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Methods Article

Efficient protocol for isolating human fibroblast from primary skin cell cultures: application to keloid, hypertrophic scar, and normal skin biopsies

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Abstract

This protocol introduces a streamlined and efficient method for isolating human fibroblast from skin primary cell culture with a specific focus on its application to keloid, hypertrophic scar, and normal skin biopsies. Additionally, the absence of suitable animal models for keloid and hypertrophic scar has led preclinical research to rely on in vitro studies using primary cell cultures. This approach addresses the challenges of existing protocols in terms of time, cost, equipment, and technical expertise required. The method involves derivation, culture, and characterization analysis including cell proliferation, migration, and fibroblastic marker (Vimentin, CD90, CD73, and CD105) expression. Our study yielded high amounts of fibroblast from tested skin explants while maintaining their in vivo-like characteristics and behaviour. Immunostaining assay confirmed that the cultivated fibroblast was positively expressed Vimentin. Flowcytometry results showed high expression of CD90 and CD73 while relatively showing lower expression of CD105. Fibroblast derived from keloid tissue showed the highest rate of proliferation and migration ability compared to the other samples. These findings suggest an efficient and reproducible technique to cultivate high qualified fibroblast from human skin in normal or pathological condition, particularly for keloid and hypertrophic scar. The application of this protocol provides a foundation for further studies to investigate the progression and potential intervention of aberrant fibrotic dermatological disorder, in vitro.

Keywords: fibroblasts; primary culture; keloid; hypertrophic scar; normal skin

Introduction

Fibroblast is a fundamental tissue component critically involved in regulating various aspects of skin biology such as wound healing, tissue repair, and maintaining skin integrity [1]. Dysregulation of fibroblast function is observed in fibrotic dermatological conditions, particularly keloid and hypertrophic scar [2]. The phenotypic alteration of keloid and hypertrophic scar-derived fibroblasts is reflected in its stronger anti-apoptosis and migration abilities compared to normal skin fibroblasts. In addition, keloid fibroblasts show higher response to growth factors that eventually contribute to high synthesis and deposit of extracellular matrix components [3–5]. The lack of suitable animal models that accurately mimic keloid and hypertrophic scar remains a significant challenge for the in vivo studies. Keloids are unique to human possibly due to differences in skin structure and immune system function compared to animal models. Consequently, most researchers utilize keloid tissue or primary cells isolated from keloid for preclinical research studies [6]. Thus, establishing a reliable method for isolating and characterizing human fibroblast from keloid, hypertrophic scar, and normal skin biopsies is essential for biomedical research. These cells serve as an ideal primary cell-based model for understanding cell signalling, extracellular matrix production, drug screening or safety testing, among others [7].

Primary cultures are ideal methods for isolating fibroblasts that represent the in vivo state of the tissue origin [8]. These explant methods are considered as the simplest techniques in primary culture and have several advantages. This approach allows for the continuation of molecular communication between tissue fragments and migrating cells by releasing cytokines and growth factors, providing a suitable environment for cell growth and proliferation without the need for exogenous supplementation [9]. Most of the existing protocols for growing and isolating fibroblast from primary cell culture of keloid, hypertrophic scar, and normal skin are complicated and expensive. These protocols often require the use of digestive enzyme (either fully or partially), such as collagenase type I, as demonstrated by Liang *et al* [10] and Nejaddehbashi *et al* [11] for isolating fibroblast from keloid,

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hypertrophic scar, normal skin samples. Furthermore, Henrot *et al* [12] and Raktoe et al [13] utilized Dispase II to separate epidermis and dermis layers of the skin in the initial step, followed by collagenase solution in the following step of their study. Besides the digestive enzyme, use of appropriate cell culture media is crucial to support cell growth in subsequent culturing phase. For instance, Kurzyk *et al*. have utilized commercially available fibroblast growth media that may be difficult to obtain in some regions [14]. This approach poses challenges for researchers with limited resources and facilities.

In this article, we proposed a streamlined and cost-efficient method for isolating and culturing human fibroblast from skin primary cell culture, with specific focus on application to keloid, hypertrophic scar, and normal skin biopsies. The fibroblasts were cultivated by explant primary culture method with cell culture basal complete media (10% serum and 1% antibiotic-antimycotic solution). This protocol aims to ensure that the fibroblast yield retains its in vivo-like characteristics and behaviour. It is expected to valuable model for investigating fibroblast behaviour in both physiological and pathological states, particularly in fibrotic skin like keloid and hypertrophic scar. By establishing this protocol, we aim to facilitate an efficient protocol for further study to explore potential intervention targeting aberrant fibrotic response in dermatological disorders.

Materials and methods

Chemicals and materials

Dulbecco's modified Eagle medium (DMEM) low glucose with glutamine and pyruvate from Thermo Scientific (11885084, New York, USA). Foetal bovine serum (FBS), antibiotic-antimycotic solution, and trypan blue from Sigma (SA F2442, St Louis, MO, USA). Phosphate buffer saline (PBS) without calcium and magnesium was purchased from Solarbio Life Sciences (P1020, Beijing, China), and accutase was purchased from Stem Cell Technology (07920, Vancouver, BC, Canada). Methanol was purchased from Merck (1.00983.2500, Darmstadt, Germany). Triton X was purchased from Invitrogen (SL038, Carlsbad, CA, USA). Normal goat serum (NGS) and dimethylsulphoxyde (DMSO) were purchased from Solarbio (D8370, Beijing, China). Vimentin monoclonal antibody and fluorescein Isothiocyanate (FITC)-conjugated goat anti-rabbit antibody were purchased from Thermo Scientific (65-6111 and SP20, Waltham, MA, USA). Antibodies against CD90, CD73, and CD105 were purchased from BD stemflow (Piscataway, NJ, USA). Mounting medium with 4′,6-diamidino-2-phenylindole (DAPI)-aqueous fluoro-shield was purchased from Abcam (ab104139, Waltham, MA, USA). Consumables such as 15 and 50 mL centrifuge tubes, 0.22 μm syringe filter, and 24-well-cell culture plate were purchased from Corning (430791 and 430829, Charlotte, NC, USA). The tissue culture flask and chamber slide were purchased from SPL Life Sciences (13485, Gyeonggi, Korea) and Lab-Tek (177445, Rockslide, Denmark), respectively.

Tissue collection

Tissue samples of keloid, hypertrophic scar, and normal skin were obtained from the abdomen skin of three patients with their consent (one sample per tissue type from three different patients). After collection, the samples were submerged immediately in transport media containing DMEM with 6.25% antibiotic-antimycotic solution. This study received approval from the Ethics Committee of the Faculty of Medicine, Universitas Indonesia (Approval No. 1206/UN2.F1/ETIK/PPM.00.02/2023).

Primary cell culture using explant method

The tissue samples were cultured on the same day within 6 hours post-surgery. They were transferred to a sterile culture plate, where the lipid layer was excised using sterile forceps and a surgical blade. The samples underwent six washes in DPBS containing 2% antibiotic-antimycotic solution, with fresh DPBS and tubes for each wash to maintain sterility. Thereafter, the samples were incubated in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic (complete media) for up to thirty minutes at room temperature inside a Biological Safety Cabinet (BSC). Ensure that the sample remains fully submerged in the media to prevent desiccation and potential tissue damage. All procedures were carried out under aseptic conditions in a pre-sterilized BSC to avoid contamination. Following the incubation, the epidermal layer (the pigmented upper side of the skin) was removed using sterile instruments within the culture dish containing complete media. The remaining dermal sample was minced into 1–3 mm in size for each fragment. Afterwards, each fragment was being placed into designated wells of a 24-well culture plate at marked. The marked area was created by scratching the surface of the well plate using a sterile blade to enhance the attachment of the explant, and then the cells could emerge from the explant more easily.

The plates were placed semi-opened in the BSC for 30 minutes for stabilization. Two hundred microlitres of complete media was gently added to each well to prevent explant detachment due to rough handling of the liquid. The explants then were incubated in a standard incubator at 37°C with 5% CO_2. The culture media was changed every 2–3 days by adding 300–350 μL complete media to each well. When cells reached 70%–80% confluence, they were detached and passaged using Accutase (Supplementary material 1).

Immune Cyto-Fluorescence imaging assay for Vimentin as fibroblast marker

Vimentin expression was assessed using an immunofluorescence imaging system with samples from passage 2 of primary cell cultures. Cells were seeded at 10,000 cells per well on chamber slides and incubated at 37°C with 5% CO_2 for 24 hours. After removing the media, each well was washed three times with 200 μL PBS. 200 μL fixation solution (methanol) was applied and incubated for 15 minutes at room temperature, followed by aspiration and three rinses with PBS. 200 μL permeabilization solution (0.3% of Triton X in PBS) was then applied and incubated for 5 minutes at room temperature before aspiration. The cells were subsequently blocked with a blocking solution (2% of NGS in PBS) for 1 hour at room temperature. Vimentin monoclonal antibodies (1:100) were added and incubated overnight at 4°C, and the cells were rinsed three times with PBS. Secondary antibodies (1:50) were added and incubated for two hours at room temperature in the dark. Following aspiration and three PBS rinses, the chamber was removed using forceps. Two or three drops of mounting media with DAPI were added and incubated for 10 minutes at room temperature. The assay area was covered with a cover glass, and fluorescence was observed using inverted fluorescence microscope (ZOE Fluorescent Cell Imager-Bio-Rad).

CD90, CD73, and CD105 expression analysis

The expression of CD73, CD90, and CD105 markers to identify fibroblast populations was analysed using flow cytometry. A total of 1.0×10^6 cells were suspended in 500 μL of DPBS. Subsequently, the suspended cells were washed twice with DPBS, and 100 μL aliquots were replaced into four new tubes. Each tube

was then incubated with anti-CD73, CD90, and CD105 antibodies, along with a lineage-negative cocktail, for 30 minutes. Following incubation, cell population analysis was performed using a flow cytometer.

Proliferation and migration assay

To assess fibroblast proliferation rates, 4×10^3 cells were seeded in each well of a 24-well plate. The cells were incubated in complete media at 37°C and 5% CO [2] for 24, 48, and 72 hours. Each sample was plated out in quadruplets for each time points. Cells were harvested every 24 hours, followed by cell number and viability measurement using the trypan blue exclusion method. The resulting cell number and viability data at each time point (24, 48, and 72 hours) were plotted to generate a cell growth curve.

We used the scratch assay to evaluate the migration rates of fibroblast from each tissue. Cells were seeded at a density of 1.0×10^5 cells per well in a six-well plate and cultured until >90% confluence. Each sample was plated out in quadruplets. The well-plate surface was scratched vertically using sterile 200 µL tips, followed by washing with PBS and changing media. The cells were incubated at 37°C for 24 hours. Cell migration toward the scratch was observed using an inverted microscope at the 0-hour and the 24-hour incubation period. The migration rate was evaluated by calculating the percentage of relative closure after 24 hours. The measurement was carried out using ImageJ software with Wound_healing_size_tool_updated.ijm plug-in. The proliferation and migration data were analysed using GraphPad Prism 9.0 Software (GraphPad Software Inc., La Jolla, CA, USA) and presented as the means ± SD (n = 4). Difference between samples were performed using ANOVA with probability values of significant differences, denoted as $^*P < .05$, $^{**}P < .01$, and $^{***}P < .001$.

Results

Primary cell culture and amplification

The present protocol allows culture and isolation of fibroblast from all samples. The primary cell culture was performed by using dermis part of normal, hypertrophic scar, keloid, and keloid-adjacent skin samples. The result showed that the fibroblast culture and isolation from all samples are relatively simple with high success rate. None of the cultures are contaminated or failed to grow (Table 1, Fig. 1). Cells are able to proliferate and propagate for subsequent assay. In addition, the cells can be used directly or be kept frozen and grow back once thawed.

The cells began to migrate from the edge of the explant between the third and sixth days after incubation. The cells from keloid and keloid-adjacent skin samples exhibited faster growth compared to those form hypertrophic scar and normal skin samples. Consistent with this result, the time to reach confluence was faster on keloid and adjacent keloid skin samples compared to hypertrophic and normal skin (Fig. 1). Cells derived from normal skin are relatively heterogeneous in morphological features (Fig. 2b), while cells derived from the other samples are homogeneous displaying fibroblastic morphology only (amorphic with single nucleus in the centre of the cell) (Fig. 2a). Interestingly, all samples, including normal skin, displayed homogeneous fibroblastic cells population right after subculturing process. These homogenous cells (passage 2) were employed for proliferation and migration analysis.

Vimentin expression assay

The harvested cells were evaluated by immune cytofluorescence imaging assay for Vimentin as fibroblast marker. Figure 3 shows that all the samples are positively expressed Vimentin in the cytoplasmic domain of the cells. Vimentin is widely acknowledged as fibroblast marker and epithelial–mesenchymal transition (EMT) [15]. This type III of intermediate filament cytoskeletal protein is expressed by epithelial cells particularly in those undergoing EMT [16].

CD90, CD73, and CD105 expression assay

Besides the Vimentin, fibroblast shared some characteristics with mesenchymal stem cell (MSC), including the expression of CD90, CD73, and CD105 surface markers [17]. Figure 4 demonstrates positive expression of CD90, CD73, and CD105 in all samples. CD90 and CD73 showed high expression levels (>99%) across all samples, whereas CD105 exhibited relatively lower expression levels (<75%).

Proliferation and migration assay

The isolated cells were examined for their proliferation and migration capability. The result identified that the cells derived from keloid had the highest proliferation rate, followed by keloid-adjacent skin, hypertrophic scar, and normal skin (Fig. 5). In line with this result, the migration assay (Fig. 6a) revealed that keloid fibroblast exhibited the highest migration rates compared to the other samples. Additionally, fibroblast derived from hypertrophic scar demonstrated higher migration rate than those from both keloid-adjacent skin and normal skin samples.

To support those results, we also measured cells' relative closure (%) after 24 hours (Fig. 6b). The findings revealed that keloid fibroblasts exhibited the highest rate of the area covered, indicating their increased migratory capacity. Fibroblasts from hypertrophic scars showed a higher area covered than those from normal and adjacent keloid skin, suggesting a higher activity level in scar tissue. On the other hand, fibroblasts from both keloid-adjacent skin and normal skin samples demonstrated the lowest percentage of closure area in the migration test, highlighting their comparatively slower migration rate.

Discussion

Skin fibroblasts are the most common type of cell playing crucial role in maintaining tissue homeostasis and wound healing process [18]. Fibroblast derived from primary cell culture is relatively closer to its in vivo conditions. Therefore, establishing a simple and affordable method for cultivating fibroblast from primary cell culture was important to obtain a perfect model for in vitro

Table 1. Comparison of keloid, adjacent skin, hypertrophic scar, and normal skin primary culture.

Property	Keloid	Adjacent skin	Hypertrophic scar	Normal skin
Contamination	No	No	No	No
Days for growth initiation	2–3	3–4	5–6	5–6
Days of reaching confluence	8–10	10–12	12–14	12–14

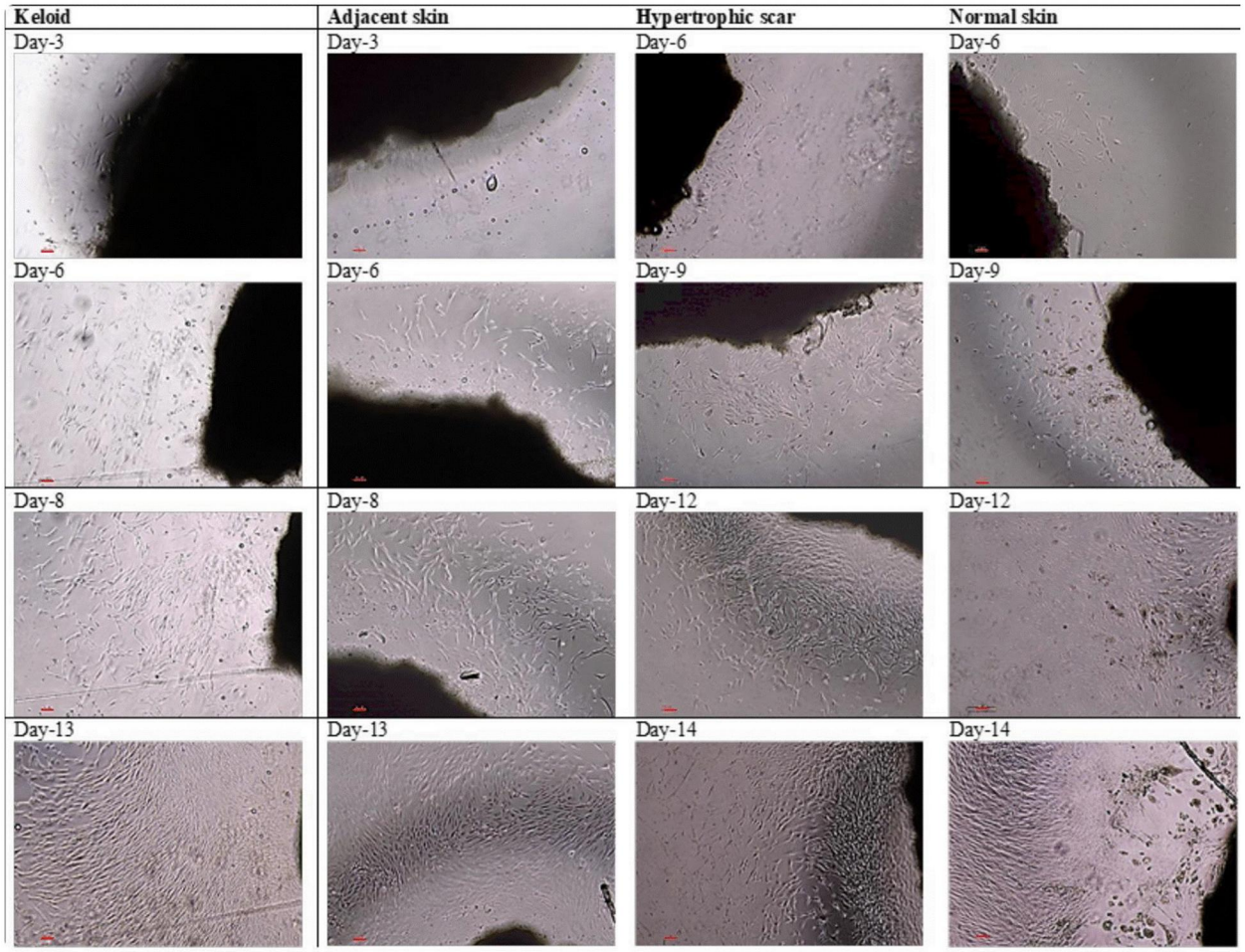

Figure 1. Human skin primary cell culture from keloid, adjacent keloid, hypertophic scar, and normal skin on day-3, -6, -8, and -13. Inverted phase-contrast microscope at 40x magnification (scale bar 100 μM).

experiment. This study established an efficient approach to cultivating fibroblast from normal, hypertrophic scar, keloid, and keloid-adjacent normal skin using the explant method without enzymatic digestion.

Many existing protocols are complex and costly, primarily due to the use of digestive enzyme in all or part of the process. Previous research utilized dispase [19, 20] or liberase [21] to separate the epidermis and dermis part of skin biopsy at the initial part of the primary culture. Followed by subsequent digestion process, collagenase was used to shred the dermis to separate the fibroblast cells from the tissue matrices [22]. However, we recognize that some studies referenced utilized enzymatic digestion to isolate diverse cellular populations, aligned with their specific research objectives. In contrast, our research focused solely on isolating fibroblast using a more efficient method. The adherent fibroblast-like cells could be observed between the third and sixth days of incubation in this study, a significantly faster compared to the previous protocol by Iannelo *et al* [23] which reported fibroblast appearance within 1–4 weeks of incubation. Additionally, the simple composition of the culture media enhances the simplicity of this protocol.

Combining the explant method and basic composition of culture media in this study has successfully reduced the risk of contamination, addressing the limitation of the explant technique. To ensure the sterility of the cultures, particularly for long-term experiments, an optional microbiological test such as Bactec system can be incorporated after the final tissue wash. Bactec is a microbial detection system that utilizes enriched culture media to detect microbial growth in samples. After the final wash of tissue fragments, a small tissue fragment can be introduced into a Bactec vial and monitored for microbial contamination. This step can be used to confirm the absence of bacteria, fungi, and other contaminants before proceeding with long-term fibroblast cultures [24, 25]. The cells could grow well in complete media containing only basic recipe of culture media (10% of FBS, and 1% of antibiotic and antimycotic solution) without any additional supplementation. Contrarily, most recent research supplemented the media with N(2)-L-alanyl-L-glutamine, heparin, epidermal growth factor (EGF), and ascorbic acid 2-phosphate (AA2P) into the fibroblast growth media [20, 26]. The omission of such supplements in the current protocol provides a viable alternative for cultivating the adherent fibroblast-like cell populations with the quality equivalent to that previous research.

Human skin fibroblast are typically characterized by their elongated, spindle, or fusiform shape with elliptical nucleus [27]. Occasionally, they can be recognized by their branched cytoplasm that form triangle, flat-star, or irregular shape [28]. Consistent with these observations, the initial cells emerging from the explant exhibited morphological heterogeneity, displaying diverse appearances like spindle-shape, triangle, flat-star, or irregular shape with single or more nucleus in the centre of the cell. The most heterogeneous cell types were identified from the normal skin sample which may be attributed to the thinner

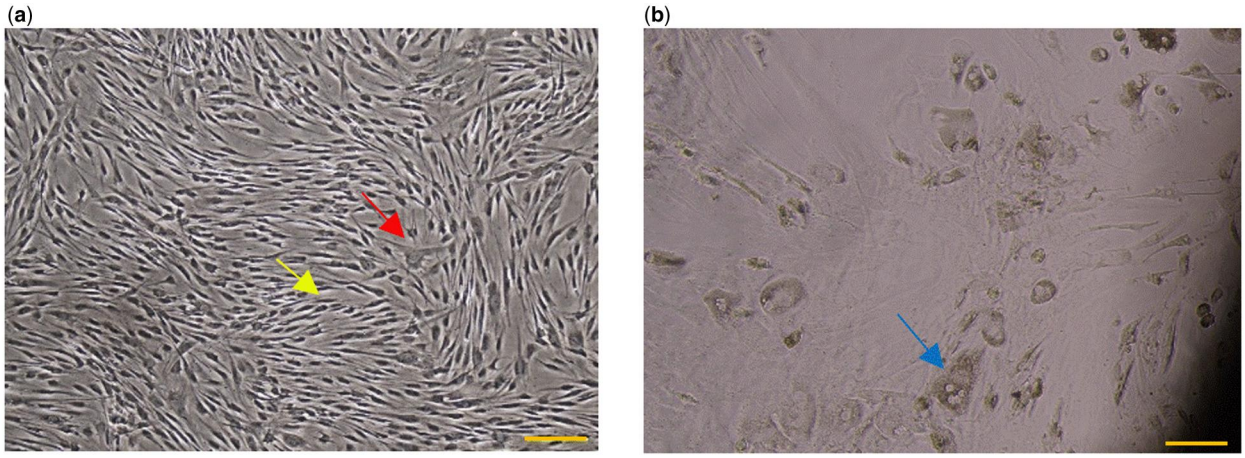

Figure 2. Phase-contrast appearance of the cells. (a) homogenous population of fibroblastic cells; yellow arrow demarks bi- and tricuspid shape and red arrow demarks stellate shape of fibroblast cell. (b) heterogenous population of cells culture; blue arrow demarks suspected brown adipocyte cell. Inverted phase-contrast microscope 100× magnification (scale bar 100 μM).

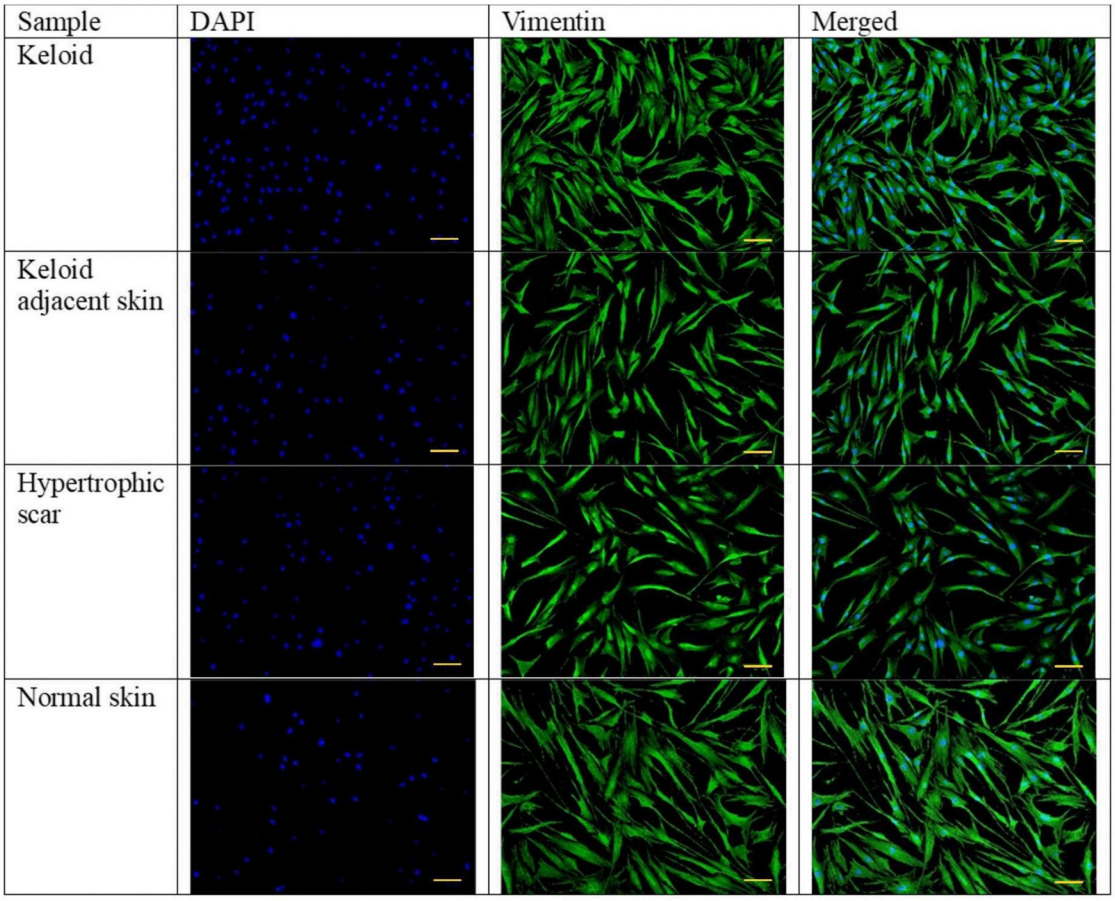

Figure 3. Immunostaining for Vimentin as fibroblast marker in keloid, keloid adjacent skin, hypertrophic and normal skin. Scale bar is 100 μm and 100x magnification.

dermis layer of this samples, leading the explant to include the cells from the reticular and dermo-hypodermis junction area. The typical cells in the reticular and dermo-hypodermis junction were more heterogenous from small tricuspid to larger stellate shapes with visible trabecular networks [28]. Additionally, dermo-hypodermis junction area lies over the hypodermis layer which is composed of adipose tissue [29], and exhibits varying cell types, including brown-coloured, differentiated adipocytes characterized by small lipid granules within their cytoplasm [30], contributing to the cellular heterogeneity (Fig. 2b).

On the other hand, the separation step of epidermis and dermis layer from keloid sample was easier than the other samples due to the thicker dermis layer of keloid, suggesting that most of the keloid explants are derived from papillary and reticular area. The identical morphology of fibroblast from these areas is thin with bi- or tricuspid shapes [28], consistent with the result to this

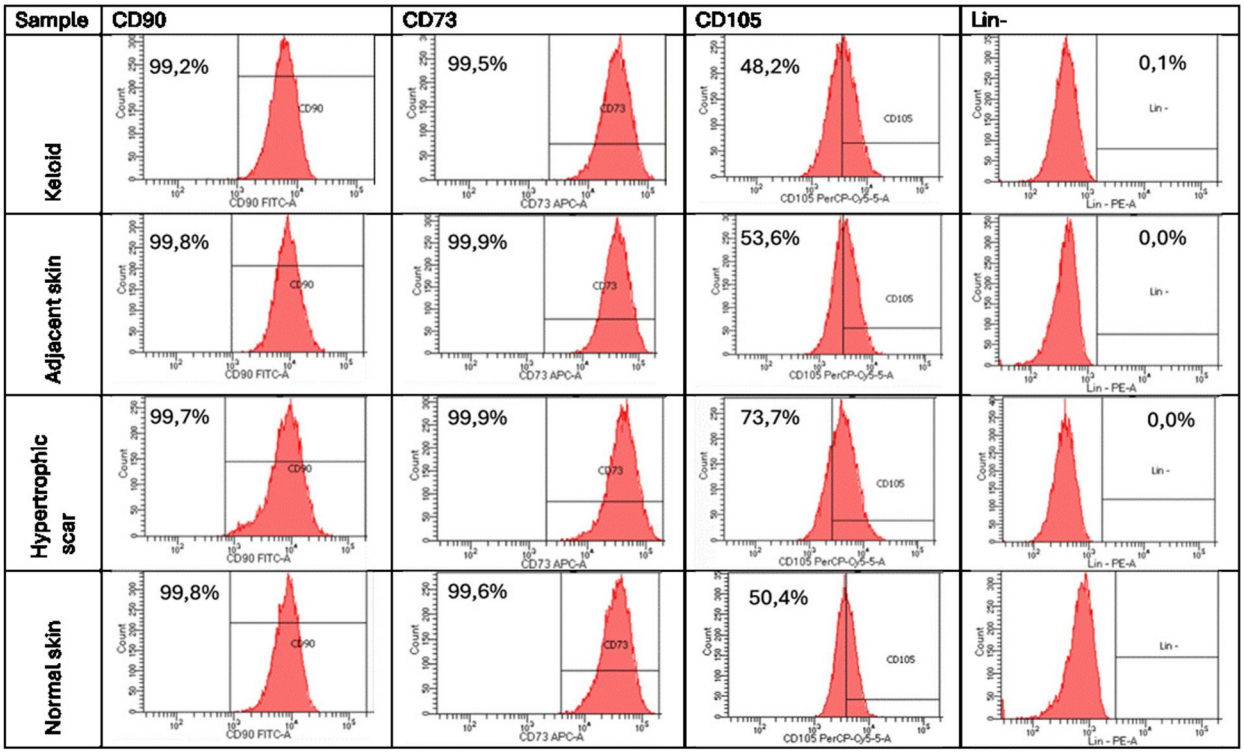

Figure 4. Flowcytometry characterization of CD90, CD73, and CD105 from keloid, keloid-adjacent skin, hypertrophic scar, and normal skin fibroblast.

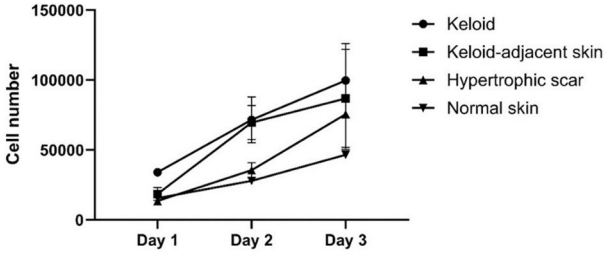

Figure 5. The proliferation rate of keloid, keloid-adjacent skin, hypertrophic scar, and normal skin fibroblast on day-1, -2, and -3.

study (Fig. 2a). Nevertheless, the same typical fusiform morphology fibroblast from all the samples in this study could be obtained even after several passaging process.

It was showed that all the cultured cells in this experiment were positively expressed Vimentin as the classic marker of fibroblast (Fig. 3). Vimentin is widely recognized marker of fibroblast and EMT. Accordingly, Vimentin tunes signalling cascade to regulate cell migration and extracellular matrix remodelling particularly collagen [15]. Despite its association with mesenchymal cells, Vimentin is also found in non-mesenchymal cell type such as epithelial cell suggested its contribution to the migratory and aggressive behaviour of epithelial tumour cells [31]. Therefore, this study utilized CD90, CD73, and CD105 as additional fibroblastic markers to further characterize the cultured fibroblasts. CD90 and CD73 showed high expression levels (>99%), whereas CD105 exhibited relatively lower expression level (<75%) in all the samples. This result aligns with findings from Sober et al [32] that showed fewer CD105-positive cells (<60%) was obtained from foreskin derived fibroblast compared with MSC (>99%). This finding could provide a useful insight to differentiate fibroblast from MSC. In line with this study, Nauli [33] demonstrated that fibroblast derived from keloid showed higher expression of CD90 and CD73 (>94%) and lower expression of CD105 (<30%). Therefore, the cultivated fibroblast using this protocol demonstrated robust expression of fibroblastic markers, including Vimentin, CD90, and CD73 supporting their identification and functional analysis.

The fibroblast yielded using this method was consistently representing their natural characteristic and behaviour. Keloid fibroblast revealed the earliest and confluence growth from the explant primary culture in this study. In accordance with the proliferation rate, keloid fibroblast showed the highest proliferation rate compared to the other samples (Fig. 5). The rapid growth of keloid fibroblast represents natural behaviour keloid formation which grows beyond the original wound boundaries [34]. Adjacent skin performs high proliferation rate after keloid, possibly due to its proximity to the keloid highly influenced by keloid microenvironment. Hypertrophic scar shows moderate increase of proliferation rate over the normal skin aligns with its characteristic of raised, but not excessively growing scar. At last, the normal skin's gradual increase serves as a baseline, indicating typical cell turnover and regeneration without abnormal growth signals.

The results are consistent with the migration assay that quantified keloid fibroblast exhibited the highest rate of migration capability by the cell scratch test (Fig. 6). This result underscores the aggressive nature of keloid fibroblast in terms of their migration ability to invade beyond the nearby normal tissue. Keloid fibroblasts are acknowledged to have higher proliferation rate that leads to excessive amounts of extracellular matrix production and deposition. In addition, keloid fibroblast exhibited an accelerated wound closure phenotype in scratch assay. Those change in cellular behaviour are intricately associated to keloid pathophysiology and other fibrotic disorder [35–37]. Similar with keloid, hypertrophic scar is known as fibrosis-associated disorder

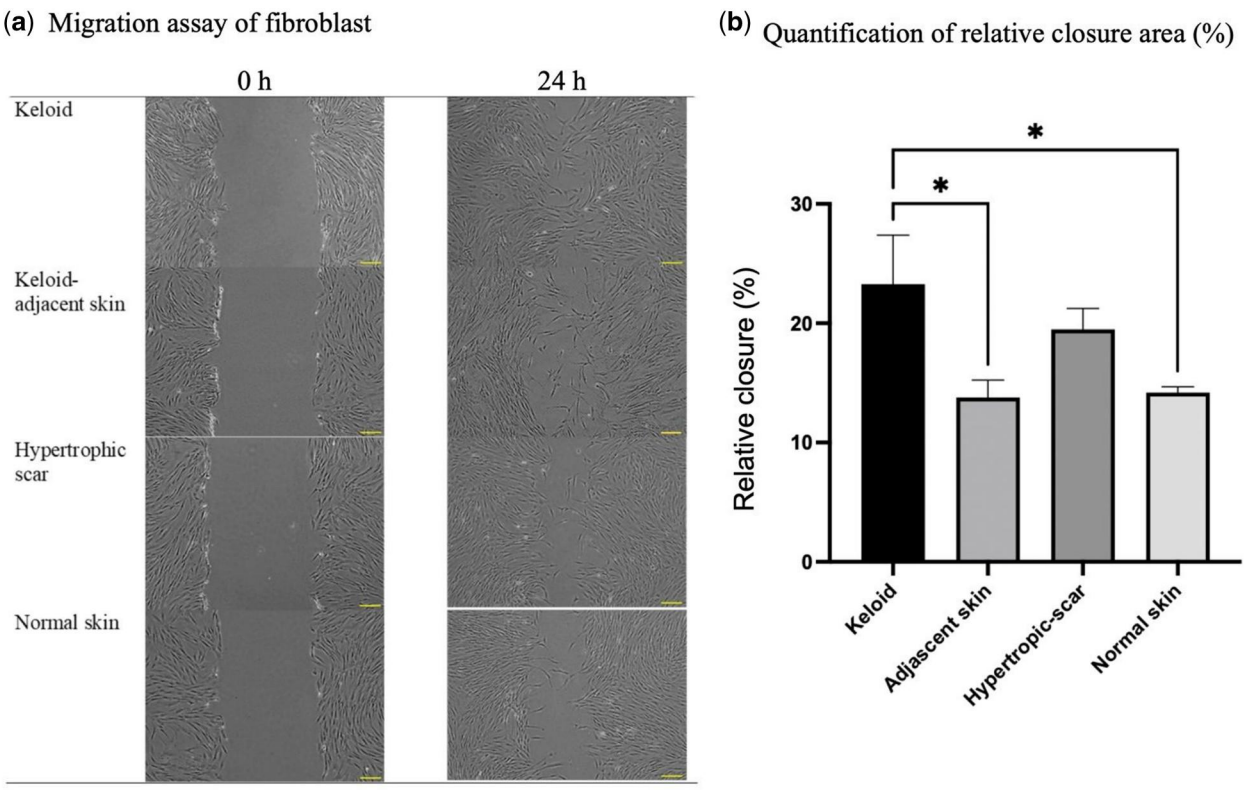

Figure 6. Migration assay visualization (a) and quantification of relative closure area of fibroblast (b). Inverted phase-contrast microscope 100× magnification (scale bar 100 μM). (ANOVA, *LSD*, n = 4, *P < .05).

[38]. Results of this study showed that hypertrophic scar-derived fibroblast exhibited high degree of migration ability, though to a lesser extent compared to keloid-derived fibroblast. Conversely, it was higher compared to normal skin from normal and keloid-adjacent skin. This finding is consistent with Fang *et al* [39] that showed keloid and hypertrophic scar-derived fibroblast possessed higher migration ability compared to normal fibroblast.

This study presents an efficient method for isolating human fibroblast from primary skin cell cultures, applicable to keloid, hypertrophic scar, and normal skin biopsies. The cost-effectiveness is achieved by reducing the reliance on digestive enzyme and media supplementation during the culture process. While this protocol efficiently cultivates fibroblast with preserve its in vivo-like characteristics and behaviour, it was not directly compared with the other existing protocols. Therefore, it is addressed as the limitation of this study. Moreover, our protocol is designed for short-term studies, we did not conduct karyotyping to assess genetic stability. We suggest conducting karyotyping for long-term experiments involving multiple passages. Lastly, we did not measure the cell yield and viability from each tissue fragment, which would provide useful data on the protocol's efficiency.

Conclusion

The current study has successfully generated a high yield of fibroblast from keloid, hypertrophic scar, and normal skin explants while maintaining their native characteristics and behaviour using this simple and efficient method. Immunostaining confirmed positive Vimentin expression in the cultivated fibroblast. Flowcytometry analysis revealed high expression levels of CD90 and CD73, with comparatively lower expression of CD105 in the isolated fibroblast. Notably, fibroblast derived from keloid exhibited enhanced proliferation and migration capabilities compared to the other samples. These findings underscore the effectiveness and reproducibility of our method for cultivating fibroblast whether in normal or pathological conditions, particularly in keloid and hypertrophic scar.

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Author contributions

Sri Suciati Ningsih (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Visualization [equal], Writing—original draft [lead]). Sri Widia A Jusman (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Visualization [equal], Supervisor [Lead]). Rahimi Syaidah Formal analysis [equal], Methodology [equal], Writing [equa] Visualization [equal], Supervisor [equal]), Raisa Nauli (Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Visualization [equal], Writing—original draft [equal. Fadilah Fadilah (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Supervisor [equal])

Supplementary data

Supplementary data are available at *Biology Methods and Protocols* online.

Data availability

All the data supporting findings of this study are available within the manuscript and/or the supplementary data.

Conflict of interest statement. None declared.

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Ethical aspects and conflict of interest

Authors declare no conflict of interest of this current study.

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