell line derived from ER $\alpha$ -positive breast cancer, knockdown of *CDK12* leads to activation of MAPK pathway, thus decreasing the requirement for estrogen/ER $\alpha$  signals, and increasing the resistance to Tamoxifen, an anti-cancer drug (Iorns et al., 2009). Last, in gastric cancer cell line, MKN7, a fusion transcript combining *CDK12* and out-of-framed *ERBB2* receptor was found, which is translated as a 15 KD deleted-*CDK12* protein (Zang et al., 2011).

Taken together, whether *CDK12* deficiency can cause cancer formation remains unclear. By using conditional knockout mice, I intended to reveal the role of *CDK12* in cancer formation.

To study whether *CDK12* deficiency can cause cancer formation, inducible Cre mouse line was used to knockout *Cdk12* in specific tissues. Unfortunately, no available inducible Cre mouse line can be used as the model of serous ovarian cancer. To investigate roles of *Cdk12* in breast cancer, two inducible tissue-specific Cre mouse line, *K5-CreER<sup>T</sup>* and *K8-CreER<sup>T</sup>*, were used to knockout *Cdk12* in basal and luminal epithelial cells in mammary gland (Liang et al., 2009; Lu et al., 2013). *K5-CreER<sup>T</sup>* mouse expresses inducible Cre activity in basal type epithelial cells in mammary gland, esophagus, forestomach, skin and thymus. *K8-CreER<sup>T</sup>* mouse expresses inducible Cre activity in luminal type epithelial cells in mammary gland, hind stomach, intestine, liver, lung, pancreas, uterus, oviduct and epididymis. Both mouse line have Cre activity in mammary gland, and can be used as the model of breast cancer.

### 1.1.2 Cdk12 in genomic stability

As the first paragraph described, previous studies reported that *Cdk12* regulates the transcription of certain DDR genes such as *BRCA1* and *ATR*, which are involved in homologous recombination repair. In *Cdk12* mutant cancer cell lines or knockdown *Cdk12*, DDR genes were down regulated and more DNA double-strand breaks (DSBs) were detected (Bajrami et al., 2013; Blazek et al., 2011; Ekumi et al., 2015; Joshi et al., 2014). These findings suggested that *Cdk12* acts as a guardian of genome, and loss of *Cdk12* causes accumulation of DNA damages. Mutated *Cdk12* were found in ovarian and breast cancers, and other major mutated genes found in ovarian and breast cancers

are *BRCA1* and *BRCA2*. Since *Cdk12* regulates the expression of *BRCA1* in transcription level, this may be the missing link of why several *Cdk12* mutants were found in cancers.

## 1.2 Skin

Besides mammary gland, most of internal and external organs are also covered by epithelial cells, so using these mouse models, potential roles of *Cdk12* can also be observed simultaneously. One of the important external organs is skin, which covers the surface of body, and it acts as the first barrier of infectious agents. Skin contain three layers: epidermis, dermis and subcutaneous fat. Outside of the skin is covered by hairs which are for body temperature maintenance. Hairs are generated by a group of specified cells developing in skin, called hair follicles. Hair follicles generate hair spontaneously depending on specific time scale, or also generate new hair while hairs are removed (Müller-Röver et al., 2001).

### 1.2.1 Hair cycle

While generating new hairs, massive signals of activators induce hair follicles start new hair cycle from the telogen phase, the resting state, to anagen phase, the growing state (Plikus et al., 2011; Schneider et al., 2009). Quiescent stem cells that reside in bulges and secondary hair germ, start to replicate and divide into several proliferating transit-amplifying (TA) cells (called matrix in hair bulb). These cells form thick hair follicles down into the fat layer, and generate new hairs inside. Meanwhile, signals of inhibitors raise. Once the signals of activators reduce, hair cycle moves to the regression part, the catagen phase. Hair generation stops, and most of TA cells that form in anagen phase undergo apoptosis. Some of them escape from apoptosis, dedifferentiate into quiescent stem cells, and migrate back to bulge and secondary hair germ. After the regression of hair follicles, hair cycle goes back to the telogen phase and halt till the next signals of activators raise. That's the reason that length of furs of rodents and other animals is kept the same.

Hair cycles of newborn mice are synchronized for the first 14 weeks (see Appendix

Figure 1(a)), thus one can investigate the hair cycle process and tell whether hair cycle is influenced under certain condition. Another way to create synchronization of the hair cycle is plucking hairs, or called depilation. After depilation, hair follicles will go into anagen phase immediately, and take 3 weeks to regenerate new hairs (see Appendix Figure 1(b)). Skin colors can be obvious objects for determining the hair cycle states, while hair cycle is in telogen phase, skin color is pink, but while in anagen phase, skin color turns dark black in C57BL/6 mice. The thickness of skin also cycles with hair cycle, it becomes thicker while hair follicles are growing, and goes back to normal in resting state.

### 1.2.2 Cdk12 in skin

According to the previous studies in our laboratory, *Cdk12<sup>del/fx</sup>* mice showed abnormal skin phenotypes like partial hair loss in aged mice (Master thesis of Yung Lin), suggesting that *Cdk12* haploid insufficiency may lead to abnormalities in skin. However this hypothesis was not yet tested, and the potential roles of *Cdk12* in skin remained unknown. Fortunately, hair follicles also contains K5<sup>+</sup> basal cells. Thus I used *K5-CreER<sup>T</sup>* mouse to knockout *Cdk12* in skin, and investigate the possible functions of *Cdk12*.

### 1.3 Aim of the thesis

In this thesis, I intended to reveal the potential roles of *Cdk12* in adulthood, especially in cancer formation and in skin. Using *K5-CreER<sup>T+0</sup>*; *Cdk12<sup>del/fx</sup>* mice and *K8-CreER<sup>T+0</sup>*; *Cdk12<sup>del/fx</sup>* mice as models, I can knockout *Cdk12* in basal and luminal type epithelial cells respectively in adulthood.

## 2. Materials and methods

### 2.1 Mouse breeding and genotyping

To generate *Cdk12* conditional knockout mice, I crossed *Cdk12<sup>del/fx</sup>* or *Cdk12<sup>fx/fx</sup>* mice with either *K5-CreER<sup>T</sup>* or *K8-CreER<sup>T</sup>*, to obtained *K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>*, *K8-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>*, and *K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>fx/fx</sup>* mice respectively. The rules of Institutional Animal Care were followed. Genotype analysis of mice was done at 3 weeks old by cutting 0.5 cm of mouse tail tip. Tail genomic DNA was analyzed by polymerase chain reaction (PCR) by following primers: Del3, Fx2, Y3, Cre-1 and Cre-β. Del3, Fx2 and Y3 primers were used for analysis of *Cdk12* alleles. Cre-1 and Cre-β primers were used for analysis of *K5-CreER<sup>T</sup>* and *K8-CreER<sup>T</sup>* alleles. Reporter mice used in this study were *CAG-CAT-EGFP* mice and *R26R-LacZ* mice, they were crossed with *K5-CreER<sup>T</sup>* and *K8-CreER<sup>T</sup>* mice respectively, and genotype of their offspring were analyzed with following primers: EGFP-1, EGFP-2, Rosa-1, Rosa-2, and Rosa-3, Sequences of primers are listed in Table. 1.

### 2.2 Drug administration

*K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>* and *K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>fx/fx</sup>* mice at 3 or 6 weeks old were injected with Tamoxifen (25 mg/Kg per injection, dissolved in 1 volume of 100% EtOH mixed with 9 volume of sun flower seed oil) intraperitoneally every other day for three times. Vehicle control mice were injected with the same volume of solvent. *K8-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>* mice were injected with Tamoxifen (50 mg/Kg per injection) intraperitoneally at 6 weeks old every other day for three times.

### 2.3 Skin surgery and depilation

Before skin surgery, mice were anesthetized with sodium pentobarbital intraperitoneally (100 mg/Kg, dissolved in 1x PBS). Operative instruments were sterilized with 70% EtOH or autoclaved. Mice were shaved, skin was cut out 1x3 cm<sup>2</sup>, and wounds were sew up with sterile Nylon needle lines. After surgery, mice were raised as one mouse per cage. Dissected skins were adhered to papers and fixed with 4% paraformaldehyde (PFA). Depilation on anesthetized mice was done with melted

wax (paraplast) around 70°C. Wax was dropped on the dorsal skin of mice with cut dropper. Wax was allowed to cool down to room temperature, and hairs were removed with wax.

## 2.4 Organ processing and sections

*Cdk12* conditional knockout mice were sacrificed and organs were fixed with 4% PFA overnight. Organs were transferred into 30% sucrose overnight for cryosection or into 70% EtOH overnight for paraffin section. For cryosections, tissues were embedded in optimal cutting compound and stored at -20°C. Samples were sectioned at 14- $\mu$ m thickness. For paraffin sections, tissues were dehydrated by a tissue processor and were embedded in paraffin, stored at room temperature. Samples were sectioned at 5- $\mu$ m thickness.

## 2.5 Hematoxylin & eosin staining

Slides were prewarmed at a 65°C oven for 1-2 hours, dewaxed with xylene, and rehydrated with EtOH and ddH<sub>2</sub>O. Slides were dipped sequentially in hematoxylin, tap water, acetate acid, lithium carbonate, eosin, EtOH and xylene, and mounted with a mounting solution.

## 2.6 Masson's trichrome staining

Slides were prewarmed at a 65°C oven for 1-2 hours, dewaxed with xylene, and rehydrated with EtOH , running water and ddH<sub>2</sub>O. Slides were placed inside the staining box and stained with Mordant reagent for 35 minutes, washed with running water for 5 minutes and ddH<sub>2</sub>O for 1 minute. Slides were stained with Weigert's hematoxylin (mix same volume of solution 1 and solution 2) for 12 minutes, washed with running water for 5 minutes and ddH<sub>2</sub>O for 1 minute. Slides were stained with 0.75% Orange G solution for 5 minutes and dipped with 1% acetic acid for 1 minute. Slides were stained with Masson B solution for 12 minutes and dipped with 1% acetic acid for 1 minute. Slides were stained with 2.5% phosphotungstic acid solution for 12 minutes and dipped with 1% acetic acid for 1 minute. Slides were stained with Alanine

Blue solution for 32 minutes and dipped with 1% acetic acid for 1 minute. Slides were dipped sequentially in ddH<sub>2</sub>O, EtOH and xylene and mounted with a mounting solution.

## **2.7 Immunostaining**

Slides were prewarmed at a 65°C oven for 1-2 hours, dewaxed with xylene, and rehydrated with EtOH and ddH<sub>2</sub>O. Slides were retrieved by antigen retrieval buffer (DAKO, pH 9.0), and being heated with a microwave at thaw power 10 minutes twice. Slides were allowed to cool down in room temperature or in cool water for 3~4 hours, and washed with 1x PBS 5 minutes twice. Slides were placed inside the staining box for blocking with 5% BSA (dissolved in 1x PBS) at room temperature for 1 hour, and then washed with 1x PBS 5 minutes for 3 times. Primary antibodies (listed in Table 2) were diluted with 5% BSA and dropped onto slides at room temperature for 1~2 hours or at 4°C overnight in the staining box. Slides were washed with 1x PBS 5 minutes for 3 times. Secondary antibodies were diluted in 1x PBS and dropped onto slides at room temperature for 1 hour, and slides were washed with 1x PBS 5 minutes for 3 times in a dark place. Slides were stained with DAPI (1:5000 dilution in 1x PBS) for 15 minutes and washed with 1x PBS 5 minutes for 3 times in a dark place. Slides were mounted with a staining mounting solution and dried at room temperature overnight.

## **2.8 Keratinocyte isolation**

Protocol of keratinocyte isolation was modified from this article (Nowak and Fuchs, 2009). Mice were anesthetized with pentobarbital intraperitoneally (100 mg/Kg, dissolved in 1x PBS), and dorsal skins were shaved twice with electric clipper and electric razor. Mice were cleaned up with 70% EtOH and sacrificed by cervical dislocation. Dorsal skins were obtained and subcutaneous fats were removed by forceps. Skins were washed by 1x PBS and placed in petri dishes with dermis on the bottom. Added 5 ml of 0.25% trypsin (EDTA free) and incubated in 37°C incubator for 30 to 60 minutes. Transferred 0.25% trypsin into another tube, epidermis were scratched by scalpel (or 15) and were cut by scissors in eppendorf. Add epidermis into tube with 0.25% trypsin, incubated in 37°C incubator for 15 minutes, and patted tubes every 5 minutes to avoid aggregation. Added 5 ml of DMEM to stop trypsin activity, and

filtered keratinocytes by cell strainers (70 and 40  $\mu\text{m}$ ). Spin down 1350 rpm in 4 °C, removed supernatant and resuspended with 1 ml staining buffer (1x PBS with 2% FBS,  $\text{Ca}^{2+}$  free) in eppendorf. Spin down 1350 rpm in 4 °C, removed supernatant and resuspended with 500  $\mu\text{l}$  staining buffer in eppendorf. Keratinocytes were diluted for 5- or 10-fold in staining buffer to 200  $\mu\text{l}$  and stained with fluorescent-labeled primary antibodies (antibodies were listed in Table 2) 30 minutes on ice and patted tubes every 5 minutes to avoid aggregation. Keratinocytes were spin down 1350 rpm in 4 °C, removed supernatant and resuspended with 500  $\mu\text{l}$  staining buffer in eppendorf and transferred to cell strainer capped round-bottom cytometer tubes (Falcon, 12 x 75 mm tube) for flow cytometry on ice.

## 2.9 Flow cytometry

Keratinocytes were analyzed by BD FACSCanto, and parameter setting for voltages were FSC: 300, SSC: 500, FITC: 520 (CD34), 478 (CD49f), APC: 448 (CD49f) and 657 (P-cadherin). Compensation was not performed since there were no significant interference of these two fluoresces. Gating all events with 20000 cells for each samples. Data analysis was operated by FlowJo V10.

### 3. Results

#### **3.1 K5- and K8-CreER<sup>T</sup> recombinase activity was successfully turned on after Tamoxifen administration in basal and luminar type epithelial cells**

*K5-* and *K8-CreER<sup>T</sup>* mouse lines were used to knockout *Cdk12* in different types of epithelial cells. To test the efficiency of CreER<sup>T</sup> recombinase in both mouse lines, mice were mated with *CAG-CAT-EGFP* and *R26R-LacZ* reporter mice respectively for examination. Mice at 6 week old were injected with Tamoxifen intraperitoneally every other day for 3 times. Sections of *K5-CreER<sup>T+/0</sup>*; *CAG-CAT-EGFP<sup>+/0</sup>* mice showed that EGFP signal was detected in basal type epithelial cells in dorsal skin, forestomach and prostate (Figure 1A-D). X-gal staining of sections of *K8-CreER<sup>T+/0</sup>*; *R26R-LacZ<sup>+/0</sup>* mice showed that β-galactosidase activity was present in luminar type cells in epididymis, prostate, stomach and intestine (Figure 1E-H). These results suggested that these two mouse *CreER<sup>T</sup>* lines are suitable tools to knockout specific genes in epithelial cells.

#### **3.2 Tumor formation was not observed in both K5- and K8-driven *Cdk12* conditional knockout mice**

Results of sections of several organs in different stages showed that loss of *Cdk12* in both basal type and luminar type epithelial cells is not sufficient for tumor formation (Figure 2A-F). In some rare cases tumors were found in *Cdk12* conditional knockout and control mice randomly, showing that these tumor formation cases were occurred spontaneously (Figure 2G-T).

#### **3.3 Several phenotypes including hair loss and less hair growth were found in K5-dirven *Cdk12* conditional knockout mice**

During the inspection period of tumor formation, other phenotypes were observed in *K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>* mice, especially hair loss (Figure 3A-H). These mice also continuously scratched their skin, and seemed to have a visual defect as they couldn't response to flash lights. Hair of aged *K5-CreER<sup>T+/0</sup>*; *Cdk12<sup>del/fx</sup>* and control mice were shaved to observe the hair growing state. Skin with growing hair would become darker than resting state, and the region of dark skin is called hair domain. Hair domains were

observed in control mice, but no obvious hair domains were found in conditional knockout mice (Figure 3I, J). Interestingly, these phenotypes are not detected in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice. These results brought out a likely scenario: loss of *Cdk12* in basal type epithelial cells influence regeneration of hair. Despite many other gene mutations showed hair growth defects (Benavides et al., 2009; Schneider et al., 2009), this was the first time that conditional knockout of *Cdk12* in skin and found hair growth defect.

### **3.4 Loss of stem cell marker and epidermal marker was found in horn cysts and hair follicle in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice**

To further understand the defect of these hair loss and hair growth phenotypes, skins were dissected and sectioned to examine the detailed morphology changes. Hematoxylin and eosin staining and Masson's trichrome staining of the skin sections of *Cdk12<sup>del/fx</sup>* mice showed that structures with epidermis, dermis and fat layer (Figure 4A-C, G), however *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice contained several onion-like cysts, thicker epidermis and melanin accumulation (Figure 4D-F, H). These cysts were called horn cysts, a serious cornified hair follicles. These findings indicated that hair follicle structures were destroyed and couldn't generate new hairs. But what is the role of *Cdk12* in this phenotype is still unclear, thus following examinations were performed.

There might be two possible ways to cause this defect. First one is to influence stem cell properties, and the other one is incorrect differentiation. I tested stem cell properties with the presence of two markers, CD34 and keratin 6 (K6). Antibodies against two molecules were co-stained to verify the stem cell population in skin. *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice showed absence of CD34 signal and increased signal of K6 in destructural hair follicle (Figure 4K), indicating stem cell properties are influenced upon deletion of *Cdk12*.

I further verified that whether this defect was also caused by incorrect differentiation, epidermis marker involucrin was used to examine. Normally, involucrin staining showed expression of in epidermis and the upper part of hair follicles (Figure 4L-M), but positive signals of involucrin were observed in horn cysts in knockout mice (Figure 4N). Enlarged figure of this hair follicle showed that involucrin signals can be

found in either horn cyst or bulge of hair follicles, indicated that incorrect differentiation occurred and differentiated into epidermis-like cells (Figure 4O). These results suggested that both stem cell property and differentiation process are influenced in the absent of *Cdk12* in basal type epithelial cells.

### **3.5 K5-driven *Cdk12* conditional knockout mice showed delay of hair regeneration after depilation**

Strong phenotypes on skin were analyzed by immunostaining, and two experiments were found to be correlated with this defect. As described in Figure 3, this hair loss phenotype was observed in 20 weeks old *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice, but not in mice at 12 weeks old, there was a long gap between Tamoxifen administration and the hair loss outcome. This delay of phenotype might be due to that at the time of Tamoxifen treatment hair follicles were in a prolonged telogen phase (Müller-Röver et al., 2001). Only after spontaneous hair regrowth, hair loss phenotype due to *Cdk12* deficiency was observed. To test this hypothesis, depilation-induced hair regeneration was applied on mice after Tamoxifen administration (Figure 5A). Interestingly, hair regeneration after depilation was delayed for 2~3 days in mutant mice in comparison to control mice (Figure 5B). And on 22 days after depilation, hairs were still scattered in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice (Figure 5B). Repetitive depilation was performed and after hair regrowth, hairs of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice were even more scattered than control mice (Figure 5C). This result indicates that loss of *Cdk12* in basal type epithelial cells in skin definitely disrupts the ability of hair follicles to regenerate new hairs.

An interesting finding was that the skin of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice showed darker color (Figure 5D), but the sections of these mice showed that their hair cycle statuses were mainly telogen (data not shown). Detailed investigation of paws also revealed that dark dots were present on *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen injection (Figure 6), indicating melanin accumulation. However, melanocytes normally locate around hair follicles, thus it is unusual to observe this phenotype.

Another way to observe hair loss phenotype is to administrate Tamoxifen before hair replacement that normally occurred in 3 to 4 weeks old mice (Figure 7A). To this

end *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice were injected with Tamoxifen in 3 weeks old and hair regeneration processes were investigated (Figure 7B). Almost half of the *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice (n= 9/15) showed a dramatic retardation of body length and body weight (Figure 7C), and some of them even died before 5 weeks old (n= 4/15). After dissection, these mice had seriously gastro-intestinal bleeding (Figure 7D), which might be another effects of *Cdk12* in gastro-intestinal epithelial cells of premature mice, though *K5-CreER<sup>T+/-</sup>* mice express *CreER<sup>T</sup>* recombinase in epithelial cells in forestomach but not in intestine. If these mice survived in the first 6 weeks, hair loss was observed in later stage (Figure 7E), suggesting the effects of *Cdk12* in hair follicles is not exhibited till hair regeneration occurred.

### **3.6 K5-driven *Cdk12* conditional knockout mice showed alterations on stem cell markers and differentiation markers after depilation**

To further analyze the underlying mechanism of *Cdk12* functions, skin sections were stained with two groups of stem cell markers CD34, K6, and K15 after depilation. CD34 signal was absent and K6 signal was increased in the hair follicles on 10, 13, 16 (anagen) and 50 (telogen) days after depilation (Figure 8C-J), but no changes were found before depilation (Figure 8A, B). These results indicated that stem cell markers are only affected after hair regeneration.

Patterns of K15 staining were the same between *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen administration and vehicle control on 10 days after depilation. A notable difference was that horn cysts located on the top layer of skin was also positive to K15 (Figure 8K, L).

To clarify whether the differentiation process was altered or not, hair follicle markers AE13 and AE15 were applied to examine whether hair follicle structure was altered. AE13 is expressed in cortex of hair, and AE15 is expressed in inner root sheath. Results of staining of AE13 and AE15 showed that hair follicle structures were not altered in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice on 16 days after depilation (Figure 9A-D). Another differentiation marker is the terminal differentiation marker of epidermis, involucrin. Involucrin was expressed in the upper hair follicles in both conditional knockout and control group (Figure 9E, F). However, in lower hair follicle, only

conditional knockout mice showed involucrin positive signals on 16 days after depilation (Figure 9G, H). This result suggested that differentiation of stem cells in hair follicle is altered and differentiate into epidermis-like cells, which might then form horn cysts in later stage.

### **3.7 K5-driven *Cdk12* conditional knockout mice showed decreasing of bulge stem cell population but no difference was found in hair germ stem cell population after depilation**

To further investigate the roles of *Cdk12* in stem cells in hair follicles and which cell populations are affected, flow cytometry were performed to clarify. *K5-CreER*<sup>T+/0</sup>; *Cdk12*<sup>f/fx</sup> mice were injected with Tamoxifen at 6 weeks old, and separated into three groups, no depilation, single depilation (performed at two weeks after Tamoxifen injection) and repetitive depilation (performed at three weeks after single depilation). Depilation was performed on Tamoxifen treated- and vehicle treated-*K5-CreER*<sup>T+/0</sup>; *Cdk12*<sup>f/fx</sup> mice, and waited for 3 weeks till hair cycle was back to telogen phase. Keratinocytes were isolated from dorsal skins, and stained with bulge stem cell markers CD34 and CD49f (also known as  $\alpha$ 6 integrin) (da Silva-Diz et al., 2013; Solanas and Benitah, 2013). The CD34<sup>+</sup>/CD49f<sup>+</sup> cell populations were not influenced in both control and knockout mice without depilation (Figure 10A, B, G), even though the CD34<sup>+</sup> cell populations were slightly right shifted in *Cdk12* knockout mice. After single depilation, the double-positive cell populations were similar (Figure 10C, D), quantification of these results were in Figure 10G. However, once knockout mice were preformed repetitive depilation, the double-positive population was decreased (Figure 10E, F). Quantification of these results were in Figure 10H. These results suggested that the roles of *Cdk12* are possibly involved in the stem cell maintenance upon hair regeneration, and loss of *Cdk12* in basal type epithelial cells would cause bulge stem cell population reduce.

To clarify whether only the maintenance of bulge stem cells was affected, another stem cell population was examined. A small cell cluster beneath the bulge is secondary hair germ (HG, also K5<sup>+</sup> cells), and it also contained stem cells called hair germ stem cells (HGSC). These stem cells showed expression of P-cadherin and CD49f, and were

important for regeneration of matrix, the transit-amplifying cell population in growing hair follicle (da Silva-Diz et al., 2013; Hsu et al., 2014; Solanas and Benitah, 2013). The results showed that P-cadherin<sup>+</sup>/CD49f<sup>+</sup> HGSC population of *K5-CreER*<sup>T+0</sup>; *Cdk12*<sup>f/f</sup> mice had no difference between Tamoxifen and vehicle groups after depilation (Figure 11A-D), quantification of these results were in Figure 11E. Thus, even these two stem cell populations both lost *Cdk12* after Tamoxifen administration, the maintenance ability is only influenced in bulge stem cells after depilation-induced hair regeneration.

### **3.8 Increase of proliferating cells were found in epidermis but not in hair follicles in *K5-CreER*<sup>T+0</sup>; *Cdk12*<sup>f/f</sup> mice**

In addition to cell stemness and differentiation ability, proliferation is also one of the important events in hair regeneration. If cell proliferation was halted upon loss of *Cdk12*, it could result in regeneration delay as well. Ki67 staining was applied to identify this event, and increase of proliferating cells were found in epidermis of *K5-CreER*<sup>T+0</sup>; *Cdk12*<sup>f/f</sup> mice on 16 days after depilation (Figure 12A, B), but no significant alteration was found in hair follicles (Figure 12C, D). Quantification of these results were in Figure 12E and F. These results suggested that loss of *Cdk12* cause increase proliferating cells in epidermis, but not in hair follicles.

### **3.9 Loss of *Cdk12* lead to an increase of DNA double strand break in basal type epithelial cells**

During development or regeneration, multiple cell divisions occurred, which demands several rounds of DNA replication. Though the rate is pretty low, spontaneous mutations may occur, which causes proliferation stress. There are several genes acting as gate keepers, to repair mutations in the genome. DDR genes are one of these guardians. According to the previous reports, *Cdk12* regulates DDR genes, such as BRCA1, ATR, FANCI and FANCD2, to repair DNA double-strand break, thus maintaining the genomic stability. To examine whether loss of *Cdk12* would down-regulate of DDR genes, thus DNA DSBs were not repaired in proliferating cells,  $\gamma$ H2AX was used to stain DSBs. Results of  $\gamma$ H2AX staining showed that the number of

cells containing more than two  $\gamma$ H2AX foci in both hair follicles and epidermis on 16 days after depilation was significantly increased (Figure 13A, B). P37  $K5-CreER^{T+/-}$ ;  $Cdk12^{fx/fx}$  mice with Tamoxifen administration at P20 also showed increase of DSBs in both hair follicles and epidermis (Figure 13C, D). Quantification of these results were in Figure 13E-H. These results suggested that  $Cdk12$  is required to maintain genomic stability in proliferating cells and lack of genomic stability may cause loss of stem cell properties and incorrect differentiation processes.



## 4. Discussion

### 4.1 Roles of *Cdk12* in cancer formation remain unclear

Cancer formation normally correlated with gain-of-function mutations of oncogenes and loss-of-function of tumor suppressor genes. Normally a solid tumor needs mutations on about 5 to 7 specific genes to generate an abnormal cell growth (Stratton et al., 2009). In this study, cancer formation was not observed in *Cdk12* conditional knockout mice. This might be due to that a single gene mutation is not sufficient to form tumors. To accelerate tumor progression, many studies crossed mice with common mutations on tumor suppressor genes, such as *Trp53* (Jonkers et al., 2001; Liu et al., 2007; Poole et al., 2006; Xu et al., 1999). In this study I tried to cross *Cdk12* conditional knockout mice with *Trp53* conditional knockout mice. However, as these two genes were on the same arm of chromosome 11, genotyping of over 30 offspring of these two mice did not give rise to any homozygous mouse. Screening of more pups might generate the double knockout mice. Due to the successful ratio was very low, this set of experiments was halted. In addition to investigate the roles of *Cdk12* in cancer formation by spontaneous mutation accumulation, introducing mutagens like 12-O-tetradecanoylphorbol-13-acetate (TPA) or diethylnitrosamine (DEN) to organs, such as skin and liver, is another way to accelerate the tumor progression.

### 4.2 Hair loss phenotypes were reported in *K5*-driven *Cdk12* conditional knockout mice

In this study, hairs in several regions of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice were lost but some of hair follicles can still generate new hairs for several times. This might be due to the efficiency of inducible conditional knockout mice, as not all of *CreER<sup>T+</sup>* cells can be induced to knockout *Cdk12*. I also tried to link this phenotype to human disease. The pathology of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice is similar to seborrheic keratosis, a human cutaneous disease with accumulation of melanin and horn cysts that generally happen in elders (Choi et al., 2007; Hafner and Vogt, 2008). However, after detailed investigation, although horn cysts are observed in both phenotypes, the position of horn cysts are different: horn cysts appear in the epidermis in seborrheic keratosis, but horn cysts are mostly in dermis in aged *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice.

Therefore the phenotypes in *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>del/fx</sup> mice are not the same as other human cutaneous diseases.

#### **4.3 *Cdk12* is required for hair follicle maintenance**

Hair loss phenotype and horn cyst formation were observed in other gene mutations. One of the most famous gene is *Hairless* (*Hr*) (Benavides et al., 2009; Liu et al., 2010). *Hr* mutation mice showed strong phenotype on skin with serious hair loss. The mechanism of its function is that apoptosis of hair follicle cells in catagen phase is incomplete, thus the bulb part of hair follicle remains in the lower part of dermis and form horn cysts in the end. This incomplete apoptosis also causes the defects of restoration of dermal papilla such that the signal from dermal papilla to trigger the next hair cycle is absent, and the ability of hair follicles to regenerate new hair is lost. Comparing to this study, after the first spontaneous hair replacement occurred on 3~4 weeks old, several cysts were observed in mostly upper part of dermis. Despite the positions of horn cysts are not exactly the same between *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>del/fx</sup> mice and *Hr* mutant mice, sections of *Hr* mutant mice under UVR-induced tumor development show similar phenotypes of severe horn cysts formation and thicker epidermis as aged *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>del/fx</sup> mice (Benavides et al., 2009). The correlation of these two genes is still ambiguous. Further tests are needed to examine whether expression level of *Hr* is affected upon loss of *Cdk12* in skin.

#### **4.4 *Cdk12* plays an important role in maintenance of stem cells**

Immunostaining of hair follicle stem cell markers showed that loss of *Cdk12* causes the alteration of stem cell markers, which might influence the ability of hair follicle stem cells to generate new cells or self-renewal. Flow cytometry also showed that bulge stem cell population was decreased upon loss of *Cdk12*. Our laboratory showed that loss of *Cdk12* in CNS can cause cell death in progenitor pool and affect neuronal migration (Chen et al., unpublished data). Another study of our laboratory showed embryo lethality in *Cdk12* deficient mice at E3.5~6.5, and caused apoptosis in inner cell mass (Juan et al., unpublished data). Previous study also reported that *Cyclin K/Cdk12* complex was important for the maintenance of self-renewal in murine

embryonic stem cells (Dai et al., 2012). Though the types of stem cells are distinct, the general roles of *Cdk12* seem to be correlated with the maintenance of stem cells.

#### 4.5 *Cdk12* acts as the guardian of genome

It's been reported that *Cdk12* can regulate DDR genes such as *BRCA1*, *ATR*, *FANCI* and *FANCD2*, which means loss of *Cdk12* reduces homologous repair, and more DSBs can be accumulated in cells (Bajrami et al., 2013; Blazek et al., 2011; Ekumi et al., 2015; Joshi et al., 2014). Here, results of  $\gamma$ H2AX staining showed that the number of cells containing DSBs was significantly increased in both hair follicles and epidermis after depilation and spontaneous hair growth. Thus these results suggest that role of *Cdk12* is to prevent accumulation of spontaneous DNA damages while proliferation through regulation of DDR genes.

Taken together, in this study I revealed the potential roles of *Cdk12* in skin, and found that loss of *Cdk12* in skin caused accumulation of DNA damages in proliferating cells, which leads to alter cell differentiation. Consequently, hair loss was observed due to failure of hair regeneration (Figure 14).

## 5. Reference

- Bösken, C.A., Farnung, L., Hintermair, C., Merzel Schachter, M., Vogel-Bachmayr, K., Blazek, D., Anand, K., Fisher, R.P., Eick, D., and Geyer, M. (2014). The structure and substrate specificity of human Cdk12/Cyclin K. *Nature Communications* 5.
- Bajrami, I., Frankum, J.R., Konde, A., Miller, R.E., Rehman, F.L., Brough, R., Campbell, J., Sims, D., Rafiq, R., Hooper, S., *et al.* (2013). Genome-wide Profiling of Genetic Synthetic Lethality Identifies CDK12 as a Novel Determinant of PARP1/2 Inhibitor Sensitivity. *Cancer research* 74, 287-297.
- Bartkowiak, B., Liu, P., Phatnani, H.P., Fuda, N.J., Cooper, J.J., Price, D.H., Adelman, K., Lis, J.T., and Greenleaf, A.L. (2010). CDK12 is a transcription elongation-associated CTD kinase, the metazoan ortholog of yeast Ctk1. *Genes & development* 24, 2303-2316.
- Benavides, F., Oberyszyn, T.M., VanBuskirk, A.M., Reeve, V.E., and Kusewitt, D.F. (2009). The hairless mouse in skin research. *Journal of dermatological science* 53, 10-18.
- Blazek, D., Kohoutek, J., Bartholomeeusen, K., Johansen, E., Hulinkova, P., Luo, Z., Cimermancic, P., Ule, J., and Peterlin, B.M. (2011). The Cyclin K/Cdk12 complex maintains genomic stability via regulation of expression of DNA damage response genes. *Genes & development* 25, 2158-2172.
- Cancer Genome Atlas Research, N. (2011). Integrated genomic analyses of ovarian carcinoma. *Nature* 474, 609-615.
- Carter, S.L., Cibulskis, K., Helman, E., McKenna, A., Shen, H., Zack, T., Laird, P.W., Onofrio, R.C., Winckler, W., Weir, B.A., *et al.* (2012). Absolute quantification of somatic DNA alterations in human cancer. *Nature biotechnology* 30, 413-421.
- Chen, H.-R., Lin, G.-T., Huang, C.-K., and Fann, M.-J. (2014). Cdk12 and Cdk13 regulate axonal elongation through a common signaling pathway that modulates Cdk5 expression. *Experimental Neurology* 261, 10-21.
- Chen, H.H., Wang, Y.C., and Fann, M.J. (2006). Identification and characterization of the CDK12/cyclin L1 complex involved in alternative splicing regulation. *Molecular and cellular biology* 26, 2736-2745.

Choi, H.-J., Yun, S.-K., Kim, H.-U.K., and Ihm, C.-W. (2007). Squamous eddies in irritated seborrheic keratosis. *The American Journal of Dermatopathology* 29, 28-31.

da Silva-Diz, V., Solé-Sánchez, S., Valdés-Gutiérrez, A., Urpí, M., Riba-Artés, D., Penin, R.M., Pascual, G., González-Suárez, E., Casanovas, O., Viñals, F., *et al.* (2013). Progeny of Lgr5-expressing hair follicle stem cell contributes to papillomavirus-induced tumor development in epidermis. *Oncogene* 32, 3732-3743.

Dai, Q., Lei, T., Zhao, C., Zhong, J., Tang, Y.Z., Chen, B., Yang, J., Li, C., Wang, S., Song, X., *et al.* (2012). Cyclin K-containing kinase complexes maintain self-renewal in murine embryonic stem cells. *The Journal of biological chemistry* 287, 25344-25352.

Ekumi, K.M., Paculova, H., Lenasi, T., Pospichalova, V., Bösken, C.A., Rybarikova, J., Bryja, V., Geyer, M., Blazek, D., and Barboric, M. (2015). Ovarian carcinoma CDK12 mutations misregulate expression of DNA repair genes via deficient formation and function of the Cdk12/CycK complex. *Nucleic Acids Research*, gkv101.

Hafner, C., and Vogt, T. (2008). Seborrheic keratosis. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft* 6, 664-677.

Hsu, Y.-C., Li, L., and Fuchs, E. (2014). Transit-Amplifying Cells Orchestrate Stem Cell Activity and Tissue Regeneration. *Cell* 157, 935-949.

Iorns, E., Martens-de Kemp, S.R., Lord, C.J., and Ashworth, A. (2009). CRK7 modifies the MAPK pathway and influences the response to endocrine therapy. *Carcinogenesis* 30, 1696-1701.

Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. *Nature Genetics* 29, 418-425.

Joshi, P.M., Sutor, S.L., Huntoon, C.J., and Karnitz, L.M. (2014). Ovarian Cancer-Associated Mutations Disable Catalytic Activity of CDK12, A Kinase That Promotes Homologous Recombination Repair and Resistance to Cisplatin and

Poly(ADP-Ribose) Polymerase Inhibitors. The Journal of biological chemistry.

- Liang, C.C., You, L.R., Chang, J.L., Tsai, T.F., and Chen, C.M. (2009). Transgenic mice exhibiting inducible and spontaneous Cre activities driven by a bovine keratin 5 promoter that can be used for the conditional analysis of basal epithelial cells in multiple organs. *Journal of biomedical science* 16, 2.
- Liu, X., Holstege, H., Gulden, H.v.d., Treur-Mulder, M., Zevenhoven, J., Velds, A., Kerkhoven, R.M., Vliet, M.H.v., Wessels, L.F.A., Peterse, J.L., *et al.* (2007). Somatic loss of BRCA1 and p53 in mice induces mammary tumors with features of human BRCA1-mutated basal-like breast cancer. *Proceedings of the National Academy of Sciences* 104, 12111-12116.
- Liu, Y., Sundberg, J.P., Das, S., Carpenter, D., Cain, K.T., Michaud, E.J., and Voy, B.H. (2010). Molecular basis for hair loss in mice carrying a novel nonsense mutation (Hrrh-R ) in the hairless gene (Hr). *Veterinary pathology* 47, 167-176.
- Lu, T.L., Huang, Y.F., You, L.R., Chao, N.C., Su, F.Y., Chang, J.L., and Chen, C.M. (2013). Conditionally ablated Pten in prostate basal cells promotes basal-to-luminal differentiation and causes invasive prostate cancer in mice. *The American journal of pathology* 182, 975-991.
- Müller-Röver, S., Handjiski, B., van der Veen, C., Eichmüller, S., Foitzik, K., McKay, I.A., Stenn, K.S., and Paus, R. (2001). A Comprehensive Guide for the Accurate Classification of Murine Hair Follicles in Distinct Hair Cycle Stages. *Journal of Investigative Dermatology* 117, 3-15.
- Natrajan, R., Wilkerson, P.M., Marchio, C., Piscuoglio, S., Ng, C.K., Wai, P., Lambros, M.B., Samartzis, E.P., Dedes, K.J., Frankum, J., *et al.* (2014). Characterization of the genomic features and expressed fusion genes in micropapillary carcinomas of the breast. *The Journal of pathology* 232, 553-565.
- Nowak, J.A., and Fuchs, E. (2009). Isolation and Culture of Epithelial Stem Cells. *Methods in molecular biology* (Clifton, NJ) 482, 215-232.
- Plikus, M.V., Baker, R.E., Chen, C.-C., Fare, C., Cruz, D.d.l., Andl, T., Maini, P.K., Millar, S.E., Widelitz, R., and Chuong, C.-M. (2011). Self-Organizing and Stochastic Behaviors During the Regeneration of Hair Stem Cells. *Science* 332,

586-589.

- Poole, A.J., Li, Y., Kim, Y., Lin, S.-C.J., Lee, W.-H., and Lee, E.Y.-H.P. (2006). Prevention of Brca1-Mediated Mammary Tumorigenesis in Mice by a Progesterone Antagonist. *Science* *314*, 1467-1470.
- Schneider, M.R., Schmidt-Ullrich, R., and Paus, R. (2009). The Hair Follicle as a Dynamic Miniorgan. *Current Biology* *19*, R132-R142.
- Solanas, G., and Benitah, S.A. (2013). Regenerating the skin: a task for the heterogeneous stem cell pool and surrounding niche. *Nature Reviews Molecular Cell Biology* *14*, 737-748.
- Stratton, M.R., Campbell, P.J., and Futreal, P.A. (2009). The cancer genome. *Nature* *458*, 719-724.
- Xu, X., Wagner, K.-U., Larson, D., Weaver, Z., Li, C., Ried, T., Hennighausen, L., Wynshaw-Boris, A., and Deng, C.-X. (1999). Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nature Genetics* *22*, 37-43.
- Zang, Z.J., Ong, C.K., Cutcutache, I., Yu, W., Zhang, S.L., Huang, D., Ler, L.D., Dykema, K., Gan, A., Tao, J., *et al.* (2011). Genetic and structural variation in the gastric cancer kinome revealed through targeted deep sequencing. *Cancer research* *71*, 29-39.

## 6. Tables

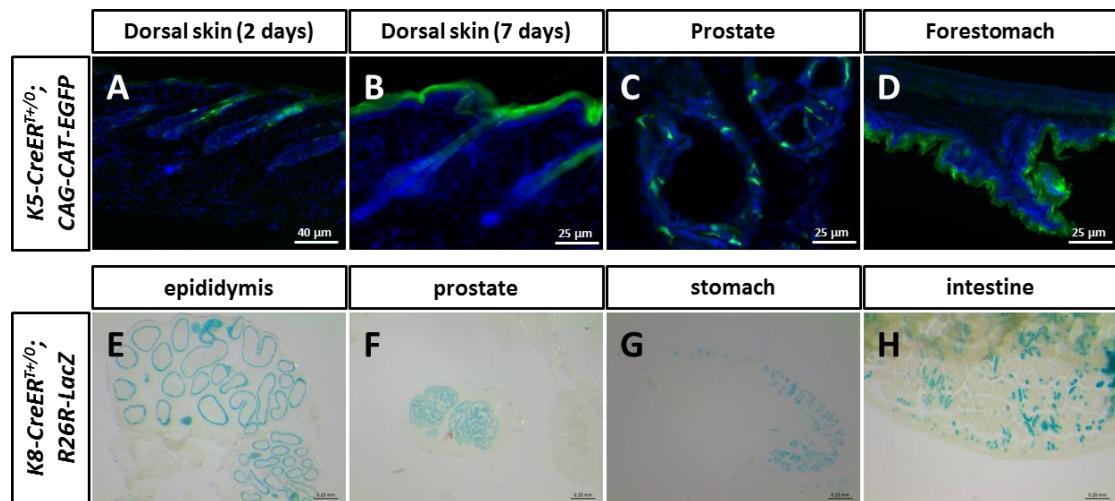
**Table. 1 Primers used in this project**

Primers	Sequence (5'→3')
Del-3	CTTCCTGCCTCCTCTTCATCATCAGGTATTG
Fx-2	GTTCAGACAGTGTCAAGACCCACCTGGAGAACG
Y-3	CCTCTGACCTCCAATGTGTGCATGACAC
Cre-1	GGACATGTTCAAGGGATGCCAGGCG
Cre-β	CGACGATGAAGCATGTTAGCTG
EGFP-1	GGTACATTGAGCAACTGACTG
EGFP-2	CTGCTAACCATGTTCATGCC
Rosa-1	GCGAAGAGTTGTCCTCAACC
Rosa-2	GGAGCGGGAGAAATGGATATG
Rosa-3	AAAGTCGCTCTGAGTTGTTAT

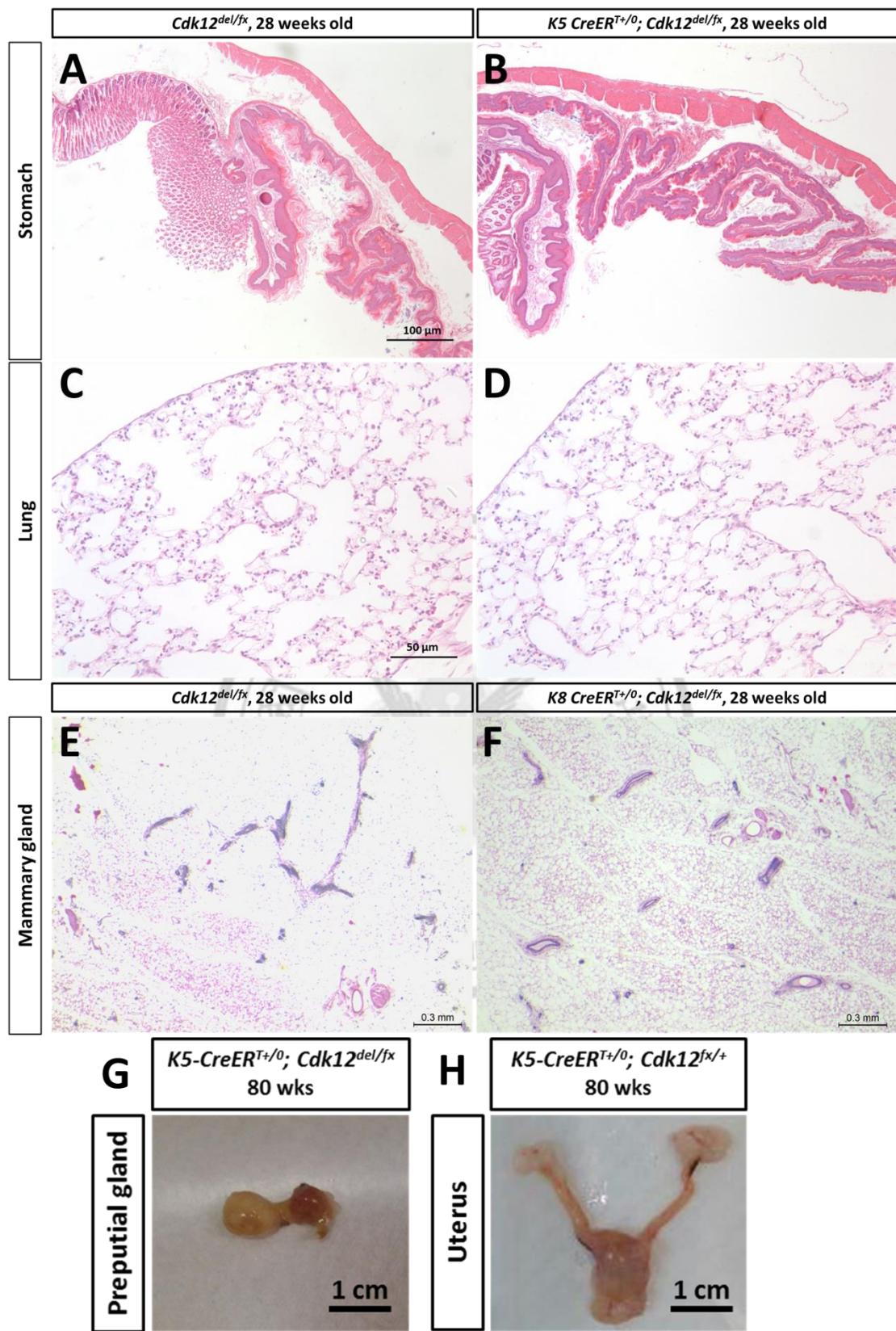
**Table. 2 Primary antibodies used in this project**

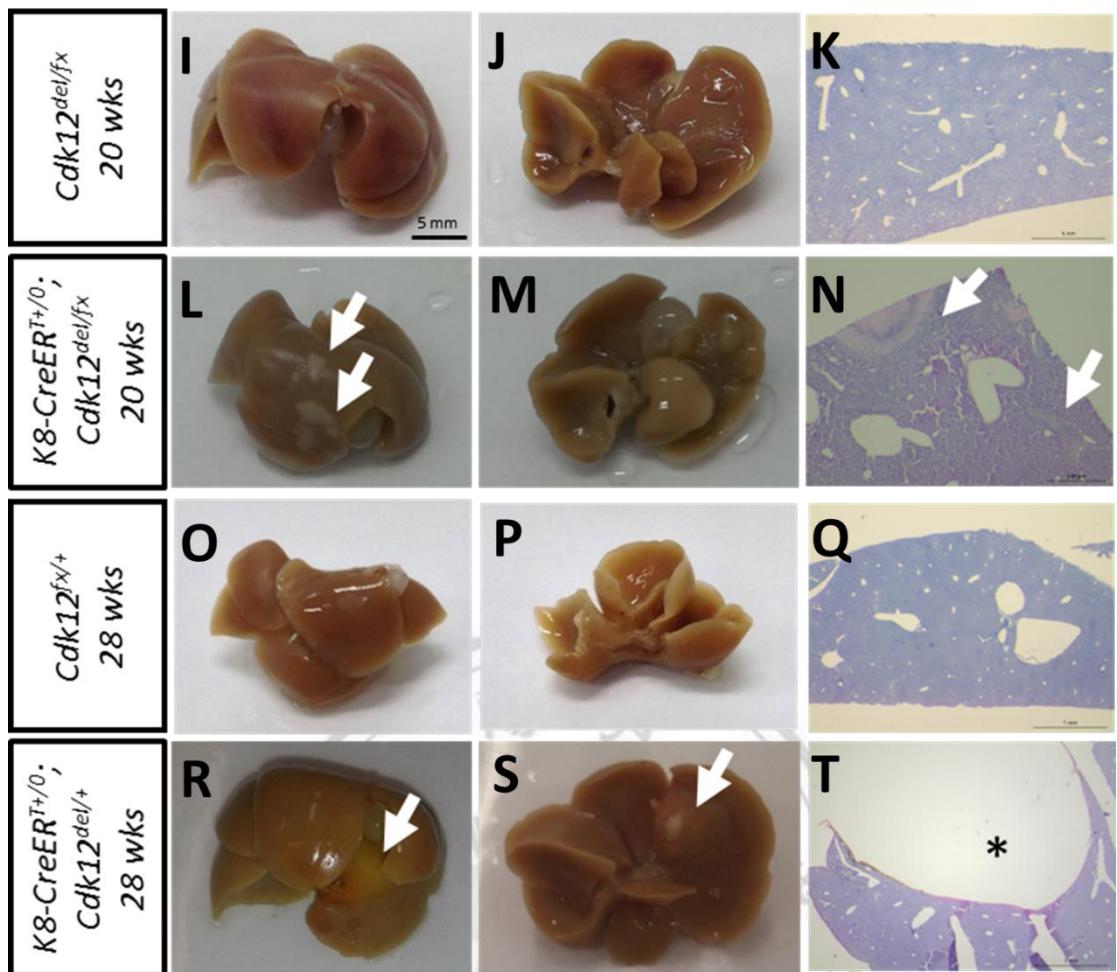
Antibody	Specie	Dilution ratio	Resource
AE13	Mouse	1:100	Abcam
AE15	Mouse	1:100	Abcam
CD34	Rabbit	1:1000	Biorbyt
CD34-FITC	Rabbit	1:50	eBioscience
CD49f-APC	Rabbit	1:40	R&D
CD49f-FITC	Rabbit	1:40	BioLegend
Cleaved Caspase 3	Rabbit	1:300	Cell signaling
$\gamma$ H2AX	Mouse	1:300	Millipore
Involucrin	Rabbit	1:1000	Abcam
Keratin 6	Mouse	1:100	Abcam
Keratin 15	Rabbit	1:500	Abcam
Ki67	Rabbit	1:500	Abcam
P-cadherin-APC	Rabbit	1:20	R&D
Sox9	Mouse	1:500	Abnova
53BP1	Rabbit	1:1000	Santa Cruz

## 7. Figures

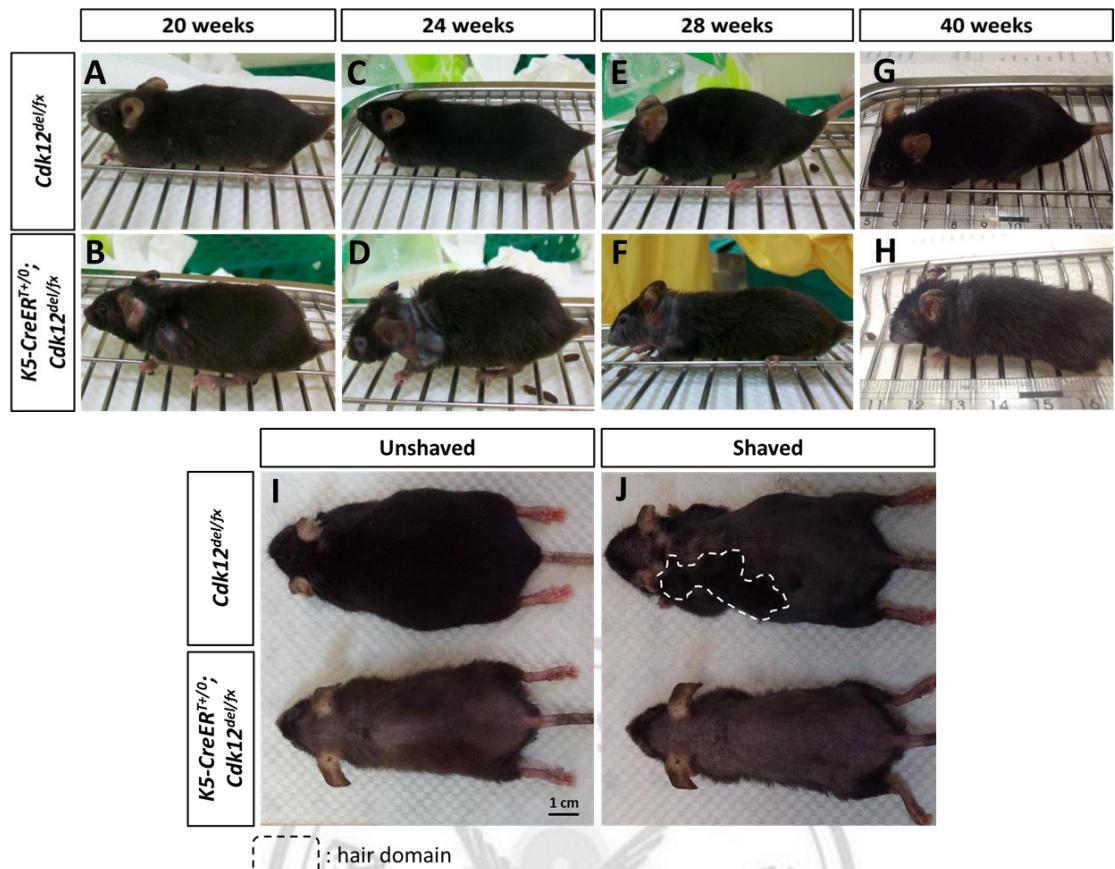


**Figure 1. *K5-* and *K8-CreER<sup>T</sup>* recombinase activity was successfully turned on after Tamoxifen administration in basal and luminal type epithelial cells.** (A-D) *K5-CreER<sup>T/+0</sup>; CAG-CAT-EGFP* mice were administrated with Tamoxifen to induce the activity of CreER<sup>T</sup> in basal type epithelial cells, EGFP signals were detected in dorsal skin, prostate and forestomach. (E-H) *K8-CreER<sup>T/+0</sup>; R26R-LacZ* mice were administrated with Tamoxifen to induce the activity of CreER<sup>T</sup> in luminal type epithelial cells, X-gal staining showed that CreER<sup>T</sup> activity can be detected in epididymis, prostate, stomach and intestine.

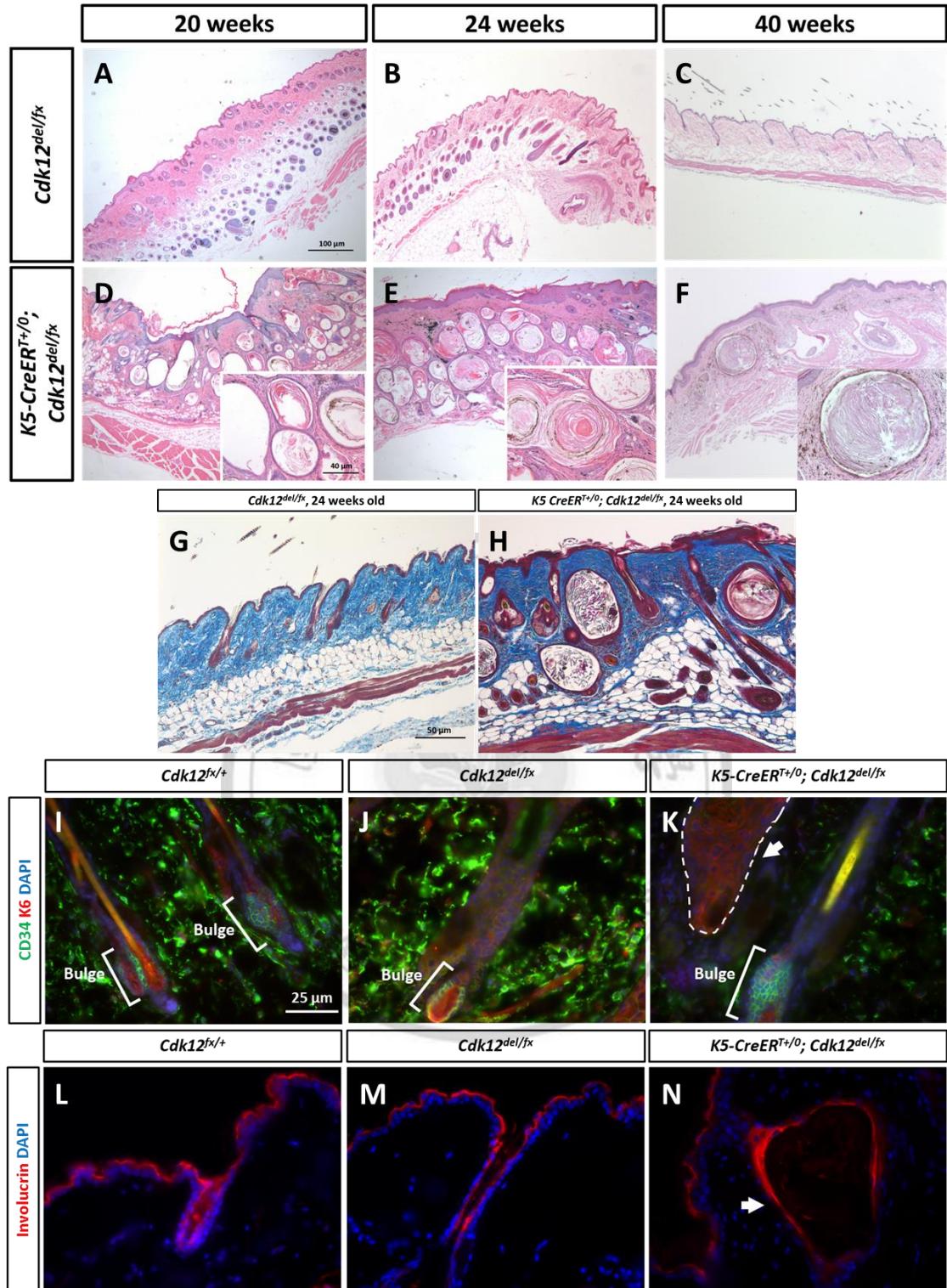


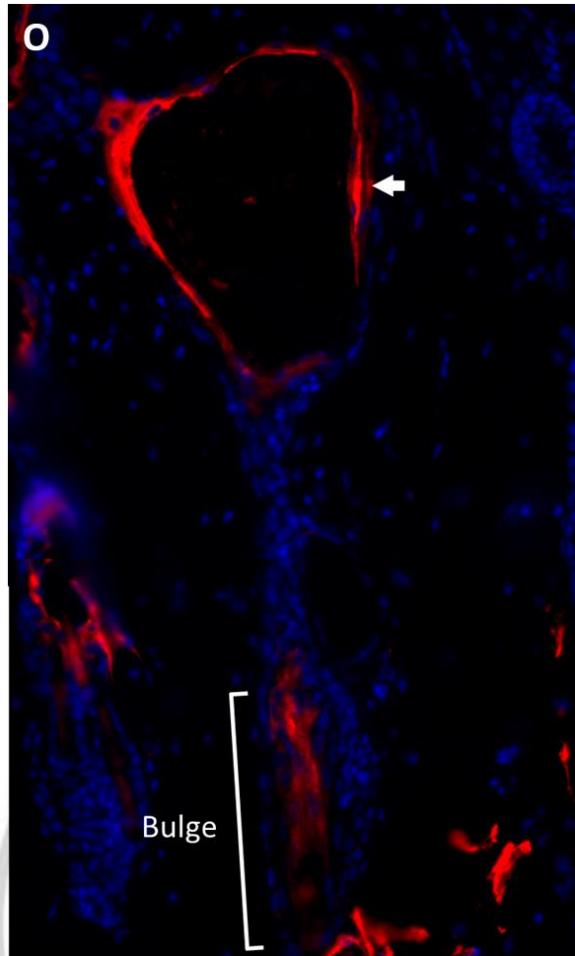


**Figure 2. Tumor formation was not observed in both K5- and K8-driven Cdk12 conditional knockout mice.** *K5-CreER<sup>T+/0</sup>; Cdk12<sup>del/fx</sup>* mice and control *Cdk12<sup>del/fx</sup>* mice were dissected and organs were sectioned. Stomach (A, B) and lung (C, D) on 28 weeks. *K8-CreER<sup>T+/0</sup>, Cdk12<sup>del/fx</sup>* mice and control mice were dissected and mammary gland (E, F) was sectioned. Tumors were found in preputial gland (G) in one *K5-CreER<sup>T+/0</sup>; Cdk12<sup>del/fx</sup>* mice and in uterus (H) in one *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/+</sup>* mice at 80 weeks old, only these two mice from over 20 mice after 80 weeks old. Necrotic cells were found in liver in one *K8-CreER<sup>T+/0</sup>, Cdk12<sup>del/fx</sup>* mice at 20 weeks old (L-N, arrow), but not in *Cdk12<sup>del/fx</sup>* mice (I-K). A cyst was found in liver in one *K8-CreER<sup>T+/0</sup>, Cdk12<sup>del/fx</sup>* mice at 28 weeks old (R-T), but not in *Cdk12<sup>del/fx</sup>* mice (O-Q, arrow and asterisk.), only these two mice from over 15 mice after 80 weeks old.

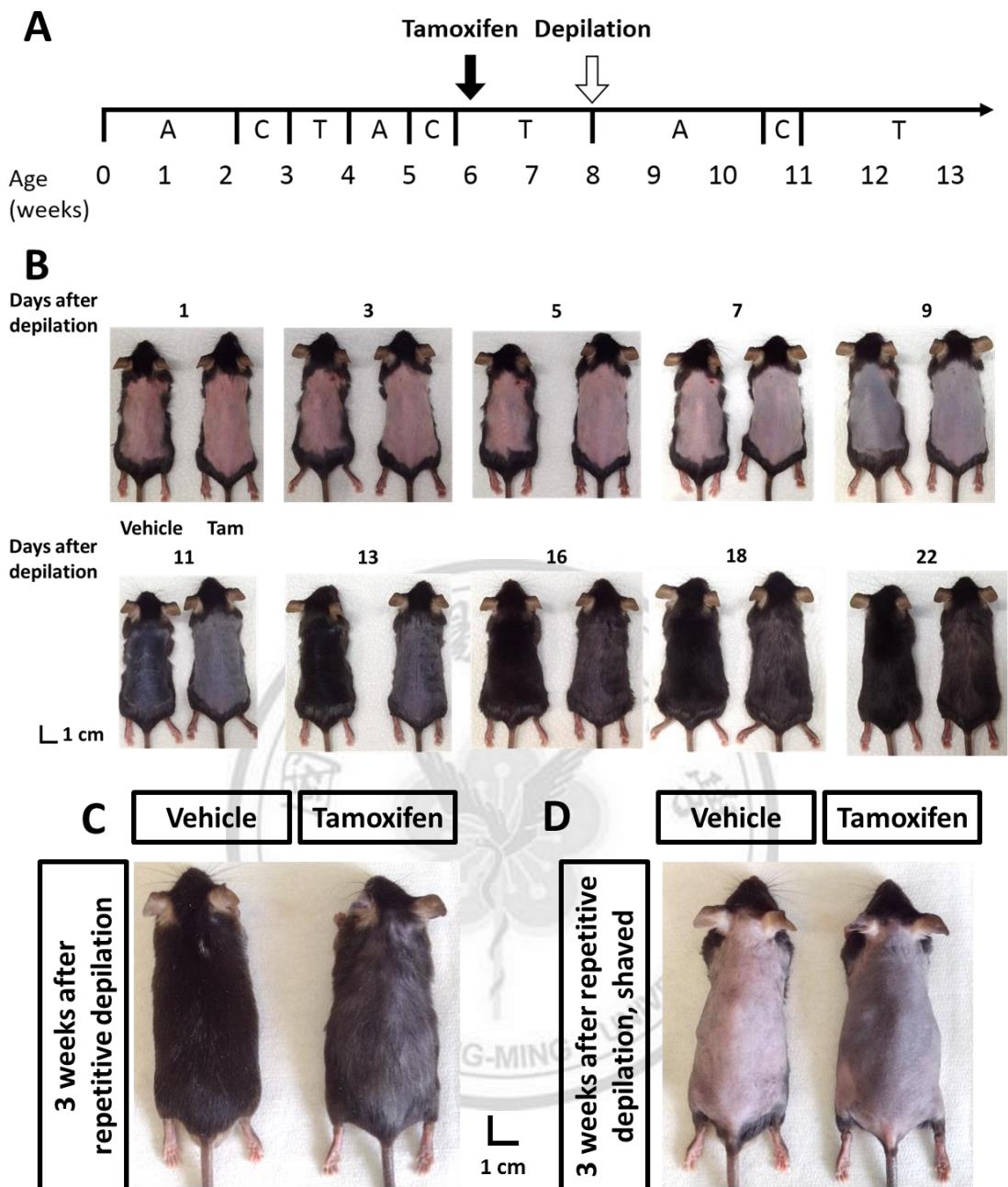


**Figure 3. Hair loss and less hair growth were found in *K5*-dirven *Cdk12* conditional knockout mice.** (A-H) Hair loss was observed in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice after 20 weeks old. (I, J) Hair of aged *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* and control mice were shaved to observe the hair growing state, and hair domains were observed in control mice, but no obvious hair domains were found in mutant mice.

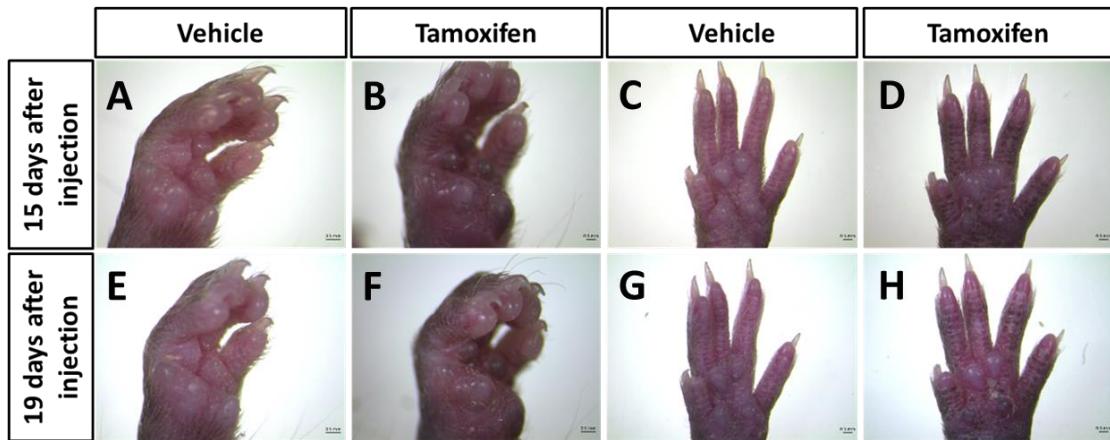




**Figure 4. Loss of stem cell marker and epidermal marker were found in horn cysts and hair follicle in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice.** (A-C, G) Skins sections of *Cdk12<sup>del/fx</sup>* mice were stained with hematoxylin & eosin staining or Masson's trichrome staining to investigate the structure of epidermis and hair follicles at 20, 24 and 40 weeks old. (D-F, H) However, *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice contained several onion-like cysts and melanin accumulation. (I-K) To test the hair follicle stem cell properties, stem cell markers, CD34 and keratin 6 (K6) were tested by immunostaining. In contrast to control mice, *K5-CreER<sup>T+/-</sup>; Cdk12<sup>del/fx</sup>* mice showed absence of CD34 signal and increased signal of K6 in destructural hair follicles (arrow, destructural hair follicles). (L-N) To test whether differentiation process was affected, epidermis marker involucrin was used to examine. Normally, involucrin staining showed expression in epidermis and the upper part of hair follicles, (M) but positive signals of involucrin were observed in horn cysts in knockout mice (arrow, horn cyst). (O) Enlarged figure of (M), positive signals of involucrin were observed in bulge.

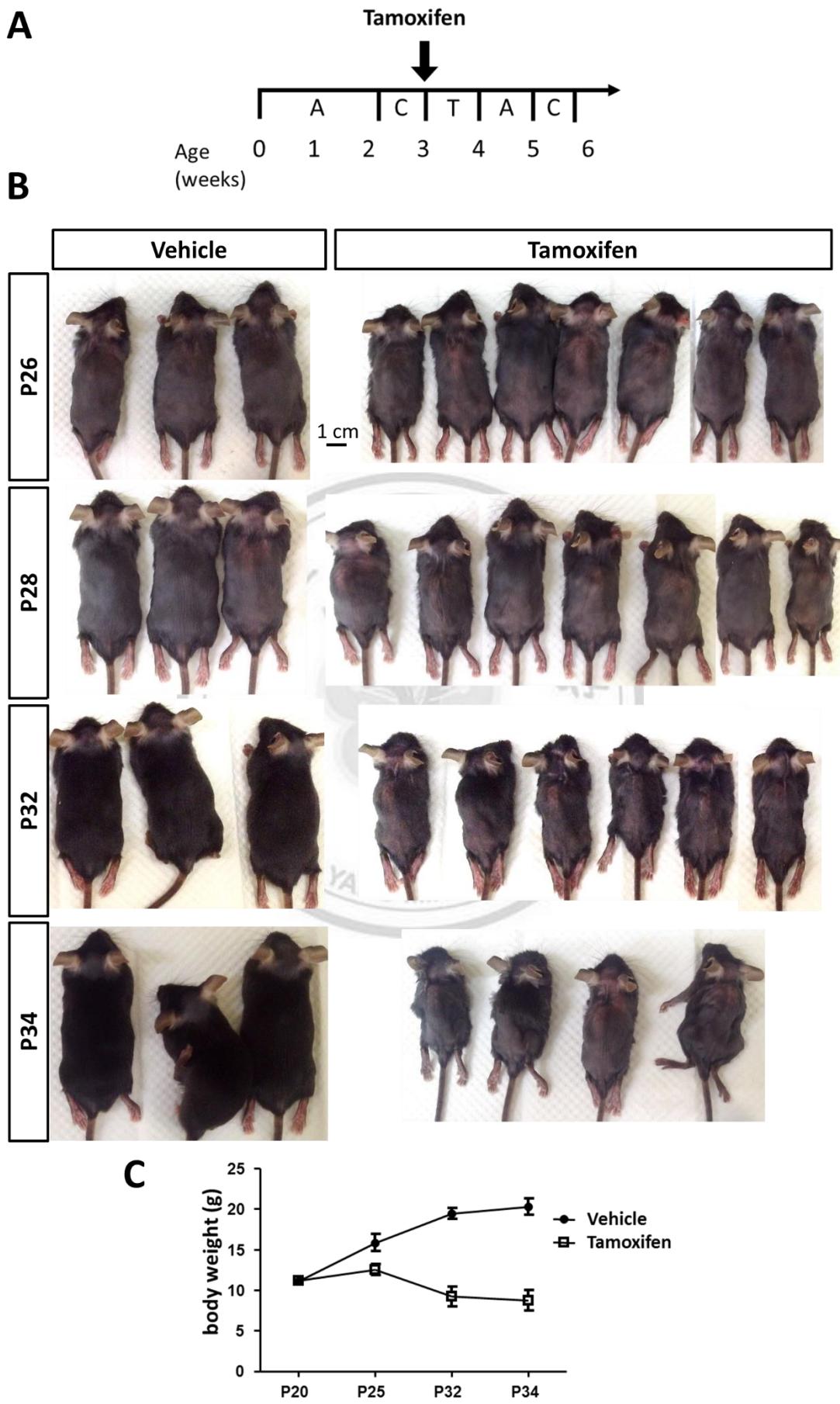


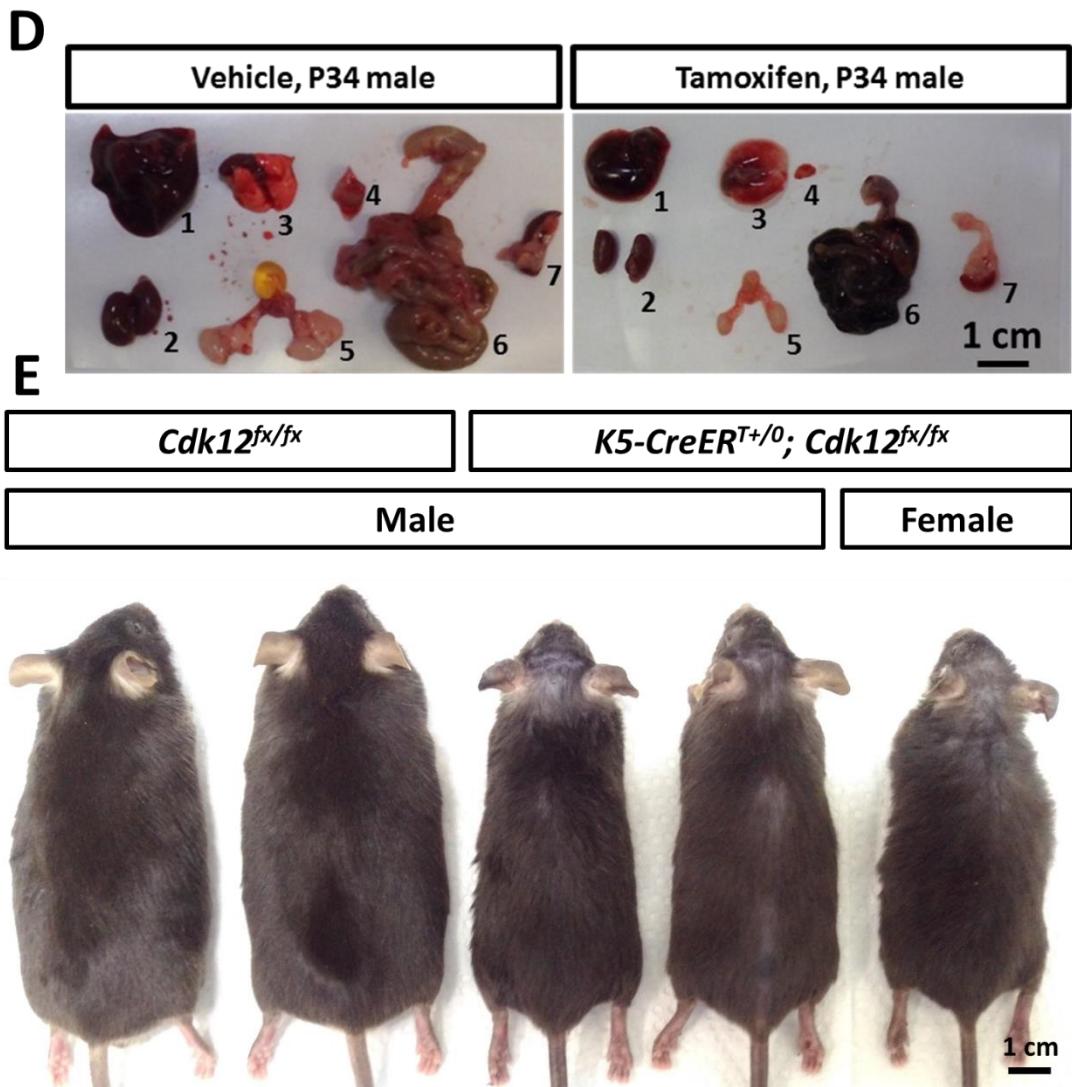
**Figure 5. Hair regeneration of K5-driven *Cdk12* conditional knockout mice after depilation.** (A) Schematic diagram of hair cycle status with depilation treatment. A: anagen, C: catagen and T: telogen. (B) Pictures of hair regeneration processes of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice and control mice after depilation. Hair regeneration after depilation was delayed for 2~3 days in mutant mice in comparison to control mice. (C) Repetitive depilation was performed after the first one to induce secondary hair regeneration. Hairs of *Cdk12* conditional knockout mice showed more severe hair loss phenotype after repeated depilation. (D) Mice were shaved to observe the skin color, and *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice showed darker skin color than control mice.



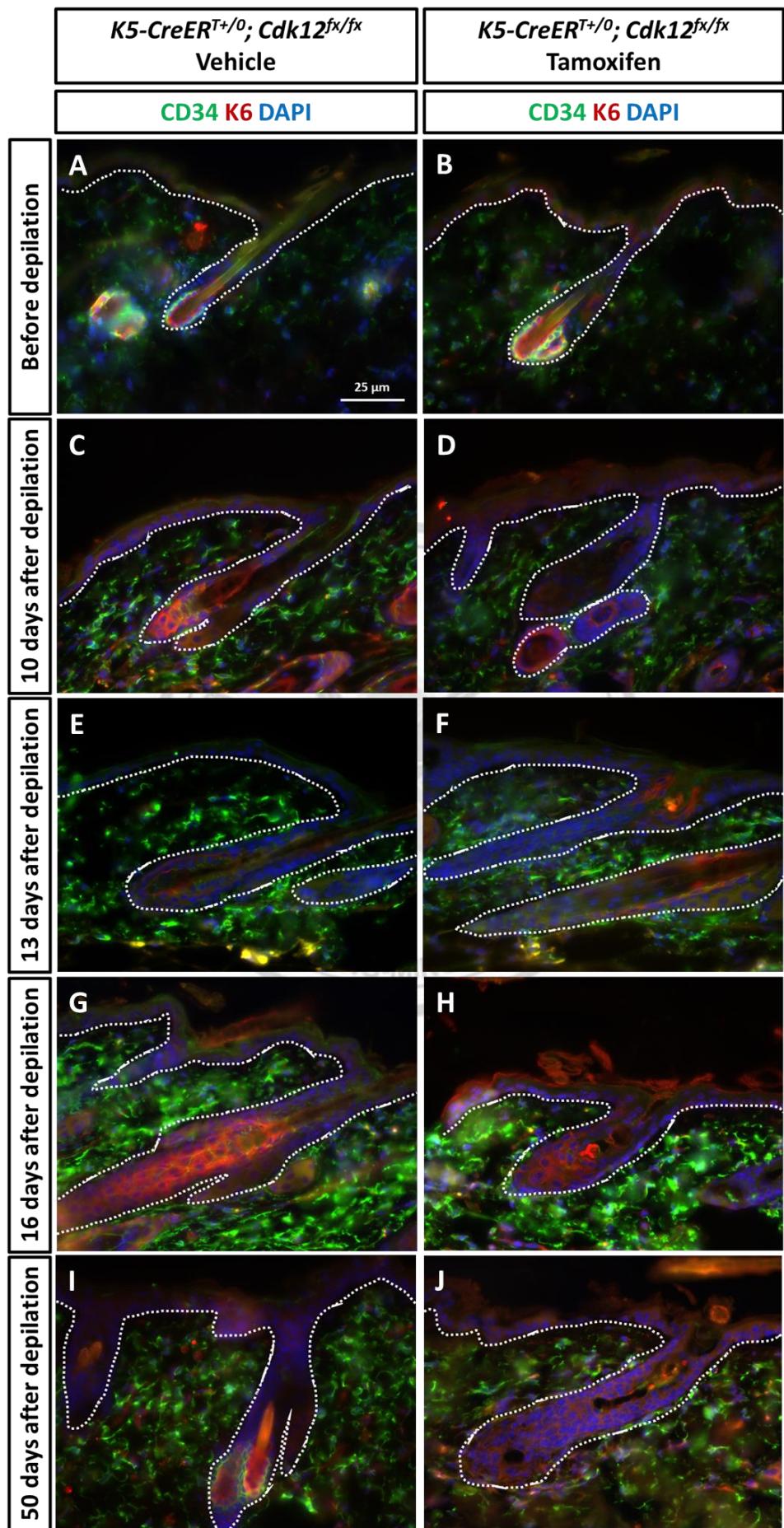
**Figure 6. Investigation of paws of K5-driven *Cdk12* conditional knockout mice after depilation.** Pictures of forepaws and (A-D) hind paws of *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice on 15 day and on (E-H) 19 days after Tamoxifen or vehicle injection, paws of Tamoxifen groups showed a darker skin and slightly sallow than vehicle control.

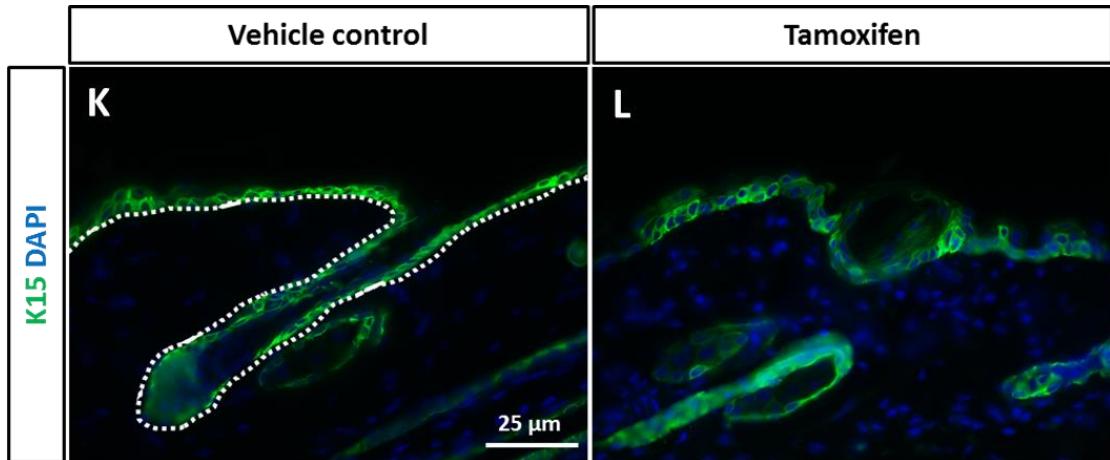




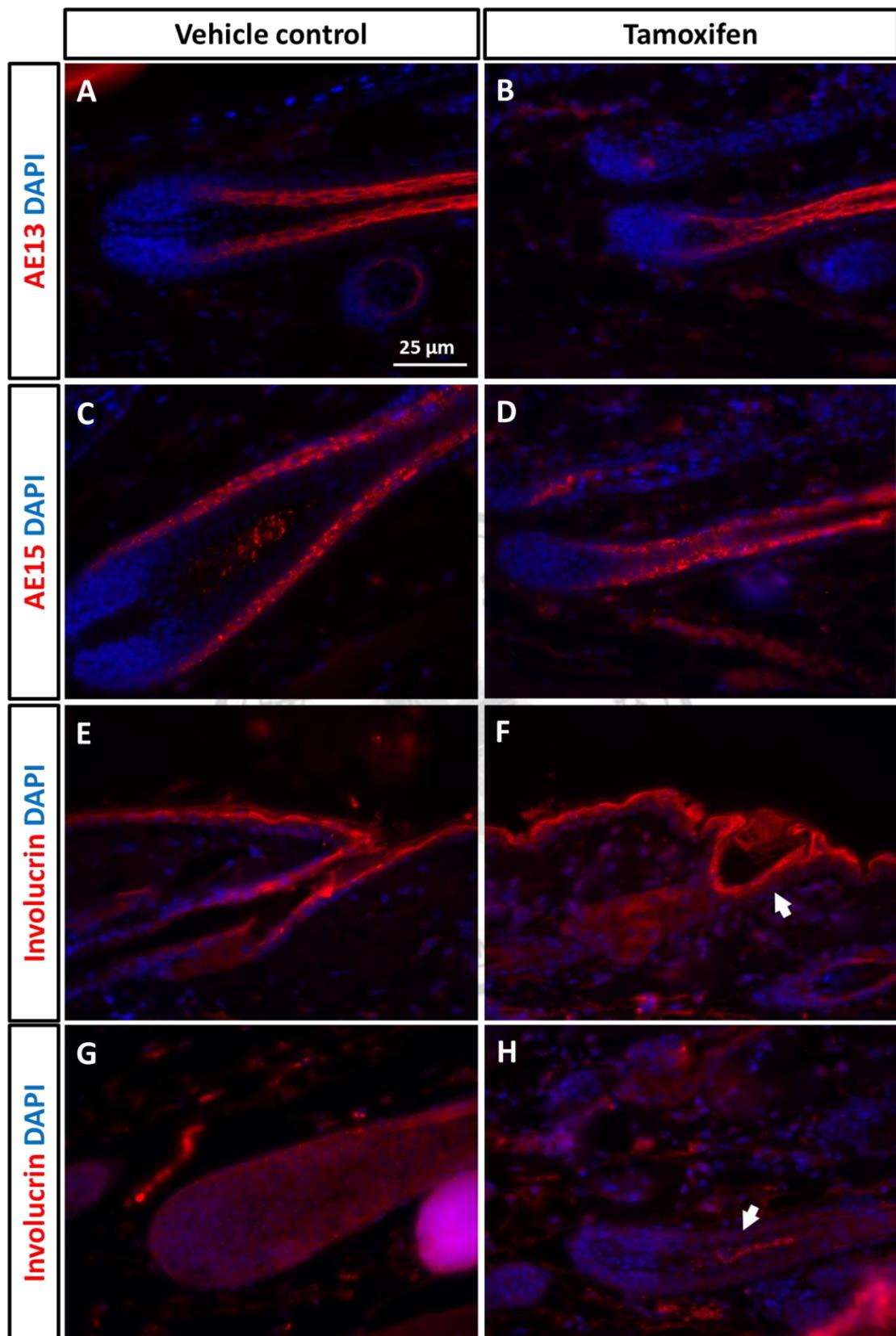


**Figure 7. Investigation of *K5*-driven *Cdk12* conditional knockout mice with spontaneous hair growth.** (A)Schematic diagram of hair cycle status. A: anagen, C: catagen and T: telogen. (B) Hair regeneration processes were investigated in *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen administration at 3 weeks old, and delay of hair regeneration were observed. (C) Dramatic body weight loss was found in *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen administration at 3 weeks old. (D) *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/fx</sup>* mice were dissected at P34 and they had seriously gastrointestinal bleeding. (1. Liver, 2. kidney, 3. lung and heart, 4. thymus, 5. reproductive organs, 6. digestive tract, 7. pancreas and spleen.) (E) Pictures of *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/fx</sup>* mice at 20 weeks old. Hair loss was observed.





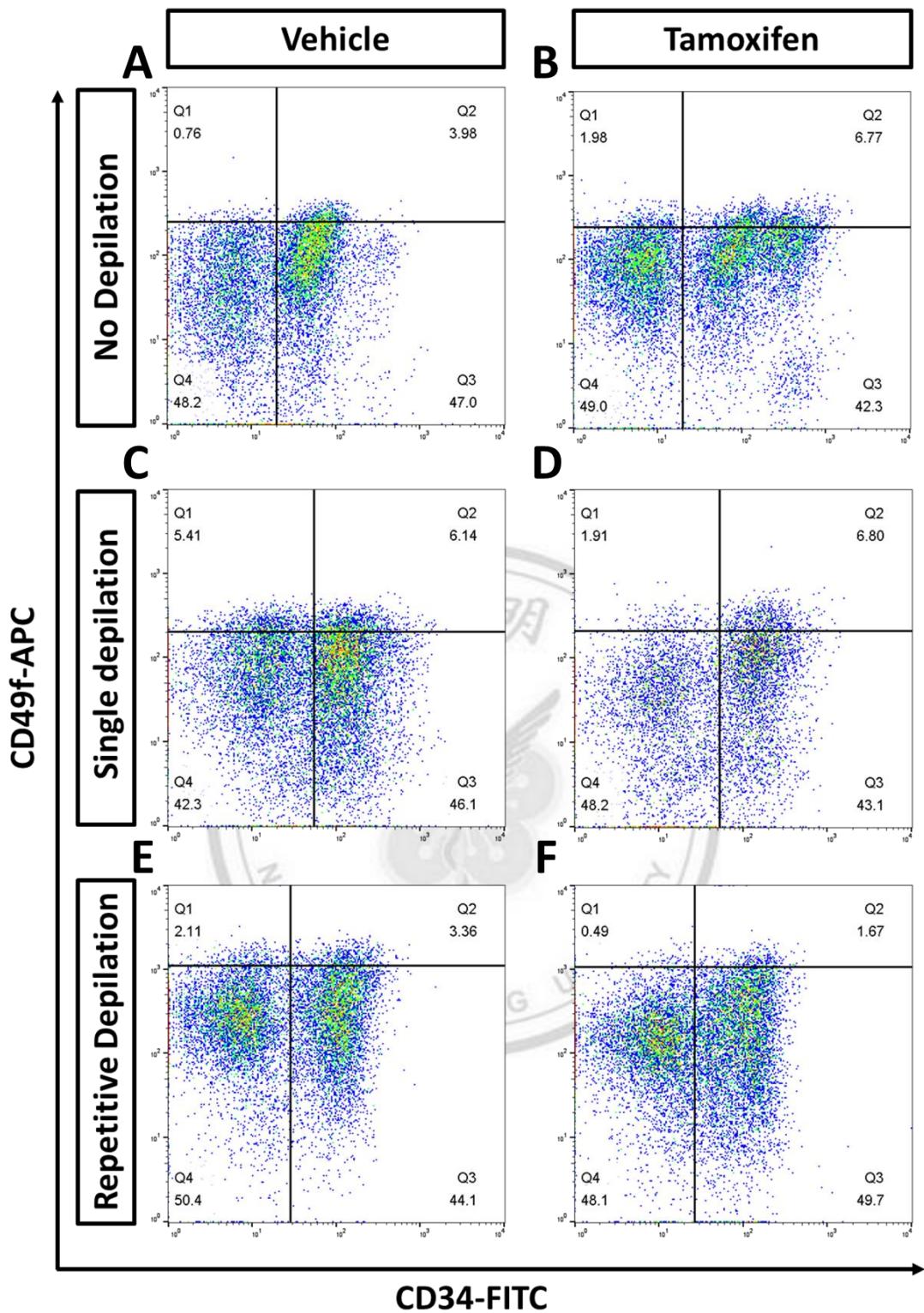
**Figure 8. *K5*-driven *Cdk12* conditional knockout mice showed alterations on stem cell markers after depilation.** *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice were injected with Tamoxifen at 6 weeks old, and depilation was operated two weeks later. Immunostaining of stem cell markers was performed to test the stem cell properties. (A-H) CD34 signal was absent and K6 signal was increased in the hair follicles in *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice on 10, 13, 16 and 50 days after depilation, but no changes were found before depilation. (I, J) Patterns of K15 staining were the same between *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen administration and vehicle control at 10 days after depilation. A notable difference was that horn cysts located on the top layer of skin was also positive to K15 stem cell markers (arrow, horn cyst). ( $n \geq 3$  for each groups)

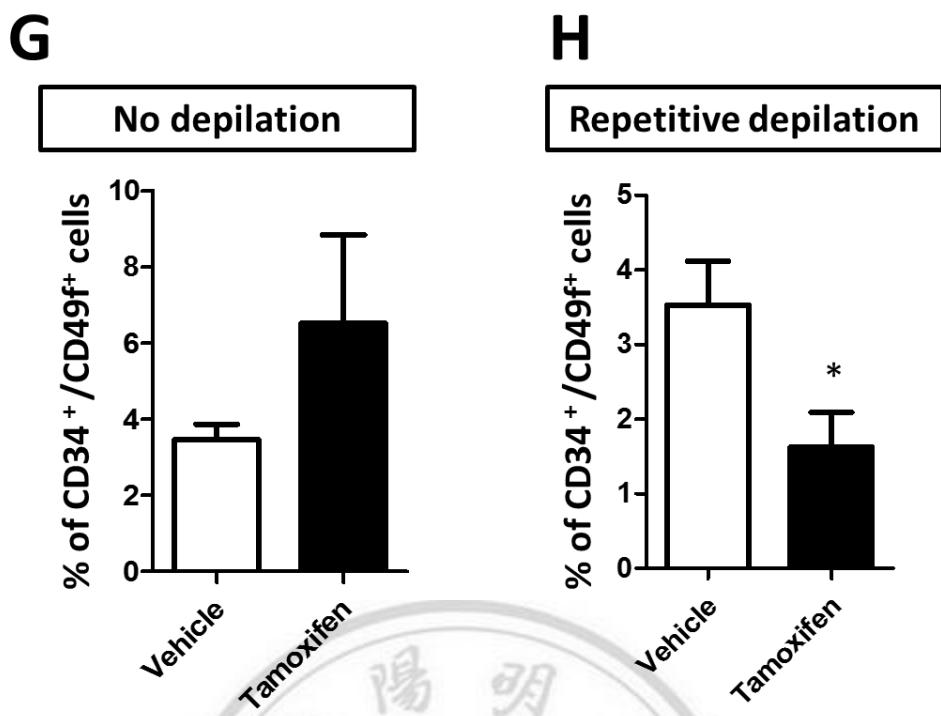


**Figure 9. K5-driven *Cdk12* conditional knockout mice showed alterations on differentiation markers after depilation.** *K5-CreER<sup>T+/-</sup>; Cdk12<sup>fx/fx</sup>* mice were injected with Tamoxifen at 6 weeks old, and depilation was operated two weeks later. (A-D) To

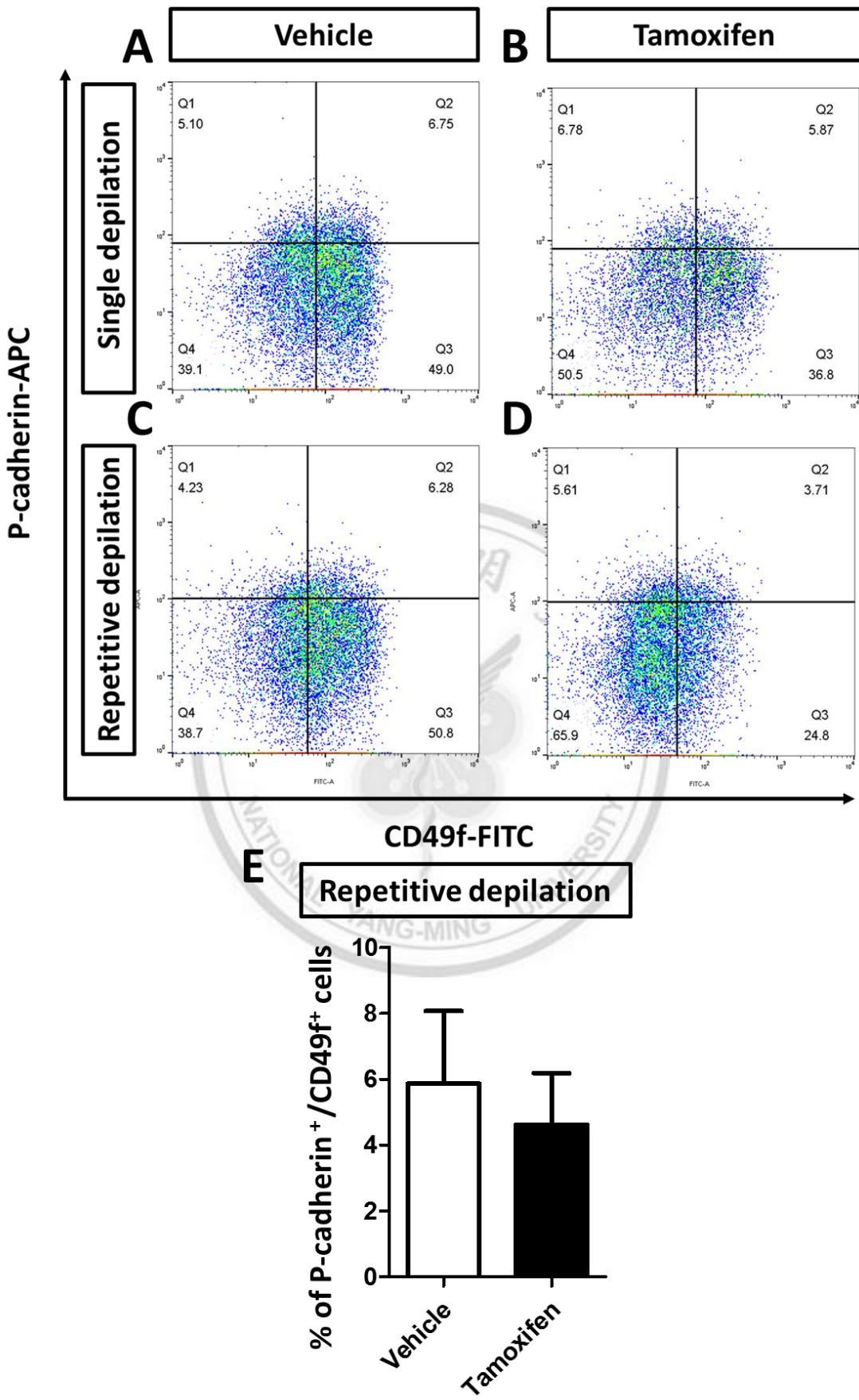
examine whether loss *Cdk12* cause defects on hair follicle structures, two hair follicle markers AE13 and AE15 were used to determine. AE13 is expressed in cortex of hair, and AE15 is expressed in inner root sheath. The results of AE13 and AE15 staining showed that hair follicle structures were not altered in *K5-CreER<sup>T+/-</sup>*; *Cdk12<sup>fx/fx</sup>* mice on 16 days after depilation. (E, F) Involucrin was expressed in the upper hair follicles in both conditional knockout and control group (arrow, horn cyst). (G, H, arrow) Conditional knockout mice showed involucrin in lower hair follicle. ( $n \geq 3$  for each groups)



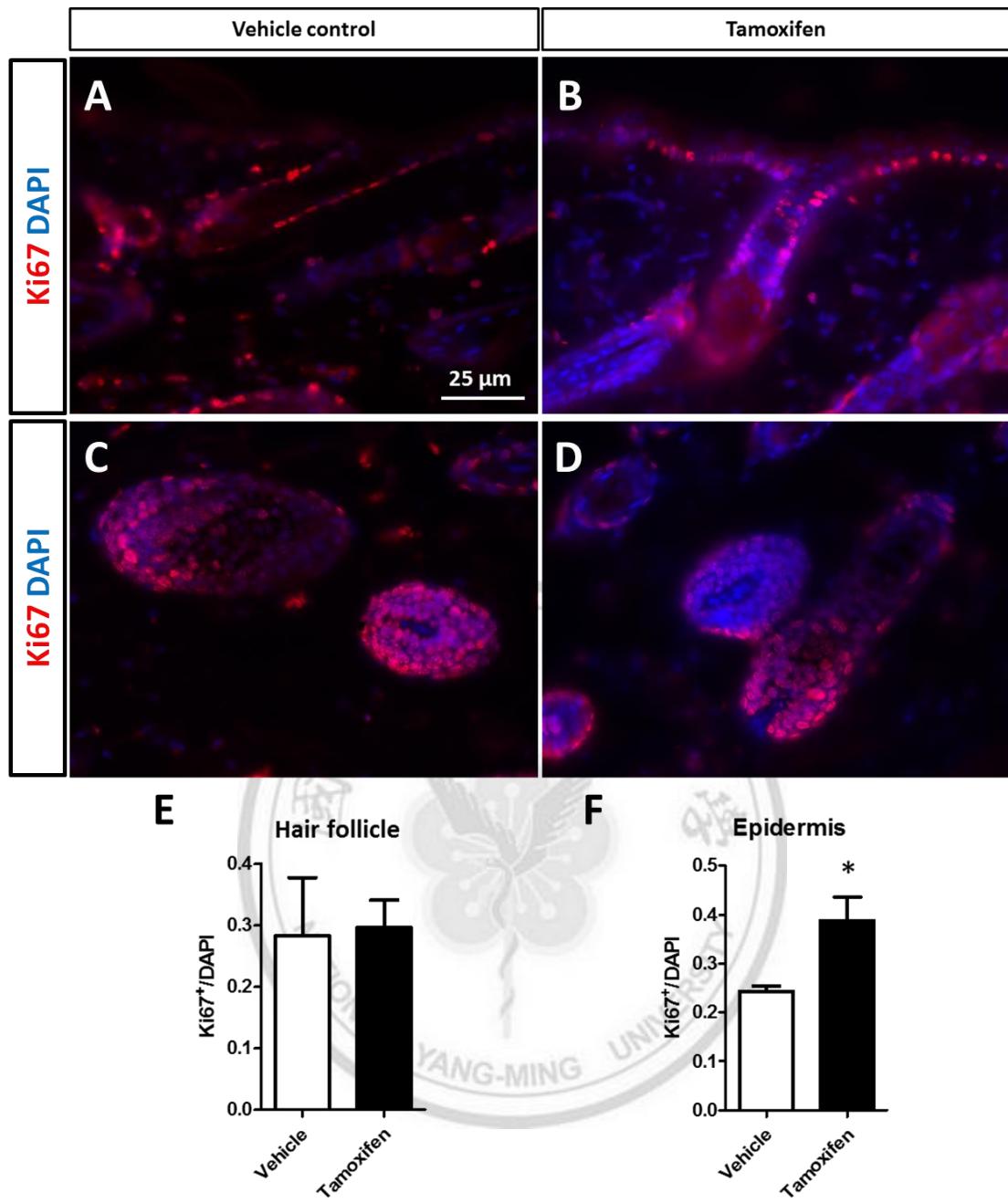




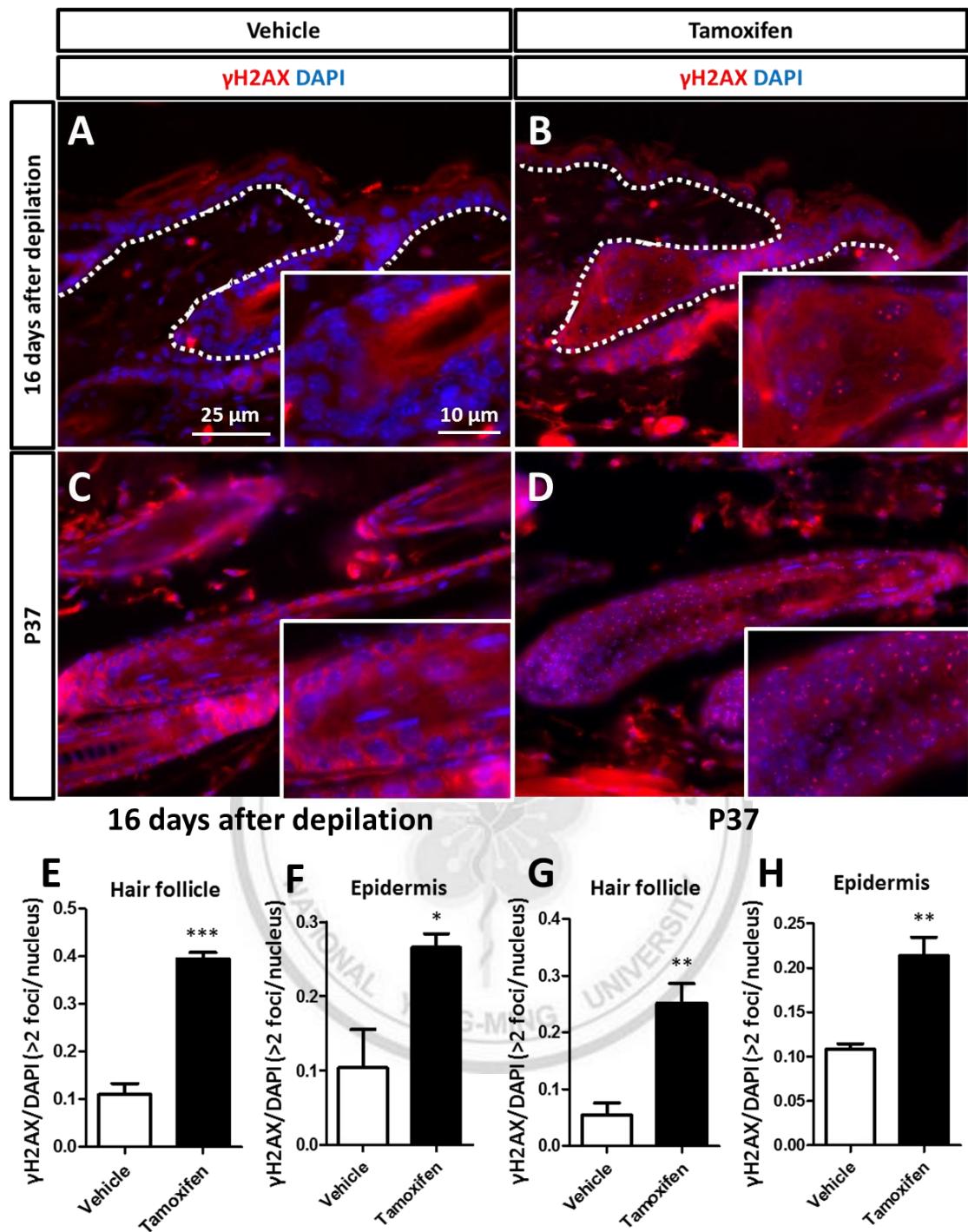
**Figure 10. Bulge stem cell population was altered in *K5*-driven *Cdk12* conditional knockout mice after repetitive depilation.** *K5-CreER*<sup>Tg1/0</sup>; *Cdk12*<sup>f/f</sup> mice were injected with Tamoxifen at 6 weeks old, and separated into three groups, no depilation (n=3 for each groups), single depilation (performed at two weeks after Tamoxifen injection, n=1 for each groups) and repetitive depilation (performed at three weeks after single depilation, n=3 for vehicle group and 4 for Tamoxifen group). Keratinocytes were isolated from dorsal skin in telogen phase, and stained with bulge stem cell markers, CD34 and CD49f ( $\alpha 6$  integrin). (A-D) Flow cytometry showed the percentage of each populations in different conditions, and the bulge stem cells located in double-positive population. The results showed that no significant difference in bulge stem cell population between Tomaxifen and vehicle groups in before depilation and single depilation. (E, F) However, after repetitive depilation, the percentage of double-positive cell populations were decreased. (G, H) Quantification results of double-positive cell populations in isolated keratinocytes shown in A, B and E, F. (\*, P value < 0.05, analyzed by T-test)



**Figure 11. Hair germ stem cell population was not altered in K5-driven *Cdk12* conditional knockout mice after repetitive depilation.** *K5-CreER<sup>T+/0</sup>; Cdk12<sup>fx/fx</sup>* mice were injected with Tamoxifen at 6 weeks old, and separated into three groups, no depilation (n=3 for each groups, antibody staining failed), single depilation (performed at two weeks after Tamoxifen injection, n=1 for each groups) and repetitive depilation (performed at three weeks after single depilation, n=3 for each groups). Keratinocytes were isolated from dorsal skin in telogen phase, and stained with bulge stem cell markers, P-cadherin and CD49f ( $\alpha 6$  integrin). (A-D) Flow cytometry showed the percentage of each populations in different conditions, and the hair germ stem cells located in double-positive population. The results showed that no significant difference in hair germ stem cell population between Tomaxifen and vehicle groups in single depilation and repetitive depilation. (E) Quantification results of double-positive cell populations in isolated keratinocytes shown in C and D.



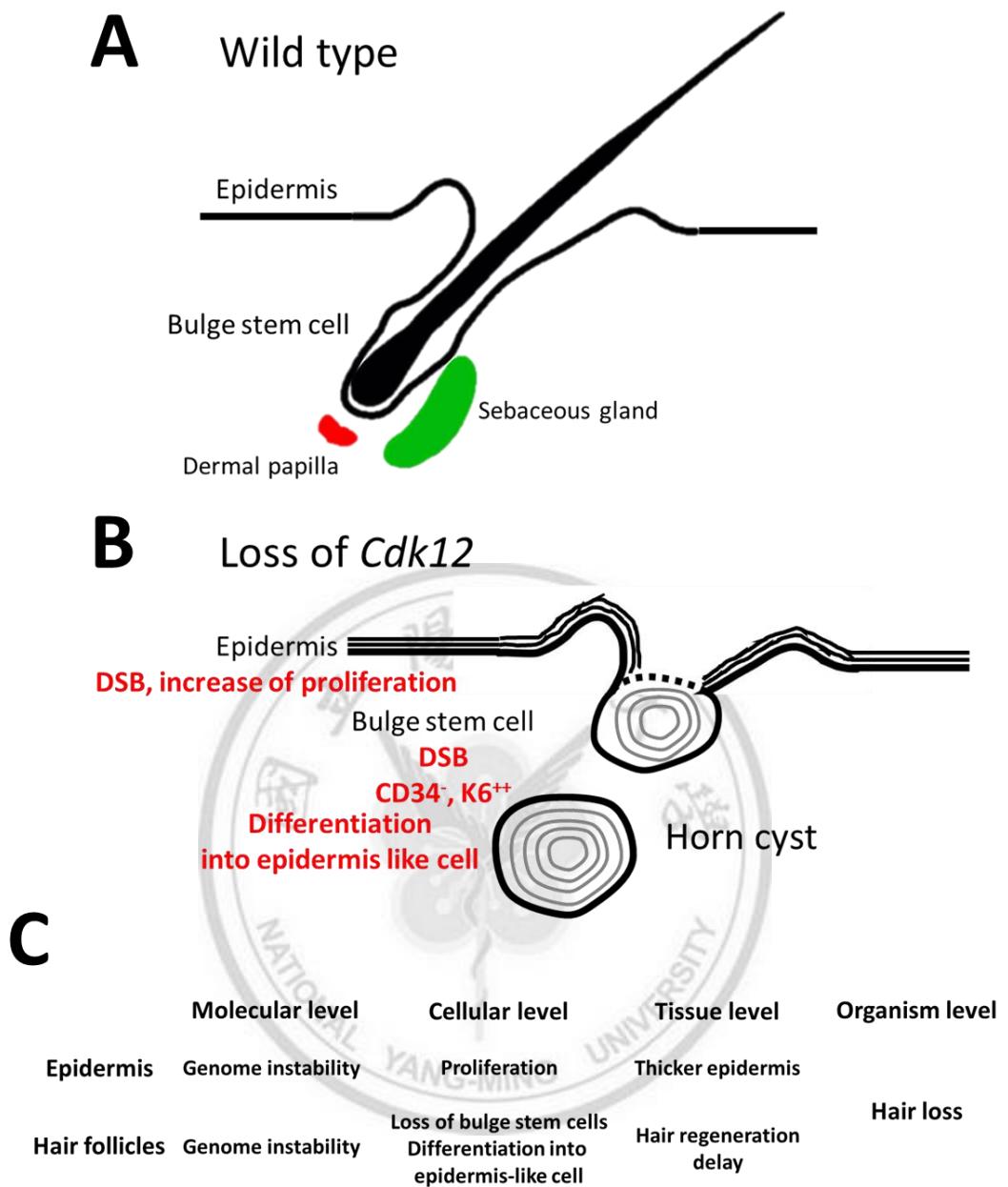
**Figure 12. Increase of proliferation cells was found in epidermis but not in hair follicles in *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>f/f</sup> mice.** *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>f/f</sup> mice were injected with Tamoxifen at 6 weeks old, and depilation was operated two weeks later. (A-D) To investigate whether cell proliferation was altered under the loss of *Cdk12*, cell proliferation marker, Ki67, was used to examine. More Ki67 staining were found in epidermis of *K5-CreER*<sup>T+/-</sup>; *Cdk12*<sup>f/f</sup> mice, but no significant alteration was found in hair follicles at 16 days after depilation. (E, F) Quantification results of proliferating cell in hair follicles and epidermis as shown in A-D. (n=3 for each groups, proliferating cells were calculated from 5 fields in one mouse. \*, P value < 0.05, analyzed by T-test)



**Figure 13. Loss of *Cdk12* lead to an increase of DNA double strand break in basal type epithelial cells.** To test whether DDR genes were altered and more DNA damages were accumulated upon depletion of *Cdk12*, DNA double strand break marker,  $\gamma$ H2AX, was used to examine. (A, B) Results of  $\gamma$ H2AX staining showed that the number of cells containing more than two  $\gamma$ H2AX foci in both hair follicles and epidermis at 16 days after depilation were significantly increased. (C, D) P37 *K5-CreER<sup>T/+</sup>; Cdk12<sup>fx/fx</sup>* mice with Tamoxifen administration in P20 also showed increase of DSBs in both hair

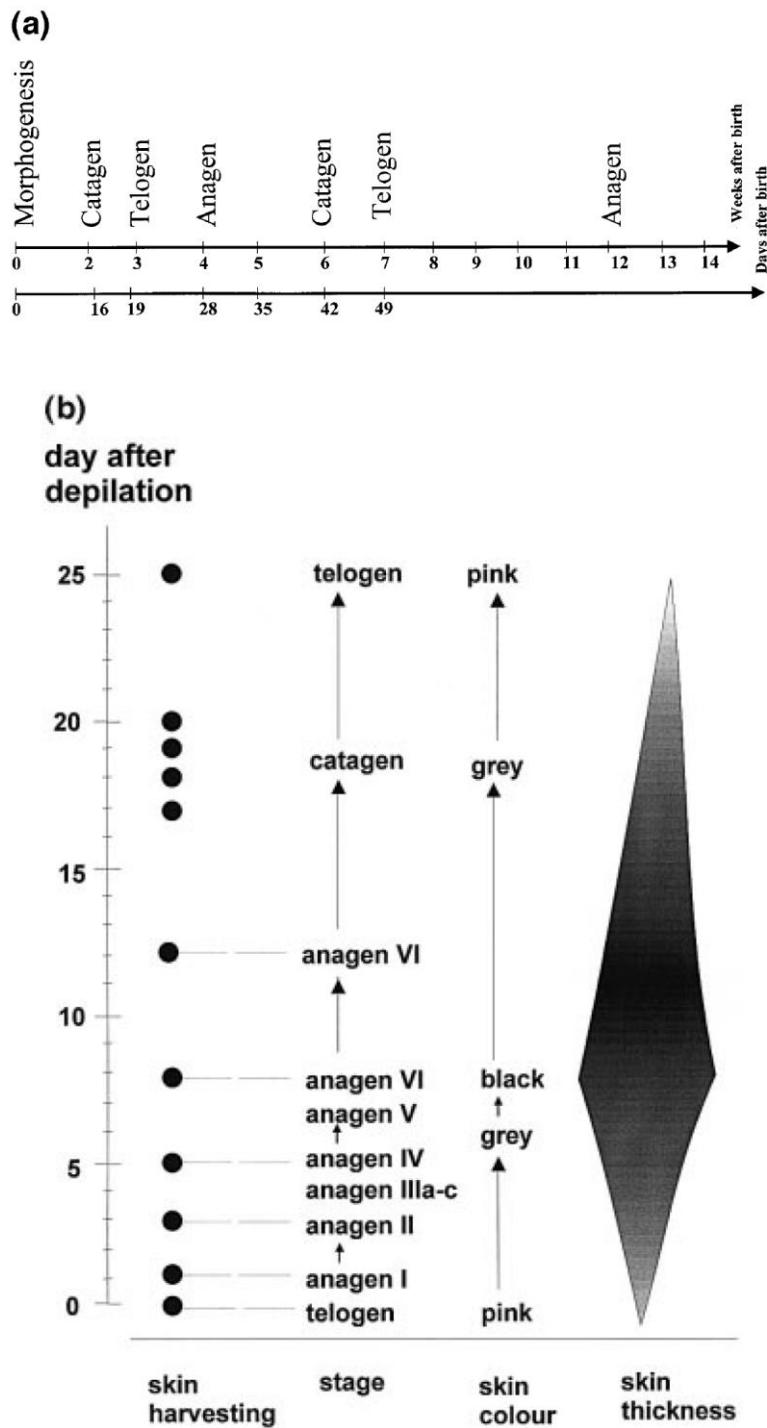
follicles and epidermis. (E-H) Quantification of A-D. (n=5 for Tamoxifen treated-depilation mice, n=3 for rest groups, DBS foci were calculated from 5 fields in one mouse. \*, P value < 0.05, \*\*, P value < 0.01, \*\*\*, P value < 0.001, analyzed by T-test)





**Figure 14. Model.** (A) Schematic diagram of a wild type hair follicle in telogen phase. (B) Schematic diagram of hair follicle upon loss of *Cdk12* in basal type epithelial cells. Loss of *Cdk12* cause several defects including increase of DSBs, increase proliferation, loss of bulge stem cells, incorrect differentiation, and cause horn cyst formation. (C) Summary of observations from molecular mechanism to hair loss phenotype in *K5*-driven *Cdk12* conditional knockout mice.

## 8. Appendix



**Appendix figure 1.** (a) Time-scale for the hair cycle in female C57BL/6 mice during the first 14 weeks after birth. (b) Time-scale for the hair cycle in female C57BL/6 mice and changes of skin pigmentation and skin thickness after depilation.

Figure was adapted from (Müller-Röver et al., 2001).