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SCARLESS WOUND HEALING: Increased Collagen Deposition Along Wound Edge in Human Fibroblasts vs. hEScells

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Abstract

Scarless wound healing is the ideal result to all who suffer from minor to major wounds. Scars occur as a result of excess collagen being assembled in a wound. Prolyl-4-hydroxylase (P4H) is the enzyme that modifies single collagen strands so that they can assemble into larger fibrils, and together with overall collagen levels determine the extent of scarring. Documenting the P4H levels in a wound and finding a more refined measurement of scar potential may give insights into wound healing. One process in which P4H was observed in wounds involved in vitro scratch assays on human primary fibroblasts containing TGFβ1. A wound was created by using a micropipette tip that was dragged across the diameter along the most confluent region of the plate of primary fibroblast cells. Immunohistochemistry (IHC) was performed on the in vitro scratch assays using antibody 5B5, a reliable marker for P4H. The staining at the edges of the scratch of the in vitro scratch assay was positive for the 5B5 marker, indicating changes in collagen chain assembly. Human embryonic stem cells (hEScells) were also used in comparison to the human primary fibroblasts cells. The IHC of the hEScells showed fluorescence of the 5B5 marker throughout the entire plate instead of just on the edges of the scratch, indicating that the signaling responsible for turning on P4H is missing in the hEScells tested. These results suggest that collagen deposition and assembly in human primary fibroblast cells are more localized compared to hES cells, and may explain in part why hES cells demonstrate the ability to heal without scarring. Our results suggest that targeting the border cells of wounds should be pursued to prevent the formation of scars.

Keywords: scarless wound healing, collagen deposition, human embryonic stem cells (hEScells), human primary fibroblasts, prolyl-4-hydroxylase (P4H), 5B5, TGFβ1, Vimentin

1. Introduction

Scarless wound healing would be the ideal result following a minor or major injury to the skin. However, in adults, scars typically follow after the complex process of wound healing. There are minor scars that appear as fine lines to major hypertrophic or keloid scars [10]. Scars not only ruin the physical aspect of the skin, but often, its normal function does not return. Scarring can also be mentally or emotionally damaging as it has been linked to anxiety, social avoidance, and even depression [11]. The goal of achieving perfect regeneration of skin using an effective scar-free wound has not yet been accomplished for adults currently. Much can be learned from the study of the structure of collagen, the distribution levels of collagen in wounds, as well as how scarless wound healing occurs in the healing of fetuses for application to adults [1]. In order to further this research, further comprehension of wound healing is required, including which factors trigger cellular responses.

Wound healing is comprised of three main phases including inflammation, proliferation, and remodeling. The first part involves the wound forming a blood clot, thus sealing the underlying tissue from the oxygen in the air [3].

Degenerating platelets release growth factors, which in turn stimulate the first phase of cell proliferation of epidermal cells, fibroblasts, and endothelial cells. Neutrophils then clear debris and bacteria, and then secrete cytokines that facilitate in activating local fibroblasts and keratinocytes. These neutrophils are later replaced by macrophages that produce cytokines and other growth factors that are essential to wound healing. TGF-β activates the fibroblasts to start proliferating and increasing collagen synthesis, which then cross link and form thick strands of collagen fibers in the extracellular matrix. (ECM) In a later phase, fibroblasts differentiate into myofibroblasts and together they contract and close the wound. Afterwards, myofibroblasts have been found to undergo apoptosis and disappear [9].

An in vitro scratch assay is a model that can help study and visualize cell migration, and in our research, collagen deposition. This method is simple, yet it can provide an infinite number of interpretations. In our research, we are using human primary fibroblasts as well as two human embryonic stem cell lines (hEScells) (BioTime). We have identified 5B5, a reliable marker for prolyl-4-hydroxylase, and seen staining at the edges of an in vitro scratch assay and may indicate changes in collagen production. There is also a positive response of Vimentin, a marker for intermediate filaments, indicating cells migrating into the scratch. In vitro scratch assays will allow us to locate where collagen production is most rampant. This in turn, can provide where collagen could be inhibited to prevent scarring. We can also compare wound scarring in human primary fibroblast cells versus human embryonic stem cells. Embryonic stem cells can provide important details that can allow us to have a better understanding of scarring in fetal skin, which can then be applied to adult skin. Adult skin lacks certain characteristics of fetal skin that results in scarring.

Collagen is a structural protein that maintains the integrity and structure of our organs and plays a significant role in wound healing. The topic of collagen deposition is primary to the understanding of scarring. Fibrosis is a result of excessive collagen deposition [4, 5, 11, 12, 13]. One explanation states that there is an imbalance between collagen synthesis and collagen degradation [13]. Another explanation is the delay of adult fibroblasts in putting down collagen as it has to proliferate before doing so, in comparison to fetal fibroblasts that are simultaneously proliferating and making collagen [11].

A more direct approach to controlling scar formation is the regulation of collagen synthesis and deposition. [5] Collagen chain assembly depends on proline hydroxylation, therefore it is facilitated by an enzyme, prolyl-4-hydroxylase, that will cause an increase in collagen chain assembly. A study using prolyl-4-hydroxylase inhibitor on a dermal ulcer wound was able to decrease the scar during the healing process. This indicates that documenting P4H levels in a wound may give insights into wound healing [5].

The idea of using stem cells and fetal skin and studying collagen deposition is a popular theme in scarless wound healing research. One study was successful in determining the origin of collagen production in dermal tissue after comparing the collagen yield of resident cells and bone marrow-derived cells. They found that resident cells provided the majority of collagen during the dermal excision healing as well as fibrogenesis [4]. The bone marrow-derived cells, believed to produce collagen I, played a minimal role in the production of collagen during wound healing and the dermal fibrogenic process [4].

Our main research method involves the use of in vitro scratch assays on human primary fibroblast cells and human embryonic stem cell lines. These two cell lines are capable of providing a plethora of information regarding scarless wound healing. With the use of advanced technologies such as immunohistochemistry and PCR, we are capable of expanding the current knowledge of scarless wound healing.

2. Methods and Materials

2.1 Cell Samples

This research uses dermal fibroblasts derived from human neonatal foreskin, passaged as P4. The medium was first vacuumed off from the flask and rinsed with PBS wash buffer three times. PBS wash buffer was added along with Trypsin-EDTA (.25%)(gibco) to dissociate the adherent cells on the flask. The flask was then incubated for 10 minutes at 37°C with 5% CO_2 (HERA cell 150). DMEM GlutaMax (gibco)+ 10% FBS without phenol red was used as the medium to suspend the cells. 15 microliters of TGFβ1 was added to the control with one-week exposure and the experimental plate with two-week exposure to the reagent. The trypsinized cells and the DMEM were thoroughly mixed in the flask by also pipetting up and down for the even distribution of the trypsinized cells in suspension. The cells were then evenly distributed onto tissue culture dishes (Falcon 60x15mm). The plates were then stored in the incubator at 37 °C with 5% CO_2 (HERA cell 150).

This research also uses human embryonic stem cell lines (hESC 6 and 28. (BioTime, Inc.) The medium was first vacuumed off from the flask and rinsed with PBS wash buffer three times. PBS wash buffer was added along with Trypsin-EDTA (.25%)(gibco) to dissociate the adherent cells on the flask. The flask was then incubated for 10 minutes at 37°C with 5% CO_2 (HERA cell 150). DMEM GlutaMax (gibco)+ 10% FBS with phenol red was used as the medium to suspend the cells. The trypsinized cells and the DMEM were thoroughly mixed in the flask by also pipetting up and down to for the even distribution of the trypsinized cells in suspension. The cells were then evenly distributed onto tissue culture dishes (Falcon 60x15mm). The plates were then stored at 37 °C with 5% CO_2 (HERA cell 150).

2.2 Wounding of the Samples

The human fibroblast cells and hESC lines 6 and 28 formed a confluent monolayer, and were then scratched. A wound was created on both the human fibroblast plates and hESC lines 6 and 28 by scratching the cells with the aid of a P200 micropipette tip. The tip was dragged across the diameter in a perpendicular fashion with consistent pressure along the most confluent region of the plate.

2.3 Immunohistochemistry

Immunohistochemistry (IHC) staining was performed on two human primary fibroblast plates after 17 days of wounding the cell. One hESC line 6, and one hESC line 28 underwent IHC staining after 8 days of wounding the cell. The medium was removed and the cell cultures were rinsed with PBS wash buffer twice. Acetone-methanol was added to each cell culture for 10 minutes at room temperature and was vacuumed and allowed to air dry for 3 minutes. A pap pen was used to mark 2 areas of interest along the wound. Normal antibody diluent (MPBioMedicals) was added to each area for 30 minutes at room temperature and vacuumed off. The first square was stained with a primary antibody, Fibroblast Clone 5B5 (1:200) (DakoCytomation; Mouse Anti-Human). The second square was stained with primary antibody, Vimentin (1:200) (Shandon Immuno, Pittsburgh, PA). The plates were then incubated at 37°C with 5% CO_2 (HERA cell 150) for 1 hour. The primary antibodies were vacuumed off. The cells were rinsed with PBS wash buffer, incubated for 5 minutes at room temperature, and vacuumed off. This step was repeated 3 times. Normal antibody diluent (MPBioMedicals) was added to each area for 15 minutes at room temperature. Antibody diluent was to reduce background levels of fluorescence. The secondary antibody, goat anti-mouse (1:200), was added to all areas of interest. The plates were then incubated in the dark at room temperature for 1 hour. The secondary antibody was vacuumed off. The cells were rinsed with PBS wash buffer, incubated for 5 minutes at room temperature, and vacuumed off. This step was repeated 3 times. Afterwards, the sides of the plates were cracked and the plates were allowed to air dry for 3 minutes. 1 small drop of Vectashield mounting medium for fluorescence with Dapi (Vector Laboratories, Inc. Burlingame, CA) was added to each area of interest and covered with a coverslip.

The plates were observed under a fluorescence microscope (Nikon Eclipse E800) after 1 week of the IHC staining and photographed using a Nikon D60. The program software, Aperture, was used to import the photographs showing a positive or negative stain.

3. Results

3.1 Human primary fibroblast signal for P4H

A flask of P4FB was split into plates containing DMEM with 10% FBS medium without phenol red and induction factor and allowing the plates to become confluent. The plates were scratched using a 200-micropipette tip with equal pressure along the diameter of the plate. Afterwards, IHC was performed using 5B5 antibody. Figure 1 shows evident fluorescence of the border cells of the in vitro scratch assay. The edge of the wound shows a positive signal for the 5B5, which marks for prolyl-4-hydroxylase. This implies that there is collagen deposition at the bordering cells. In the negative control, shown in Figure 2, the border cells showed a negative response to 5B5 with no fluorescence.

3.2 hEScell *signal for P4H*

Flasks of stem cell lines 6 and 28 were split into plates containing DMEM with 10% FBS medium with phenol red. Once the plates were confluent, they were scratched using a 200-micropipette tip with equal pressure along the diameter of the plate. Afterwards, IHC was performed using the 5B5 antibody. The P4H signal was present throughout both stem cell plates line #6 and line #28 as seen in Figure 3 and Figure 4.

3.3 hEScell signal for Vimentin

Flasks of stem cell line #6 and line #28 were split into plates containing DMEM with 10% FBS medium with phenol red. Once the plates were confluent, they were scratched using a 200-micropipette tip with equal pressure along the diameter of the plate. Afterwards, IHC was performed using the Vimentin antibody. In Figure 5, there was a positive signal for Vimentin in both plates. Cells along the border contain intermediate filaments and migratory cells moving into the scratch. At higher magnifications, faint striations could be seen on the filamentous cells.

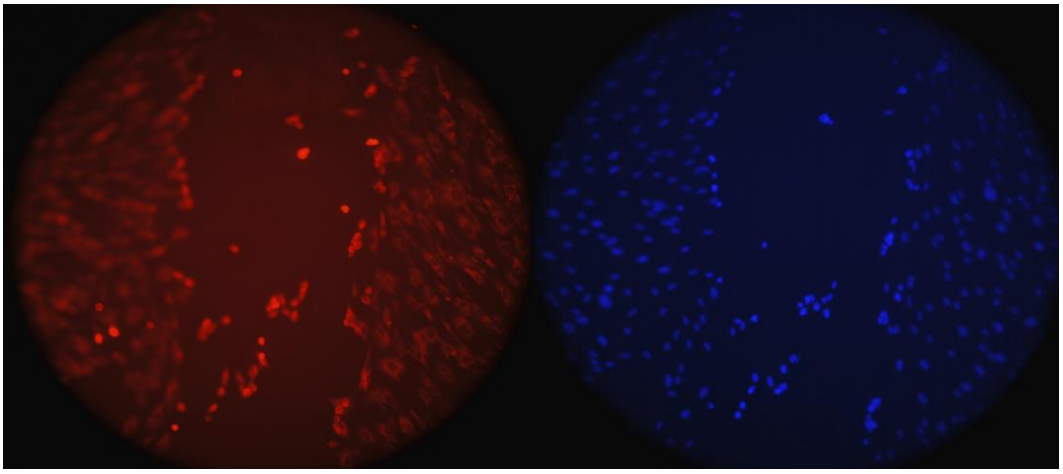

Figure 1. (L) Immunofluorescence staining of P4FB (+ TGFβ1) with 5B5 marker under 20x magnification in Texas Red. 5B5 marker is positive along the edge of the in vitro scratch assay. (R) Dapi staining of P4FB.

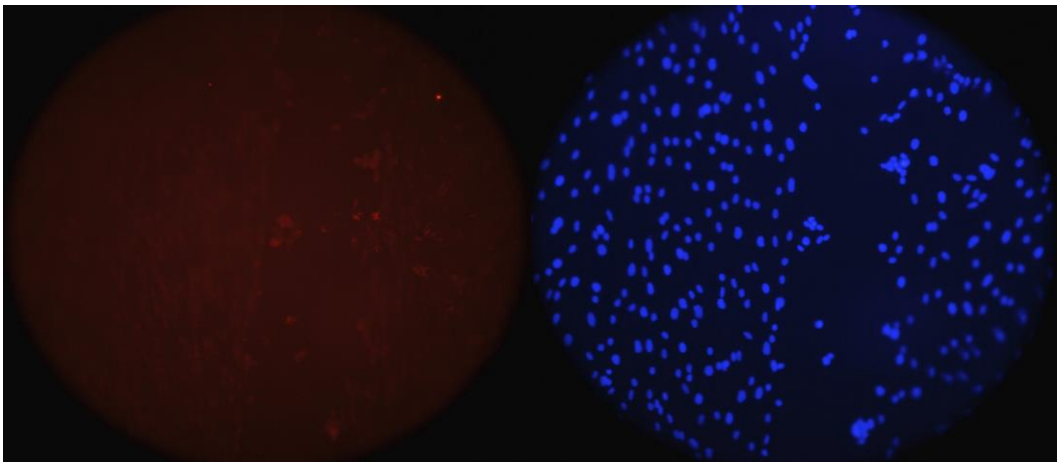

Figure 2. (L) Immunofluorescence staining of P4FB (- TGFβ1) with 5B5 marker under 20x magnification in Texas Red. 5B5 marker is negative in the in vitro scratch assay. (R) Dapi staining of P4FB.

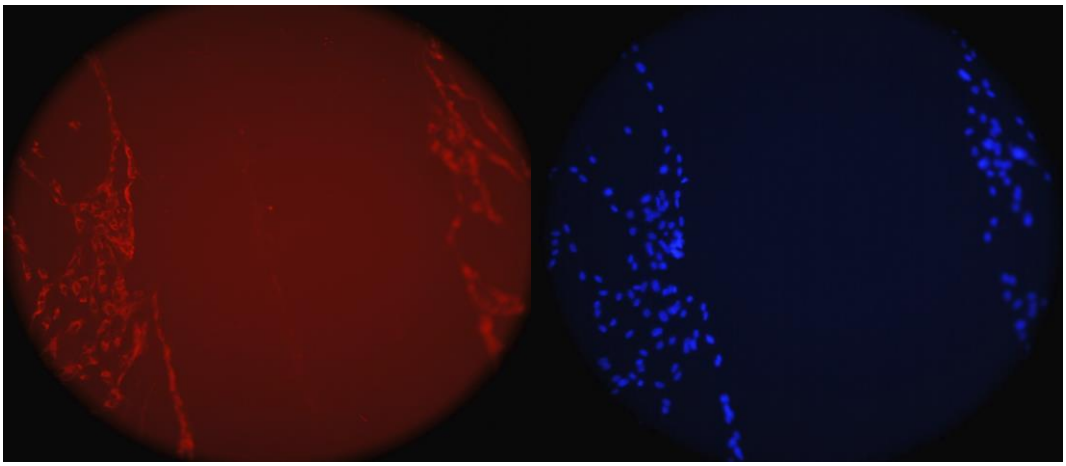

Figure 3. (L) Immunofluorescence staining of hEScell Line #6 with 5B5 marker under 20x magnification in Texas Red. 5B5 marker is positive all over the plate. Cells are also lining up along the edge of the in vitro assay. (R) Dapi staining of hEScell Line #6.

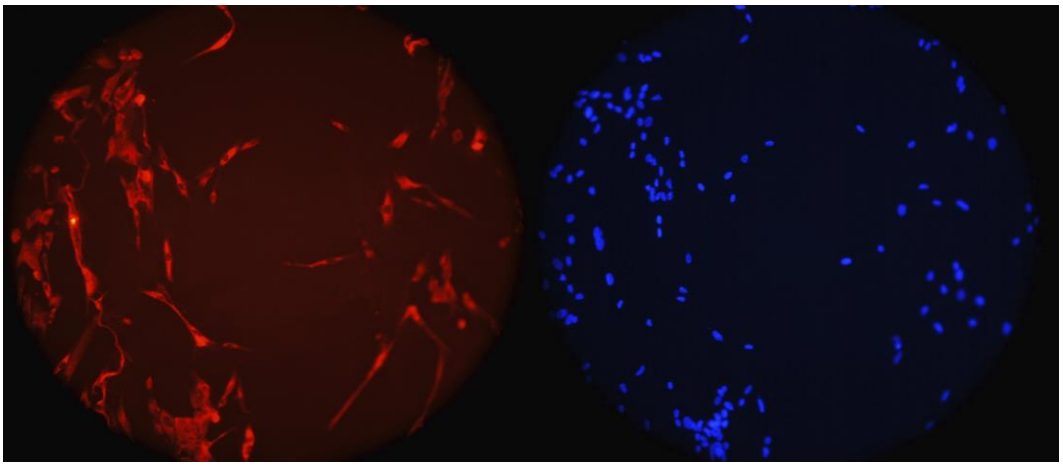

Figure 4. (L) Immunofluorescence staining of hEScell Line #28 with 5B5 marker under 20x magnification in Texas Red. 5B5 marker is positive all over the plate. Cell migration is random and occurred at a faster rate compared to hEScell Line #6. (R) Dapi staining of hEScell Line #28.

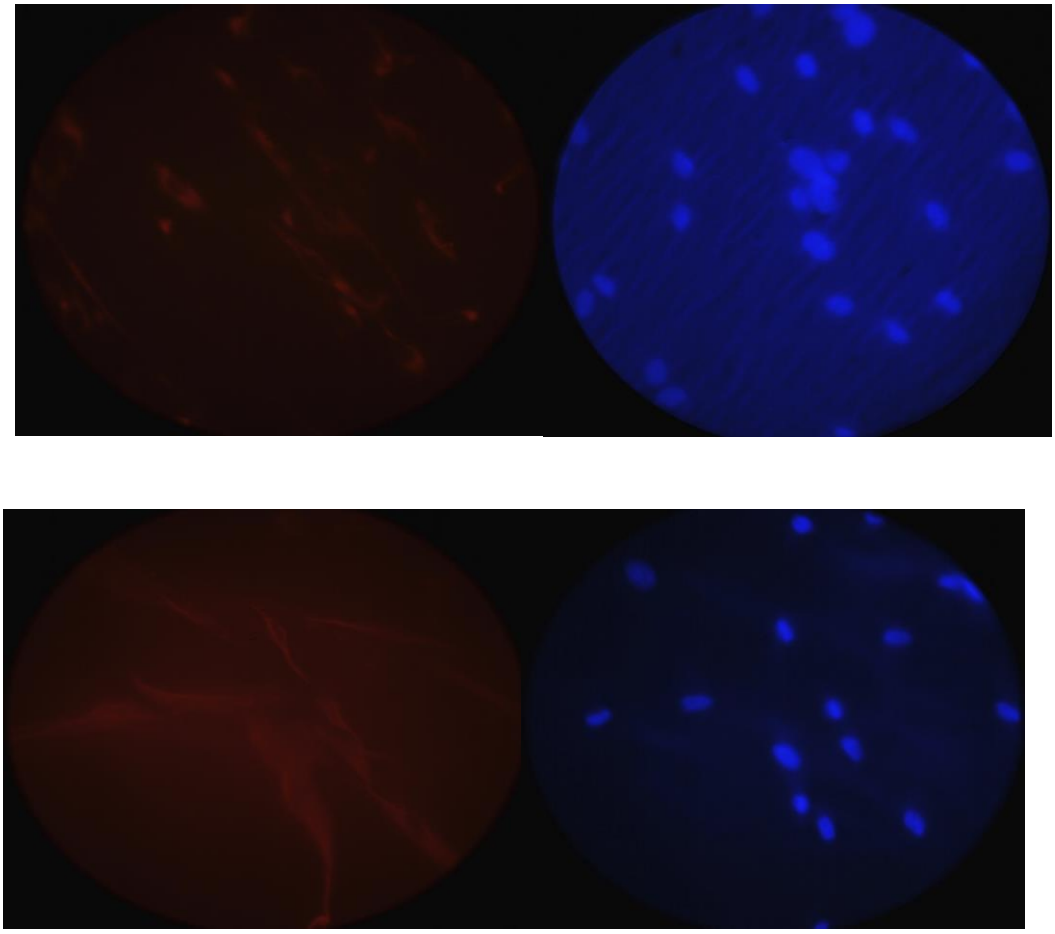

Figure 5. (Top Left, Bottom Left) Immunofluorescence staining of hEScell Line #6 and #28 with Vimentin marker under 60x magnification in Texas Red. At a higher magnification, Vimentin stained cells have a filamentous appearance with faint striations that are visible at higher magnifications. (Top Right, Bottom Right) Dapi staining of hEScell #6 and #28.

4. Discussion

This present study focuses on collagen deposition in both human primary fibroblasts and human embryonic stem cells. According to our results, collagen deposition in human primary fibroblasts differs greatly in comparison to human embryonic stem cells, not only in quantity, but also in location.

There was a positive response to the 5B5 marker for P4H on the edges of the wound in our positive control plate, P4FB (+ TGFβ1). A positive signal for P4H correlates with collagen assembled fibers. It can be deduced that there was collagen assembly occurring along the border cells. TGFβ1 plays a role in this event. TGFβ1 is one of the secreted cytokines that are involved in several processes, including wound healing, specifically in growth, proliferation, differentiation, and apoptosis [6]. One role of TGFβ1 is the stimulation of the production of extracellular matrix components such as collagen. It has been studied that TGFβ1 supplementation has proven to increase collagen production from cells of mesenchymal origin [14]. In this study, the addition of TGFβ1 to the human primary fibroblasts could have increased the cells' ability to produce more collagen when wounded and to make the location of the collagen deposition more evident. With the collagen being more evident, it allows the

localization of the assembly process. In the case of our negative control, P4FB (- TGFβ1), there was no response to the 5B5 marker for P4H. This suggests that without the growth factor, the collagen assembly is not evident.

In the case of the hEScells, there was a positive response for the 5B5 marker for P4H. The P4H signal was present on the entire plate as opposed to just the border cells of the human primary fibroblasts. There is a large difference between collagen deposition in fetal skin and adult skin. There are several factors in the fetus that differ from the adult, including the fact that there is more type III collagen than type I, the patterns of collagen deposition are neater and more organized, and the collagen fibers are thinner and smaller [1]. Adult skin is characterized by type I collagen, which results in a disorganized thick collagen fibers and more collagen cross-linking [1, 11, 13]. There is a correlation between scarring and the arrangement and presence of specific types of collagen [1]. In this study, the fact that hEScells have collagen production everywhere, could suggest that there is even distribution of collagen, which can then prevent scarring. In the case of human primary fibroblasts, collagen production is localized and is therefore overproduced in the edges of the wound, forming a scar.

Another positive response from the hEScells was for Vimentin. The border cells of the hEScells showed striations when seen at a high power objective on the fluorescent microscope. Vimentin is a marker for intermediate filaments and is seen in mesenchymal cells, or cells that are transitioning from epithelial to mesenchymal cells [7]. The positive response for Vimentin shows that the hEScells are perhaps undergoing transition as they migrate into the wound.

In summary, the findings in this study present that collagen deposition in human primary fibroblasts are found along the edges of the wound using P4H as the determining factor. The P4H levels account for collagen-assembled fibers and is therefore a more refined measurement of scar potential. Another finding suggests that adult skin is often prone to scarring due to the localization of P4H along the edge of the wound, causing uncontrolled collagen production. By understanding the cellular process of the border cells, studies can expand on the prevention of scarring. The findings in this study could explore the different possibilities in reaching technology towards scarless wound healing.

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