



Research Paper

P63: A marker of the proliferative basal layers of skin epithelia and the hair follicle

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ABSTRACT

This study was conducted to investigate the variation of P63 protein in normal hair cycle and hair regeneration process after hair follicles were thoroughly destroyed by cyclophosphamide. Regeneration of the destroyed cells is an optimal model for the research of cell proliferation. In the present study, BrdU was used to identify which part of skin was destroyed and the new cells regenerated (without or with new cells) in this CYP-injection induced dystrophy. The new cells in hair cycle were also labeled by BrdU. P63-positive cells and new cells were identified at basal layers of skin epithelia and outer root sheath of hair, which provided an evidence for the research result that the p63 is a marker of the proliferative basal layers of skin epithelia. IHC results showed that less p63-positive cells appeared in the resting phase (telogen), and new cells also significantly decreased in this phase. Much more p63-positive cells appeared in later anagen than early anagen, but reverse situation occurred in the high proliferative cells (marked by BrdU). Most p63-positive cells congregated along the inner side of the hair matrix, whereas the new cells scattered in the hair matrix at anagen phase. These results suggested that p63 is not sufficient to be a marker of high proliferative cells of the hair follicles.

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INTRODUCTION

Hair, as a unique organ, could repeat regeneration through the entire mammalian life. Hair, its stem cells located in the follicle bulge and regulated by the surrounding microenvironment, is an important model for organ regeneration (Plikus et al., 2008). The stem cells are also the source of high proliferative cells. The process of hair regeneration in wound healing is a typical model of organ regeneration. Originating from cells outside of the hair follicle stem cell niche, new follicles will form at the middle of wounded part. The new follicles arose from cells in the epidermis and/or upper portion of the follicle (infundibulum). These areas are occupied by epidermal stem cells that normally undergo epidermal differentiation (Ito et al., 2007).

The processes of normal hair cycle and wound-induced hair regeneration are related with the epidermal stem cells, transiently amplifying cells and differentiating

keratinocytes. Here, we simulated the process of hair regeneration in wound healing by the recovery process which is induced by cyclophosphamide (CYP). Cyclophosphamide has been listed as one of the most careladen side effects of cancer therapy by the National Coalition for Cancer Survivorship and it could induce the organ apoptosis including hair loss (Chemotherapy-induced alopecia) (Wang et al., 2006). Few days after drug administration, new hair would grow out even without any treatment. The process of hair regeneration is a valuable model for investigating the relation between hair regrowth and epidermal stem cells. In this experiment, p63 that is strongly expressed in the epithelial cells was used as a marker of the proliferative basal layers of skin epithelia, including the hair follicle (Senoo et al., 2007). P63 has been reported to be essential in the development of epithelia and is mainly expressed by basal and myoepithelial cells (Ivan

et al., 2005), which is also expressed in many normal human tissues including prostate basal cells, uterine cervix or urogenital tract (Burstein et al., 2006). At the gene level, p63 belongs to the p53 family of transcription factors and shares high homology with the other family members (Sayan et al., 2007). It contains two different promoters and expresses at least six major transcripts that lead to two fundamentally different kinds of proteins. Three of the p63 isoforms (TAp63) encode proteins with roles of transactivation and induction of apoptosis, other three isoforms (Δ Np63) lack the acidic amino (N)-terminal transactivation domain and exert inhibitory effects (Muskhelishvili et al., 2003).

BrdU immunohistochemistry (IHC) was performed to detect the proliferative cells in basal layers and hair follicle in chemotherapy induced alopecia. P63 IHC result was also checked. Hematoxylin and eosin (H&E) staining was used to investigate the morphology change of the damaged hair follicles.

MATERIALS AND METHODS

Main experiment process

In this experiment, 8-week-old B57CL/6 mice that were purchased from Danhan Biolink Inc. (South Korea) were used. Mice were divided into 4 groups numbered #1, #2, #3 and #4. Each group comprised 4 mice. Groups of #1 and #2 were control groups and the other two groups were experimental groups. At 10 days post depilation, the two experimental groups were injected with cyclophosphamide (200 mg per kilogram body weight). Mice of control groups were sacrificed at 10, 15, 20 and 25 days post depilation and mice of CYP-injection group were sacrificed at 15, 20, 25 and 30 days post depilation. The full-thickness back skins were taken for experiments. Mice of #1 and #3 groups were served as western and immunohistochemistry (IHC). The other two groups were served as BrdU IHC. Two hours before killing the mice of #2 and #4 groups, we injected 5-bromodeoxyuridine (BrdU) into mice to detect cell proliferation. BrdU is efficient in identifying S-phase cells (Muskhelishvili et al., 2003). The number of labeled cells was calculated. Hematoxylin and eosin (H & E) staining was used to observe the appearance of skin section.

Hematoxylin and eosin staining

Mice were sacrificed and skin samples were fixed with 0.4% formalin and 80% ethanol dissolved in distilled water, at 4°C. Two hours later, samples were transferred in turns into the following solutions: 90% ethanol, 100% ethanol, half ethanol and half xylene (v/v), half xylene and half paraffin (v/v), paraffin. Each step lasted for 1 h. Embedding and slicing were subsequently performed.

Sections were dyed based on the following steps: stained with hematoxylin solution (Sigma, USA), rinsed in distilled water, differentiated with 0.1% hydrochloric acid, rinsed in distilled water, dipped into saturated Li_2CO_3 for 30 s and stained with eosin for 1 min.

Incorporating BrdU and immunohistochemistry

Mice were injected intraperitoneally with BrdU in single doses of 200 mg per kilogram body weight (Sams et al., 2001). IHC (Immunohistochemistry) steps were done as follows: Tissue sections were deparaffinized, rehydrated, and equilibrated in PBS for 10 min at room temperature. Sections were blocked with 10% normal cow serum (Santa Cruz Biotechnology, Inc. USA) at room temperature for 20 min (Soma et al., 2002) and then incubated with anti-p63 (A4A) dissolved in PBS (0.05% tween 20, pH7.4), at 37°C for three hours. After rinsing three times with PBS, biotinylated rabbit anti-mouse IgG (Santa Cruz Biotechnology, Inc. USA) was used as a secondary antibody. Finally, 3,3'-Diaminobenzidine (DAB) (Sigma, USA) was used as a color-developing reagent (Soma et al., 2002).

RESULTS

H&E staining

The most easily detectable and intuitive morphological sign of CYP-induced dystrophy of hair follicles is the disruption of melanin accumulation and transfer (Bodo et al., 2007). Five days post depilation, mice treated with CYP showed broadened out root sheath, tortured hair shaft and blurred hair follicle. At 15 days post depilation, hair follicles showed shortened hair length and decrease in size of the hair bulb due to the massive apoptosis in the hair matrix (Figure 1E) (Sharov et al., 2004). Hair follicle apoptosis was induced by the CYP which blocked nutrition transportation and thoroughly destroyed the hair follicles (Figure 1F) at day 20 post depilation. Nearly all hair shafts were destroyed and faded away, and the withered hair follicles with clumping of melanin inhabited in the middle part of skin. Much more apoptotic hair follicles in CYP-induced alopecia than the withered hair follicles which resided in telogen of normal hair cycle (Figure 1B and F).

At day 25 post depilation, many hair follicles consisted of larger hair bulbs, internal root sheaths, and thicker outer root sheaths, which indicated that the structure of hair follicles recovered (Figure 1G) (Nakashima-Kamimura et al., 2008). The CYP-treated mice experienced a shorter interval from hair apoptosis to maximal regrowth (Figure 1E, F and G) than the control (Figure 1A, B, and C) (Duvic et al., 1996). Ectopic melanin appeared in or out of the hair follicles, even the hair shafts obtained a good recovery situation (Figure 1H).

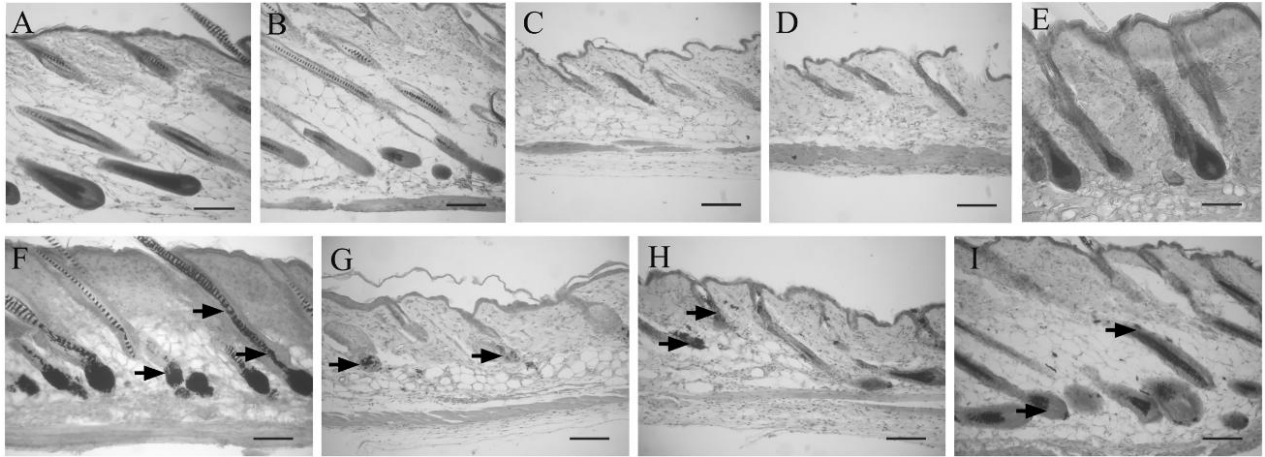


Figure 1: Representative of histological sections at day 10, 15, 20, 25 post depilation. A, B, C, D are control group, they are histopathologic sections at day 15, 20, 25, 10 post depilation in turn. F, G, H, I are CYP injection group, they are histopathologic sections on day 15, 20, 25, 30 post depilation in turn. A and I are skin histological sections of middle anagen, B and F are histological sections of catagen, D and G are histological sections of telogen, E and H are histological sections of early anagen. 5 days after CYP injection hair follicles were dystrophic; they shed 10 days after injection. Hair follicles recovered within 15 days. Arrows indicated clumping of melanin and abnormally dilated hair canals containing the tortured hair shaft (F) (Ohnemus et al., 2004). Destroyed hair bulbs and smeared melanin appeared in telogen (G). Ectopic distribution of melanin as a sign of dystrophy can be seen in H and I. (bars: 200 μ m)

Incorporating BrdU and immunohistochemistry

The number of BrdU-positive cells had no significant difference between control and CYP-injection groups in each growth phases; most of the positive cells were located in stratum granulosum, stratum spinosum and basal stratum of epidermis (arrows in A, D, G, J and M, P, S, V of Figure 2). BrdU-positive cells scattered in the hair matrix and were also observed in out root sheath at the catagen, new anagen and later anagen. It was widely observed that a population of multipotent stem cells was localized to the bulge region of the hair follicle and protected from external damage, those stem cells participated in normal follicular growth/rest cycling (Trempus et al., 2007). We could not found positive cells in the middle part of hair follicles at telogen; those apoptotic hair follicles which moved upward had no labeled cells (arrows in E and Q of Figure 2). The arrow in Figure 2Q is the ectopic melanin.

New cells resided in the hair follicle were impaired in the CYP-injection group at 15 days post depilation (catagen) because CYP metabolites acted as alkylating agents of cross-linking of DNA strands (Figure 2M and N) (Bodo et al., 2007). BrdU-positive cells were destroyed in the bottom of hair follicles at 20 days post depilation (Figure 2R). Surprisingly, BrdU-positive cells appeared in new hair follicles (Figure 2N), which means new cells did not come from the former hair follicles which were utterly damaged.

The average number of BrdU-positive cells per hair follicle was calculated, the result is shown in Figure 2Y. The number of those positive cells decreased with the aggravation of CYP-induced damage and responded to the damage at catagen (15 days post depilation) and telogen

(20 days post depilation). CYP-injection group had more BrdU-positive cells than the control group in new anagen and in later anagen.

P63 immunohistochemistry

The result showed that for epidermis labeled with p63, those p63-positive cells were observed in the basal and suprabasal layers of epidermis (Kim et al., 2009). The number of p63-positive cells in epidermis did not vary even in the control CYP-injection groups (Figure 3A, D, G, J, M, P, S and J). Although CYP induced dystrophy and hair follicle regression (Muller-Rover et al., 2000), epidermis of CYP-injection group still usually possessed the p63 protein (Figure 3M, P, S and V). P63-positive cells were detected in hair follicle epithelial and also observed in the sweat glands and the root sheaths (arrows in Figure 3B, E, H and K) of the control group (Fiuraskova et al., 2006). Positive cells that resided beside the middle part of hair shaft were also intact in the control and CYP-injection groups (arrows of Figure 3B, E, H, K, N, Q, T and W). Contrary to the p63-positive that existed in hair matrix in normal hair cycle, p63-positive cells of CYP-injection group located in hair matrix were destroyed in catagen (Figure 3O) and telogen (Figure 3R). Those cells nearly obtained complete recovery at new anagen (Figure 3U) and later anagen (Figure 3X). Some cells possessed p63 in out root sheath and apoptotic dermal papilla (arrows in Figure 3F) in telogen phase of control group, this illuminated p63 participation in the activation process of hair follicle. High density of p63-positive cells was found in hair matrix especially beside the

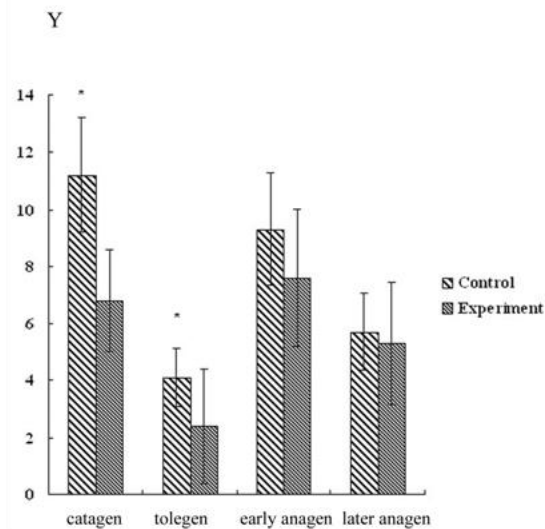
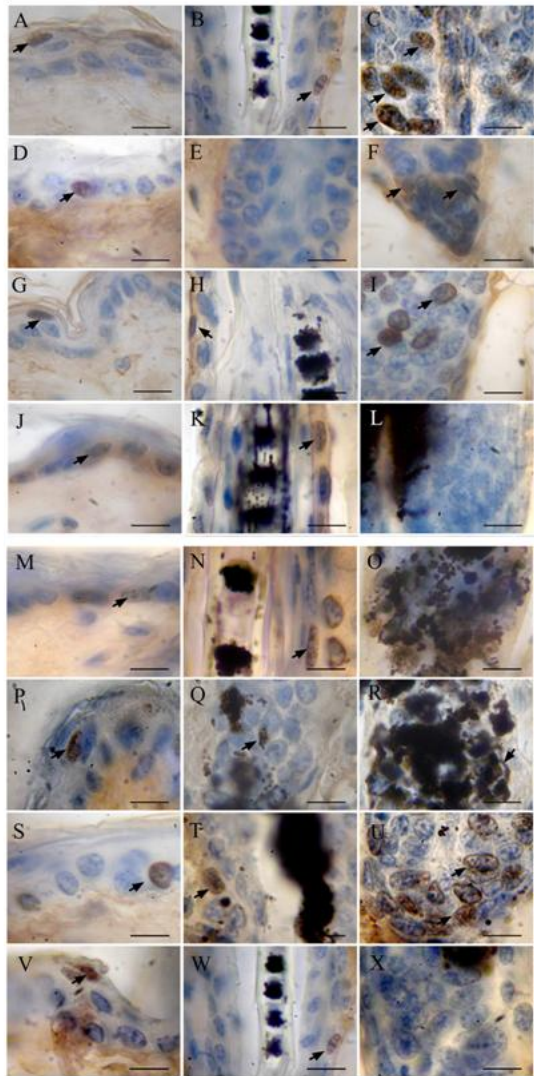


Figure 2: Cell proliferation of hair follicle and epidermis of control group is shown in Figure 2A-L). Each rank is different part of skin, first rank is epidermis, second is middle part of hair follicle, and

third is hair bulb. Histological sections of A, B and C are mouse skin at 15 days post depilation (catagen), BrdU-positive cells were observed in hair bulb. Few cells were labeled in the skin sections of 20 days post depilation (D, E, and F). Histological sections of G, H and I are mouse skin at 25 days post depilation (new anagen). Histological sections of J, K and L are 10 days post depilation (later anagen). (Bars: 10 μm)

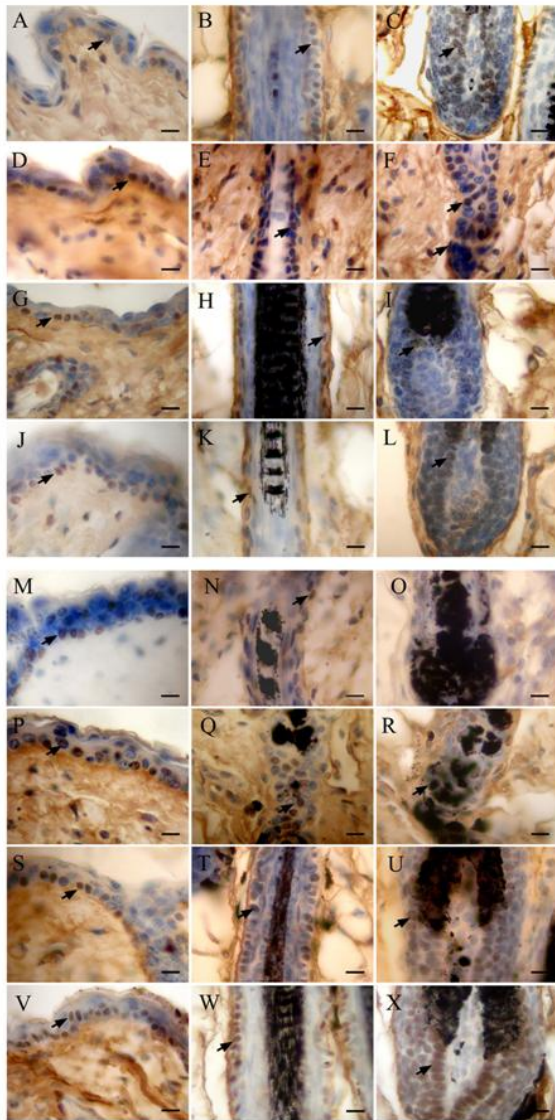
Cell proliferation of hair follicle and epidermis of CYP-injection group is shown in Figure Figure 2M-X. Each rank is different part of skin, first rank is epidermis, second is middle part of hair follicle, and third is hair bulb. Histological sections of M, N and O are mouse skin at 15 days post depilation (catagen); BrdU-positive cells were destroyed in hair bulb. Few dystrophic cells were labeled in the skin sections at 20 days post depilation (P, Q, and R), most of labeled cells resided in the hair bulbs. Histological sections of S, T and U are 25 days post depilation (new anagen). Histological sections of V, W and X are mouse skin at 30 days post depilation (later anagen). (Bars: 10 μm). Average number of BrdU-positive cells per hair follicle is shown in Figure 2Y. There is significant difference between control and CYP injection groups in catagen and telogen. ($P < 0.05$)

edge of dermal papilla in catagen, early anagen, and later anagen in the control groups. P63-positive cells had the same position in early anagen and later anagen in the CYP-injection groups. It is impossible for the thoroughly destroyed papilla to give birth to new hair in our experiment, so mesenchymal and bulge (Figure 3O and R) which obtained some p63-positive cells were the source of new hair follicle.

The average number of p63-positive cells per hair follicle was calculated to represent the degree of p63 recovery (Figure 3Y). Number of p63-positive cells of CYP-injection group was significantly different at four cycle phases. Hair follicles of CYP-injection group rapidly obtained more p63-positive cells than control group at the anagen phase.

DISCUSSION AND CONCLUSION

15 days after CYP-injection, hair follicles were thoroughly destroyed by cyclophosphamide (200 mg per kilo gram body weight). New hair follicles that originated from the bulge produced the new intact hair with ectopic melanin beside the hair shafts 25 days after injection. Not all the p63-positive cells at the upper part of skin were destroyed. P63-positive cells appeared in the new hair follicles at 20 days post depilation. BrdU was used to identify the new cells and the positive cells scattered in the hair matrix at catagen and early anagen in the control groups. The high density of p63-positive cells was found in hair matrix especially beside the edge of dermal papilla in catagen, early anagen, and later anagen in the control groups. The new cells appeared not in the same position with p63-positive cells, so p63 is a marker of the proliferative basal



3A-L). Each rank is different part of skin, first rank is epidermis, second is middle part of hair follicle, and third is hair bulb. Histological sections of A, B and C are mouse skin at 15 days post depilation (catagen), most positive cells were observed in hair bulb. Labeled cells also resided in the interfollicular epithelium between hair bulb and dermal papilla at 20 days post depilation (D, E, and F). Histological sections of G, H and I are mouse skin at 25 days post depilation (new anagen), less cells were labeled in this growth phase. Histological sections of J, K and L are 10 days post depilation (later anagen). (Bars: 20 um)

P63-positive cells in CYP-injection groups (Figure 3M-X). Each rank is different part of skin, first rank is epidermis, second is middle part of hair follicle, and third is hair bulb. Histological sections of M, N and O are 15 days post depilation (catagen). P63- positive cells were destroyed in hair bulb. Most labeled cells of hair follicle were destroyed in the skin sections at 20 days post depilation (P, Q, and R) especially in the hair bulbs. Histological sections of S, T and U are mouse skin at 25 days post depilation (new anagen). Histological sections of V, W and X are mouse skin at 30 days post depilation (later anagen), at this growth phase p63 recovered. (Bars: 20 um). Average number of p63-positive cells per hair follicle is shown in Figure 3Y. Numbers of p63-positive cells were significantly different between control and CYP injection groups in all of four growth phases. ($P < 0.05$).

layers of skin epithelia, not including the hair follicle in the hair cycle. In the CYP-injection groups, BrdU-positive cells were scattered in hair matrix in early anagen, but no positive cells were detected in later anagen at the same position in later anagen. Most p63-positive cells congregated at the inner edge of the hair matrix (arrow of Figure 3X), but new cells (labeled by BrdU) were scattered in hair matrix. The p63 was not a proper marker of high proliferative cells for hair follicles, it was just a marker of the proliferative basal layers of skin epithelia, including outer root sheath.

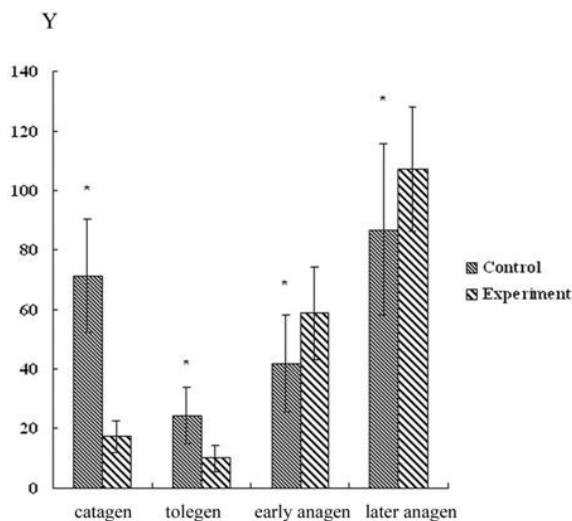


Figure 3: p63-positive cells of control groups (Figure

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