Research Article

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A Mouse Model for Studying Stem Cell Effects on Regeneration of Hair Follicle Outer Root Sheaths

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Abstract: Objective: Stem cells hold promise for treating hair loss. Here an *in vitro* mouse model was developed using outer root sheaths (ORSs) isolated from hair follicles for studying stem cell-mediated dermal papillary regeneration. Methods: Under sterile conditions, structurally intact ORSs were isolated from hair follicles of 3-day-old Kunming mice and incubated in growth medium. Samples were collected daily for 5 days. Stem cell distribution, proliferation, differentiation, and migration were monitored during regeneration. Results: Cell proliferation began at the glass membrane periphery then spread gradually toward the membrane center, with the presence of CD34 and CD200 positive stem cells involved in repair initiation. Next, CD34 positive stem cells migrated down the glass membrane, where some participated in ORS formation, while other CD34 cells and CD200 positive cells migrated to hair follicle centers. Within the hair follicle matrix, stem cells divided, grew, differentiated and caused outward expansion of the glass membrane to form a dermal papillary structure containing alpha-smooth muscle actin. Neutrophils attracted to the wound site phagocytosed bacterial and cell debris to protect regenerating tissue from infection. Conclusion: Isolated hair follicle ORSs can regenerate new dermal papillary structures *in vitro*. Stem cells and neutrophils play important roles in the regeneration process.

Keywords: hair follicle outer root sheath, EdU, α -SMA, CD200, CD34, LY6G, regenerative stem cells

1 Background

Hair follicles are mammalian skin appendages which possess periodic and typical regenerative capacity to continuously produce new hair throughout life [1, 2]. The regenerative ability of hair follicles relies on the presence of stem cells and other cell populations, as well as non-cellular components such as molecular signaling factors and extracellular materials [3]. Because stem cells have the ability to self-renew and differentiate into specific functional cell types, they are key to tissue development, regeneration, and disease development [4, 5]. Meanwhile, hair follicles have become a powerful model system for the study of stem cell biology, since a hair follicle is a self-sufficient organ with a fixed population of stem cells that periodically regenerates to produce new mature hair shafts throughout the life of an organism [6].

The regeneration of hair follicles is directly related to the proliferation and differentiation of resident stem cells that can be monitored using various markers. CD34, a type I transmembrane glycoprotein surface antigen expressed by a variety of cell types, including hematopoietic progenitor cells, endothelial cells, and mast cells [7], can serve as a mouse hair follicle bulge stem cell marker [8]. As demonstrated by Trempus et al [9], this marker is especially useful, since CD34 positive cells possess both CD34 tag-retaining properties and large proliferative potential. CD200, another useful bulge stem cell marker, was identified by Ohyama et al [10], and cells expressing this marker play an important role in hair follicle regeneration. Ohyama's group has also found that neutrophils play an essential role in various autoimmune and inflammatory disorders. The monoclonal antibody LY6G has been used frequently for neutrophil detection and for studying inflammatory responses [11].

In this work, we established an *in vitro* model to study regeneration of outer root sheaths of hair follicles of KM mice by observing morphological changes during ORS regeneration. Next, this model was used to explore the effects and significance of hair follicle stem cells on the regeneration process. Using the fluorescent cell

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proliferation marker 5-ethynyl-2'-deoxyuridine (EdU), hair follicle cells surrounding the root sheath were monitored for proliferation as part of a larger preliminary analysis conducted to assess the significance of hair follicle stem cells in ORS regeneration. Using LY6G, the role of neutrophils in the regeneration of outer root sheath of hair follicle was studied *in vitro* as well. This work provides a foundation for further study of wound healing and tissue regeneration as well as new insights to guide future studies to explore tissue regeneration mechanisms.

2 Materials and Methods

2.1 Materials

2.1.1 Mouse

Male and female three-day-old healthy Kunming (KM) outbred milk mice were provided by the Experimental Animal Center of the Fourth Military Medical University (SCXK 2012-0007). All mice were raised under specific pathogen-free conditions that included clean food and housing, following guidelines outlined in the China Laboratory Animal Rights Protection Act.

2.1.2 Main reagents

The EdU keyFluor488 Fluorescence Detection Kit and primary anti- α -SMA antibody were purchased from Wuhan Sanying Biotechnology Co., Ltd. (China). Anti-CD200 and LY6G primary antibodies and secondary antibody 594 were purchased from Beijing Boasens Biotechnology Co, Ltd.(China). Goat blocking serum, optimal cutting temperature compound (OCT) frozen embedding agent, hematoxylin, eosin, and bovine serum albumin (BSA) were purchased from Gibco or Sigma (USA). Other biochemical reagents were purchased from companies in China and were of analytical grade and included 4% paraformaldehyde (PFA), anti-fluorescence quencher, Dulbecco's phosphate-buffered saline (DPBS), absolute alcohol, and Triton X-100 stock solution.

2.1.3 Main instruments

Stratospheric ultra-clean bench (Germany), Surgical anatomic microscope (Germany), Leica cryostat (Germany), McAudi visual binocular microscope (China), and a Zeiss inverted visualization fluorescence microscope (Germany).

2.2 Method

2.2.1 Establishment of a mouse model of scarless healing

Thirty male 3-day-old KM mice were randomly selected and sacrificed by cervical dislocation. Microscopic surgical ophthalmic scissors were used to isolate 30 hair follicles from mouths of mice and specimens were viewed during processing using a surgical dissection microscope. Complete hair follicles were micro-surgically isolated and the tops of the hair follicles, hair bulbs, and inner root sheaths were removed without damaging the outer root sheath bulge structures. Isolated outer root sheaths of hair follicles were placed into 25-mm sterile plastic culture dishes and 3 ml of 5% FBS + DMEM (with added streptomycin and cell proliferation assay culture medium containing EdU at a concentration of 20 μ mol/L) per dish was added. Specimens were immediately placed in a humidified incubator at 37°C with 5% CO₂ and incubated for 5 d in triplicate. Triplicate samples were removed daily and prepared for microscopy using OCT frozen embedding agent, stored frozen at -80°C for 7 d, and then sectioned using a cryostat (Germany Leica) to generate 8- μ m thick slices. Frozen slices were then affixed to slides using a positive charge anti-offset slide adsorption method. Slides were stored frozen at -20°C until needed. Each culture was repeated in triplicate for each culture duration to establish reproducibility of the scarless healing model used to study hair follicle outer root sheath regeneration (Figure 1).

Ethical approval: The research related to animals use has been complied with all the relevant national regulations and institutional policies for the care and use of animals.

2.2.2 Conventional pathological staining

The frozen slides were dried at room temperature for 10 min, placed on the biological tissue sheet roasting machine (Leica, Germany) for 30 min, and then wetted by distilled water for 2 min. The slides were stained with Hematoxylin for 3-5 min and rinsed with distilled water for 1 min to clean the excess dye off the slide. A 1% hydrochloric acid alcohol solution was applied for 5s (for differentiation) and rinsed with distilled water for 1 min. Lastly, the slides were stained with Kay red for 20~30 s, rinsed with distilled water for 1 min, and sealed with neutral gum. The experiment was repeated three times.

Figure 1. Diagram of regeneration and culture of outer root sheath. Note: HS: hair shaft, SG: sebaceous gland, BG:bulge, GM: glass membrane, ORS: external root sheath, DP: dermal papilla, IRS: internal root sheath, RCS: regenerated cells. In this process, the top of the hair follicle and the hairball part were firstly removed, the inner root sheath and hair shaft extracted, and 3D culture was carried out at the gas-liquid interface outside the body. New cells were regenerated inside the outer root sheath.

2.2.3 Immunohistochemistry

The slides were naturally dried at room temperature for 40 min and fixed in 4% paraformaldehyde for 20 min. The slides were washed with PBS twice for 5 min each. A permeabilized 0.1% Triton X-100 (PBS dilution) was applied to the slides for 30 min. Slides were then washed with PBS for five min each. A mixture of 200 μ l 3% BSA (PBS dilution) and 200 μ l 20% FBS (PBS dilution) was added to the slides and incubated at room temperature for 1h. The slides then were washed with PBS twice for five min each. 200 μ l primary antibody (IF=1:200) was added to each slide, and the slides were then placed in 4°C wet box for the night. Slides were then washed with PBS three times for 5 min each. 200 μ l fluorescence secondary antibody diluent (IF=1:150) and 4 μ l DPAI was added to each slide and then washed three times for 5 min each. Lastly, 2.5 μ l of anti-fluorescence quenching agent was added to each slide, avoiding light. The experiment was repeated three times.

3 Results

3.1 Observation of hair follicle and ORS of normal mice

The normal hair follicle structure of the 3-day-old KM mice, the basic structure of the hair follicle outer root sheath, inner hair root sheath, connective tissue sheath, dermal papilla and hair shaft can be clearly seen (Figure 2A). The outer root sheath structure of the hair follicle of the 3-day-old KM mice, the inner root sheath and the hair shaft have been removed via microscopic dissection.

3.2 Morphological observation of hair follicle regeneration process

The hair follicle outer root sheaths were cultured *in vitro* for 1 to 5d, sampled daily, embedded in OTC, and stained with HE, and then observed for regeneration of the outer

Figure 2. The structural of mouse follicle and ORS. Note: Figure 2A shows HE staining ($\times 100$) structure of normal hair follicle; Figure 2B shows HE staining ($\times 100$) structure of outer root sheath of hair follicles. ORS: outer root sheath; GM: glass membrane; IRS: inner root sheath; DP: dermal papilla; curve box is bulge (bulge area); Scale = 5 μm .

root sheath of hair follicles. When the outer root sheath of the hair follicles cultured for 1d (Figure 3A), the results showed that a small number of cells regenerated inside the glass membrane. With the increase of culture time, the internal cells of the glass membrane increased continuously, and evolved into the original morphology of the root sheath of the hair follicle (Figure 3B-D). When the outer root sheath of the hair follicle was cultured for 5d (Figure 3E), the regeneration pattern of the inner root sheath was the best. The structures of hair follicles appeared complete.

3.3 Characteristics of α -SMA expression in hair follicle regeneration area

Because alpha-smooth muscle actin (α -SMA) is a special cytoskeletal protein that plays an important role in the tissue cell regeneration process, α -SMA expression was used to monitor tissue cell regeneration via an α -SMA immunofluorescence assay of frozen hair follicle outer root sheaths on day 5 of culture. The results showed that α -SMA was positively expressed in the outer root sheath of hair follicles (Figure 4A-D). Because skeletal proteins are interconnected, the structures of outer root sheaths of hair follicles tended to remain intact after regeneration, demonstrating that the hair follicle ORS possesses stem cell regeneration potential. The immunofluorescence

staining of α -SMA inside the outer root sheath of hair follicle at the early stage of culture is shown in Figure 4a-c. PBS was used in place of primary antibody to serve as a negative control (Figure 4A-C).

3.4 EdU labeled cell proliferation and dynamic migration

The results of the EdU labeling to detect cell proliferation demonstrated that at 1 d of culture (Figure 5A), proliferation of cells inside the external follicle was mainly concentrated outside the glass membrane, with only a small number of proliferating cells present inside. By 2 d (Figure 5B), cells inside the glass membrane of the outer root sheath of the hair follicle began to proliferate and reconstruction of internal hair follicle tissue had begun. At this time, a large number of new green fluorescence-emitting cells inside and outside the glass membrane could be clearly seen. By 3 d (Figure 5C), the number of cells proliferating in the region of the outer part of the glass membrane had gradually decreased, with new cells mainly concentrated within the interior of the glass membrane. By 4 d (Figure 5D-E), proliferation of cells within the glass membrane and expansion of the glass membrane was apparent. At this time, green fluorescence of new cells in the glass membrane was enhanced and the initial morphology of the root sheath inside the hair follicle was constantly evolving.

3.5 Localization and dynamic distribution of CD200 in follicular regenerated stem cells

At 1 d of culture (Figure 6A), the stem cell marker CD200 observed within the region of the bulge began to be visible in abundance, with cells exhibiting high expression gradually approaching the glass membrane. At 2 d of culture (Figure 6B), cells expressing stem cell factor CD200 were visible within the interior of the glass membrane and remained there for a short time before further migration. From 3d to 4d of culture (Figure 6C-D), CD200 was visible at the bottom of the glass membrane as migration stopped and the glass film expands from the inside out. At 5 d (Figure 6E), restoration of the structure of the regenerated outer root sheath of the hair follicle appeared to be complete, with cells expressing CD200 migrating and differentiating upward from the bottom of the glass membrane and guiding new cells to extend inward toward the center of the hair follicle. Figure 6F shows the control, where PBS was used in place of monoclonal antibody.

Figure 3. HE staining of hair follicle ORS regeneration process. Note: Figure 3A-E shows HE staining ($\times 100$) of a hair follicle outer root sheaths cultured *in vitro* for 1 to 5d as the regeneration model; 3F shows a microscurgically isolated hair follicle outer root sheath immediately after isolation (control group). ORS: outer root sheath; GM: glass membrane; RCS: regenerative cells; Scale = 5 μm .

Figure 4. Expression Characteristics of α -SMA in Follicle Regeneration Region. Note: Figure 4A shows the expression of cytoskeletal protein α -SMA during hair follicle regeneration as indicated by green fluorescence. Figure 4B shows intact cell nuclei as indicated by blue fluorescence staining with DAPI. Figures 4C-D shows the superposition of α -SMA onto DAPI immunofluorescence (4a-c is the control); The white curve is glass film (GM); The white arrow indicates expression of α -SMA within the regeneration region. Scale: Figure 4D = 5 μm ; Figure 4A-C = 10 μm .

Figure 5. EdU -Labeled Cell Proliferation and Dynamic Migration. Note: Figure 5A-E shows green fluorescence highlighting the proliferation of cells in the external root sheath of hair follicles cultured from 1-5 d *in vitro* with EdU labeling. In the figure, the white curve is the glass membrane (GM) and the white arrow shows the dynamic distribution of proliferating cells. Figure 5F shows the negative control without EdU; Scale = 20 μm .

Figure 6. Localization and Dynamic Distribution of CD200 in Follicular Regeneration Region Note: Figure 6A-E shows the dynamic distribution of CD200 within the regenerated region within stem cells in the outer root sheath of the hair follicle during *in vitro* culture for 1-5 d and visualized with immunofluorescence staining. The white dashed line (GM) marks the glass membrane structure and the white arrow indicates the dynamic distribution of CD200 in stem cells. Figure 6F shows the negative control. DAPI is blue fluorescence and CD200 is red fluorescence. Scale for Figure 6A-F = 20 μm .

3.6 Localization and dynamic distribution of CD34 in follicular regenerated stem cells

Stem cell CD34 immunofluorescence results showed that when the ORS of the hair follicle was cultured *in vitro* for 1 d (Figure 7A), cells expressing CD34 were visible at the periphery and those cells gradually migrated to the interior of the glass membrane. At 2 d (Figure 7B), cells expressing CD34 began to be visible inside the glass membrane, with CD34 expression visible at sites arranged mainly along the glass membrane. At 3 d (Figure 7C), cells with CD34 expression extended downward along the glass membrane and were involved in regeneration of hair papilla. At 4 d (Figure 7D), CD34 was continuously expressed along the glass membrane and a ring of cells was visible in the central part of the root sheath outside the hair follicle. At 5 d (Figure 7E), at the center of the hair follicle ORS, regeneration was apparent and new hair follicle structures were basically formed, with CD34 expression visible by immunofluorescence at the bottom of the glass membrane and surrounding the new hair papilla. This result suggests that regeneration began at the bottom of the glass membrane then underwent an

outward expansion. In addition, some CD34 was observed to be concentrated in the center of the regeneration site, indicating CD34-positive cells there were likely involved in further new hair shaft regeneration. In the control (Figure 7F), antibody was replaced with PBS.

3.7 Dynamic distribution of neutrophils in the regeneration region of ORS

Hair follicle ORS cells are shown after culture for 1 d (Figure 8A). Immunofluorescence results show glass membrane inflammation at trauma sites where neutrophils stained with LY6G begin to accumulate due to chemokines that attracted them to the inflammation site; at 2 d (Figure 8B), neutrophils at the inflammatory site are present in large numbers and scattered around the site where they can be seen engulfing dead cells; at 3 d (Figure 8C), the repaired area has initially formed within the hair follicle root sheath where neutrophils in the center of the new hair parts continue to exhibit activity but are in relative decline as they appear to engulf foreign bodies created by apoptosis; at 4 d (Figure 8D) within

Figure 7. Localization and dynamic distribution of CD34 in follicular regenerated stem cells. Note: Figure 7A-E shows the location and dynamic distribution of CD34 in regenerated regional stem cells when the hair follicle ORS was cultured *in vitro* for 1-5 d and labeled using immunofluorescence staining. The white dashed line (GM) represents the glass membrane structure while the white arrow indicates the dynamic distribution of CD34 in the stem cells. Figure 7F is a negative control using PBS in place of antibody. DAPI staining is indicated by blue fluorescence and the presence of CD34 by red fluorescence. Scale: Figure 7A-F = 20 µm.

Figure 8: Dynamic distribution of LY6G in the regenerated region of the ORS. Note: Figure 8A-E shows neutrophils within the regeneration area of the hair follicle outer root sheath cultured for 1-5 d *in vitro* using immunofluorescence staining; the white dashed line (GM) is the follicular glass membrane; the white arrow shows the neutrophil expression area. Figure 8F: negative control (PBS in place of antibody). DAPI (blue fluorescence) showing cells with intact nuclear DNA and LY6G showing neutrophils (red fluorescence). Scale: A-E = 20 μ m.

the hair follicle regeneration area, immunofluorescence shows neutrophil cell lysis (dotted box) and gradual reduction of the inflammatory reaction; at 5 d (Figure 8E), immunofluorescence shows an absence of neutrophils in the hair follicle as regeneration of the hair follicle is almost complete. Meanwhile, a protective film is visible on both sides of the middle part of the glass membrane that formed to protect the hair follicle structure. In the control (Figure 8F), PBS was used instead of monoclonal antibody.

4 Discussion

In vitro regeneration of the hair follicle outer root sheath requires two basic conditions: first, stem cells must be made available to promote regeneration of wounded tissue; second, a similar culture environment *in vitro* must be established to replicate the *in vivo* environment within mouse tissues. Numerous studies have demonstrated that hair follicle cells of the outer root sheath bulge region and cells of the hair bulb region must interact to achieve regeneration of the hair follicle [12-15]. Recent preliminary studies have suggested that stem cells normally reside in the carinal part of the hair follicle [16, 17]. In agreement

with this finding, Taylor et al [18] found that the cells within the protuberance at the lower end of the hair follicle regenerated new hair follicle cells to repair the structure when it was damaged. Notably, Oshima et al [19] found that the carina of the hair follicle could regenerate the entire hair follicle, including the outer root sheath, the inner root sheath, and the hair stem, as well as all components of the epidermis and sebaceous glands. In addition, a number of molecular signaling pathways also have been implicated in the activation of hair follicle stem cells that play dominant roles in inhibition of BMPs [20, 21] and enhancement of Wnt expression [22]. For example, during the process of hair follicle regeneration, Wnt activates protuberant stem cells through signal transduction, causing them to migrate downward and differentiate and ultimately complete hair follicle regeneration [23, 24].

Because hair follicle regeneration *in vitro* could be viewed as a form of wound repair, we developed a model of hair follicle regeneration based on this concept. In our model, the outer root sheath of each hair follicle was isolated microsurgically and separated from the inner root sheath, hair stem and lower end of the dermal papilla, while preserving the complete outer root sheath protuberance. By monitoring proliferation of cells using EdU labeling and identifying cell types

using immunofluorescence staining, active proliferation of hair follicle protuberance cells was monitored. Next, regeneration of initial hair follicle morphology was observed after cells migrated to the wound site inside the hair follicle. Using fluorescence labeling, after induction of hair follicle trauma, the locations of hair follicle cells expressing CD34 and CD200 stem cell markers were tracked as the hair follicle microenvironment changed and repair was initiated [25]. The stem cells involved in regeneration began expression of these markers. After activation, stem cells migrated to the site of trauma, remained at the trauma site briefly, dividing many times, then migrated to the hair bulb to form a new hair matrix region. Within the new hair matrix region, stem cells divided, expanded and differentiated to gradually form the dermal papilla structure. Finally, the dermal papilla acted as a signal source to perpetuate regeneration, stem cell migration, proliferation, differentiation, and final completion of regeneration of the trauma site [26].

Immediately after harvest of hair follicle outer root sheaths, inflammatory stress reactions were observed at hair follicle wound sites, as noted by the presence of neutrophils, active immune cells that can change shape to engulf and phagocytize bacteria and foreign bodies [27]. As a well-established fact, neutrophils are attracted to inflammatory sites by chemoattractants where they are activated to become neutrophilic granulocyte phagocytes that kill pathogenic microorganisms during the inflammatory stage. As the LY6G immunofluorescence results demonstrated, neutrophils were present during wound healing of hair follicle ORS, but they did not appear to play an important role in regeneration, as morphological changes suggestive of activation were not observed. However, apoptosis of neutrophils was detected after phagocytosis of bacteria and cellular debris [28, 29], in agreement with numerous studies that had demonstrated that neutrophil phagocytosis of foreign pathogenic microorganisms could lead to their apoptosis and subsequent phagocytosis by macrophages [30, 31]. Therefore, during the inflammatory response stage, it is possible that both neutrophils and macrophages are needed to promote regeneration and repair of wound tissue, but only neutrophils were present in our model.

In conclusion, the mechanism of hair follicle outer root sheath regeneration is a complicated process involving a change of the hair follicle microenvironment that induces stem cells to alter their normal behavior [32-35]. However, the mechanisms involved in cell division, differentiation, and migration of stem cells caused by changes in the hair follicle microenvironment are unclear, as are interactions between cells during stem cell proliferation

and migration. In this study, the process of regeneration of outer root sheath of hair follicle *in vitro* was explored. The knowledge gained provides a solid foundation for further study of biological mechanisms of stem cell-based repair of hair follicles, as well as a theoretical basis for tissue engineering and regeneration.

5 Conclusion

Our study finds that the basic morphology of the hair follicle has been formed by the 5th day of mouse outer root sheath culture. In this experiment, we found a dynamic, annular cell layer during the inner regeneration of the outer root sheath, which we defined as the glass membrane. The cytoskeleton of the new cells was remodeled well. The new cells first appeared in the protuberant part of the hair follicle and migrated to the inner part of the glass membrane during the process of regeneration. Under the chemokine action of inflammatory chemokines, Neutrophils migrated to the wound site and initiated the inflammatory response. In the later stage of regeneration and remodeling inside the outer root sheath, the neutrophils formed a cell layer outside the glass membrane, which aided in the protection of new structures.

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