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Comparison of Primary Keloid Fibroblast Cultivation Methods and the Characteristics of Fibroblasts Cultured from Keloids, Keloid-surrounding Tissues, and Normal Skin Tissues

Comparación de los Métodos de Cultivo de Fibroblastos Queloides Primarios y las Características de los Fibroblastos Cultivados a partir de Queloides, Tejidos Circundantes Queloides y Tejidos Cutáneos Normales

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SUMMARY: The establishment of primary keloid fibroblast culture has always been a fundamental measure for studying mechanisms of keloid disease. The quality of the primary cell culture can directly affect the results of further experiments. This study was performed to investigate the optimal growth conditions, including the optimal storage time and collagenase treatment time, for in vitro cell culture models and the suitable methods for epidermis-dermis separation in different tissues. Keloid tissues, keloid-surrounding tissues, and normal skin tissues were collected from patients, for primary fibroblast culture. Two methods, tissue explant and collagenase digestion, were deployed and compared. Expression levels of the keloid-related genes α-SMA, Col1, and Col3 were assessed in cells cultured using both methods, to verify the qualities of the primary cells. A comparative analysis was conducted between the two methods and among the three different tissues used. Bacterial and lipid contamination was immediately minimized after the samples were processed. Different methods of epidermis removal and different durations of collagenase digestion were required in different tissues to generate optimal results. Real-time PCR results showed that the mRNA expression levels of keloid-related genes in cultured fibroblasts correlated to their in vivo expression profile, as previously reported in other studies. The results of this study have revealed several key points in the culture of primary keloid fibroblasts and demonstrated the correlation in gene expression between in vivo keloid fibroblasts and in vitro primary keloid fibroblasts.

KEY WORDS: Keloid; Normal skin; Fibroblasts; Primary cell culturing; α-SMA.

INTRODUCTION

Keloid is a type of fibrotic dermal tumor characterized by excessive accumulation of extracellular matrix (ECM) and overgrown dermal fibroblasts after wound healing (Andrews *et al*., 2016). Quality of life can be hugely impaired due to the skin damage and high rate of recurrence after current treatments (Andrews *et al*.). The recurrence rate can be as high as 70 %-100 % with simple surgical excision, and a larger lesion usually forms on recurrence. Combination treatment with surgical excision and other treatments like radiation therapy, pressure therapy, or cryotherapy can significantly lower the chance of recurrence but still not to an ideal range (Berman *et al*., 2017; Jaloux *et al*., 2017; Jones *et al*., 2017). Thus, an effective treatment remains to be developed.

So far, little is known about the etiopathogenesis of keloid disease (Deodhar, 1999; Shih & Bayat, 2010). Due to the lack of appropriate animal models, in vitro or ex vivo cell models are still the main study methods for elucidating keloid formation mechanisms (Supp, 2019). Since the biological characteristics of primary cells are the best model to represent the in vivo environment compared to other cell models, primary cell culture has been a crucial method for keloid studies (Tucci-Viegas *et al*., 2010). In addition, dermal fibroblasts were shown to be the primary ECM producers—a process thought to be a key factor for the onset of keloid forming—so the cultivation of primary keloid-derived fibroblasts became the most widely used model (Shi *et al*., 2019; Kang *et al*., 2020). Thus, the establishment of a reliable

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primary dermal fibroblasts cultivation method is pivotal for studying keloids.

Current studies usually use normal skin-derived fibroblasts (NF) (Fischer *et al*., 2020) as a control to compare to keloid-derived fibroblasts (KF) (Katayama *et al*., 2020). This makes it important to comprehend the cultivation methods of both NF and KF. The keloid tissues are highly fibrotic, and they biologically differ from normal skin tissues. Moreover, the differences are reflected in the culturing methods of the primary fibroblasts from the two tissues. Fibroblasts belong to the family of connective tissue cells and the primary fibroblasts are mainly cultivated via two methods, tissue explant and enzyme digestion, which further includes collagenase digestion and trypsin digestion (Tucci-Viegas *et al*.; Kisiel & Klar, 2019).

The α-smooth muscle actin-(SMA), collagen 1 (Col1), and collagen 3 (Col3) are highly expressed in myofibroblasts, which are transdifferentiated from fibroblasts when the skin is injured. These cells are critical in speeding wound healing, but their persistence was shown to be crucial in keloid formation (Hinz, 2010; Sidgwick & Bayat, 2012; Klingberg *et al*., 2013). Although the mechanisms of the transition from fibroblasts to myofibroblast and the regulations of these fibrosis-related proteins are not fully understood, myofibroblasts and these proteins were considered as hallmarks for keloids and potential therapeutic targets (Bai *et al*., 2016). Here, α-SMA, Col1, and Col3 were used to assess whether the cultured primary fibroblasts still retained the in vivo features and if the cells are suitable models for further keloid studies.

MATERIAL AND METHOD

Sources of samples: Normal skin, keloid, and keloid-surrounding tissue specimens were obtained from patients (18-30 years old) undergoing plastic surgery at the China-Japan Union Hospital of Jilin University. The patients had no organic lesions, and the skin at the sampling sites had no infections or ulcerations. All patients had signed the informed consent form.

Primary culture: Keloid and normal skin tissues were collected by surgical excision, and the subcutaneous tissues were cut off with scissors. Subsequently, the tissues were cut into squares or strips with a scalpel, disinfected with alcohol gauze, and transferred into a sterile cell culture bottle containing DMEM supplemented with 10 % fetal bovine serum (FBS, Biological Industries, Israel) and 500U/ml penicillin-streptomycin (Gibco, USA). Each bottle was sealed and temporarily stored at 4 ºC before using a tissue explant or enzyme digestion method. Cell morphology, bacterial contamination, and epidermal cell intermixing were monitored during the culturing process.

Tissue explant method

Keloid-derived fibroblasts culture. Skin tissue was first transferred from the bottle to a sterile petri dish and washed twice with the D'Hanks solution (HyClone, USA) with 100U/ml penicillin-streptomycin. The keloid epidermis layer was cut off as much as possible using a surgical scalpel, and the center part of the keloid tissue was kept for further processing. Tissues were washed twice with D'Hanks solution before cutting into 1-3 mm fragments with scissors. Tissue fragments were transferred into sterile culture bottles using a 1 ml sterile syringe needle. The tissues were laid as flat as possible to increase the adherent area. The interval between the tissue fragments was approximately 0.3-0.5 mm. Tissue adherence was promoted by incubating the culture bottles inverted in a cell incubator for 2 h. When the tissue fragments were dried and adhered to the bottle, the culture bottles were inverted back. Next, the culture bottles were incubated with the addition of culture media submerging the tissue fragments for two days. If there were no bacteria growing around the fragments and the cells on the edge of the tissues grew outward, the media were replaced on the fifth day after inoculation. The media were replaced every one or two days. After two or three weeks, the cells formed a monolayer and were sub-cultured at a split ratio of 1:2 or 1:3 by trypsinization with 0.2 % trypsin after reaching approximately 100 % confluency.

Normal skin fibroblast culture. The normal skin fibroblast culture method was similar to the description in Keloid-derived fibroblasts culture method. However, due to the thinness of normal skin tissue, there were differences in the process:

(a) Removal of subcutaneous tissues as thoroughly as possible until observing an exposed granular sebaceous gland layer.
(b) Separation of epidermis and dermis using dispase II: Tissues were digested with 2.5 mg/ml dispase II (Coolaber, China) overnight at 4 ºC and washed with PBS twice after discarding the dispase II. Epidermis was peeled off with tweezers or scraped off with sterile surgical blades and before washing with PBS twice to ensure no remaining epidermis.

Enzyme digestion method. The methods of separating NF and KF epidermis were the same as described in section Tissue explant method. Dermal tissue was transferred to a six-well plate and cut into pieces as fine as possible before

adding 1 mg/ml of collagenase I. Digestion was performed at 37 ºC for 1-3 h with gentle shaking for digestion, and five times volume of stop solution (serum culture medium:PBS=1:1) was added.

The above solution containing cells were treated in three ways:

1) The mixture was directly transferred to a six-well plate for further culturing, and the culture medium was replaced with fresh medium the next day. When cell clusters were observed, tissue fragments were carefully removed by a pipette. Cell clusters or adherent single cells were kept to grow.
2) The mixture was filtered through a sterile 70 μm filter mesh. The filtered solution was transferred into a six-well plate for culturing, and culture medium was replaced with fresh medium after 48 h.
3) The mixture was centrifuged for 8 min at 1000 × g. The supernatant was discarded, and the remnant cells were resuspended in fresh media and counted using a hemocytometer. The cells were cultured in a culture dish, and the adherence of the cells was monitored after 48 h. When adherent cell clusters or cells were observed, the culture media were removed and replaced with fresh media. The cells were passaged according to cell growth conditions.

Identification of cell model

Immunofluorescence (IF) staining. Primary KF, keloid-surrounding tissue-derived fibroblasts (KSF), and NF were inoculated into a six-well plate at a concentration of 1×10^5 cells/well. When the cells reached ~60 % confluency, the cells were fixed and stained with primary antibody, rabbit anti-Vimentin antibody (1/200 dilution) (Bioworld, USA) or rabbit anti-a-SMA antibody (1/100 dilution) (Abcam, USA). This was followed by secondary antibody staining with Alexa Fluor®594 goat anti-rabbit IgG (1/200 dilution). DAPI was used to stain the cell nucleus. Samples were kept in the dark. Cell morphology was examined under fluorescence microscopy. Vimentin positive cells were counted.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol from KF, KSF, and NFand quantified spectrophotometrically. A RevertAid First Strand cDNA Synthesis kit (Takara Biotechnology Co., Ltd., Dalian, China) was used for the generation of cDNA according to the manufacturer's protocol. The thermocycling conditions were as follows: 42 ˚C for 60 min; 70 ˚C for 5 min; and maintenance at 4 ˚C. A PrimeScript™ RT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.) was used according to the manufacturer's protocol with a CFX Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.) for qPCR. The thermocycling conditions were as follows: Initial denaturation at 95 ˚C for 3 min; and 45 cycles of denaturation at 95 ˚C for 15 sec, annealing at 6˚C for 20 s and extension at 72 ˚C for 30 s. The primers used are demonstrated in Table I. Gene expression was normalized to the level of GAPDH in a given sample. The expression level of genes and gene alternative splicing products were calculated and analyzed using the $2^{-\Delta\Delta Cq}$ relative quantification method (Livak & Schmittgen, 2001). Each experimental treatment was conducted in triplicate. All data are presented as mean ± standard deviation. Data was analyzed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). Analysis of variance followed by Dunnett's test was performed. $P<0.05$ was considered to indicate a statistically significant difference.

Table I. Primers and conditions for polymerase chain reaction analysis.

Gene	Direction	Primer sequences
α-SMA	Forward	GACAATGGCTCTGGGCTCTGTAA
	Reverse	TGTGCTTCGTCACCCACGTA
Col1	Forward	GAGGGCAACAGCAGGTTCACTTA
	Reverse	TCAGCACCACCGATGTCCA
Col3	Forward	CCACGGAAACACTGGTGGAC
	Reverse	GCCAGCTGCACATCAAGGAC
GAPDH	Forward	GTGAAGGTCGGAGTCAACG
	Reverse	TGAGGTCAATGAAGGGGTC

α-SMA, alpha-smooth muscle actin; Col1, collagen type 1; Col3, collagen type 3.

RESULTS

Morphology and contaminations of bacteria, lipids, or epithelial cells

Tissue explant method. After cultivating the keloid tissue fragments for three to five days, a few cells started to grow out of the fragment. Some spindle cells could clearly be observed on the edges of the tissue after five days. More cells could be observed after 12 days, and the cells began to disseminate. The gap between tissues became narrower while cell density increased. KF cells grew faster and dissociated from the tissue at an earlier time point compared to NF. KF cells could occupy all free spaces between tissues at around 12-15 days (Fig. 1).

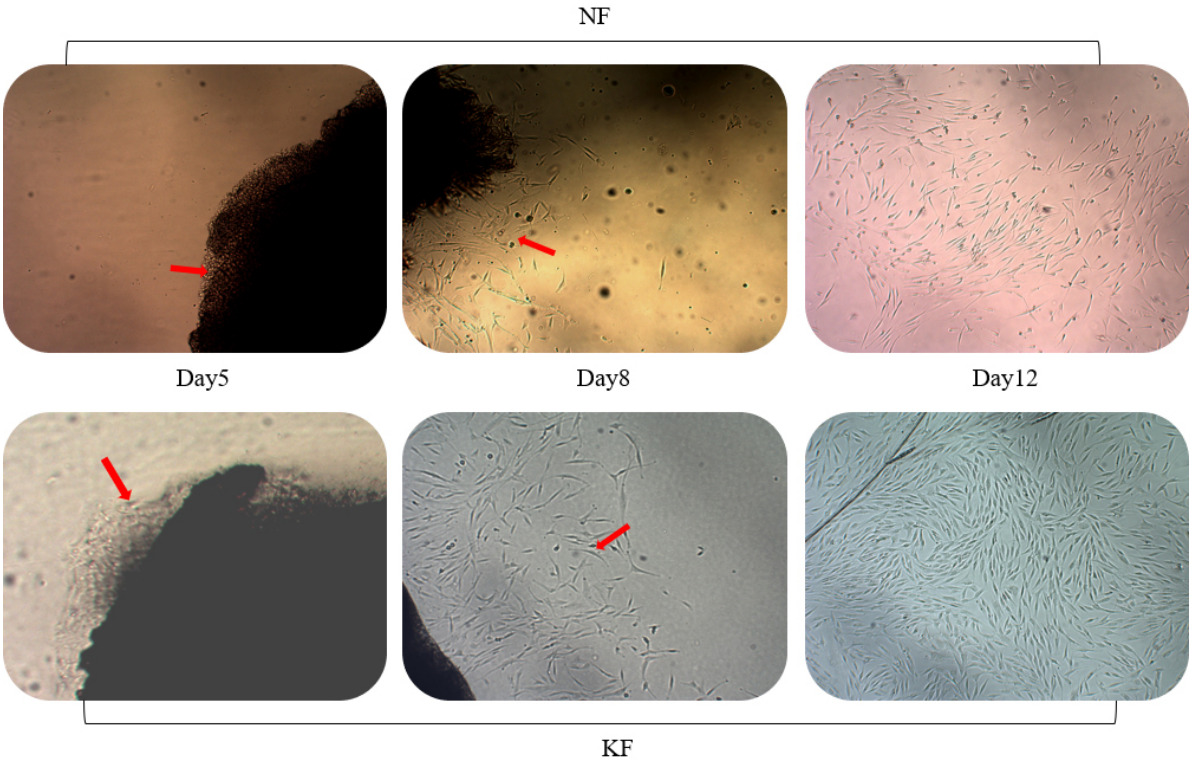

Fig. 1. Cell growth of NF and KF in tissue explant method. Normal skin and keloid tissue fragments were cultivated via tissue explant method after surgical excision. Cell dissociation and growth can be observed under the microscope during the time

The cultivations were usually successful if the tissues were processed within 4 h after surgical excision. The chances of bacterial contamination increased when storage time exceeded 4 h. Cells were found with difficulty to disseminate and replicate and lipid contamination could often be observed when the storage time was longer than 24 h. Cells contaminated with bacteria or lipids demonstrated low chance to be rescued, which could lead to unsuccessful cultivation (Fig. 2).

Collagenase I digestion method. The cells formed small clusters and adhered to the bottom of the plates or petri dishes, and the shape of the cells started to elongate at 48 h after keloids were digested and cultivated. At 72 h, cells were observed to morph from a round to a spindle shape. On the fifth day, the cells clearly turned into a spindle shape. Cell replication could be observed from day five, and KF were confluent and aligned in parallel clusters on day 10. The adherence of cells cultivated from normal skin tissue took longer compared to cells from keloids. Cell adherence was observed at 72 h, and cell replication was observed on the fifth day. NF cells had fully spread out on the tenth day, but they were not as confluent as KF (Fig. 3).

The collagenase digestion time and the separation of epidermis and dermis required for keloids and normal skin were quite different. Since the keloid tissues are thicker and denser, cutting off the thicker epidermis layer would cause less contamination of epidermal cells than using dispase II to remove epidermis. On the contrary, normal skin is thinner, which brought a challenge to completely separate epidermis and dermis via the cutting method. Although neither of the methods can avoid the contamination of epidermal cells in the NF cultivation, dispase II digestion was more suitable to remove epidermis for normal skin tissues. Due to the differences in thickness and density, keloids needed to be treated with collagenase for a longer time compared to normal skins. The number of fibroblasts generated from keloids improved with the increase in collagenase digestion time. However, 1 h digestion worked the best for normal skin, and the fibroblast numbers decreased if the tissues were treated with collagenase for longer than 2 h (Fig. 4).

Assessment of the primary fibroblasts from different tissues

Cell morphology. Cells cultivated from keloids, keloid-surrounding tissues, and normal skin all matched the phenotype of fibroblasts when observed under optical microscope, but there were still differences. NF cells were like standard fibroblasts with a spindle shape, branched cytoplasm, and even distribution. KF cells lacked contact

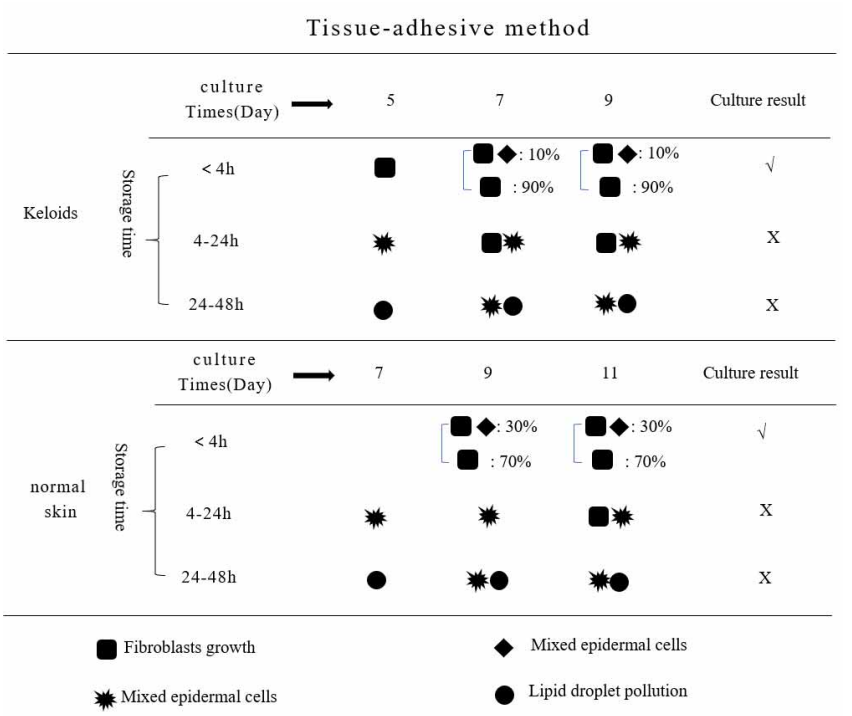

Fig. 2. Storage time is a crucial factor in primary fibroblast cultivation. Long storage time may result in bacterial or lipid contamination in the cell culture which further resulted in unsuccessful primary fibroblast cultivation.

inhibition; therefore, some cells overlapped with others and the cell alignment was disordered. KSF cells were either spindle-shaped or triangle-shaped, and they formed large nest-like clusters during cultivation (Fig. 5A).

Fibroblast purity. Fibroblast cells are derived from primitive mesenchyme, and they express a high level of vimentin (Cheng *et al*., 2016). The third passage of primary KF, KSF, and NF were labelled by immunofluorescence. DAPI-stained nuclei were shown in blue to indicate the total cell number count, and vimentin positive cells were shown in red. The ratio of vimentin positive and DAPI positive cells in each group were all close to 100 %, indicating that cells were nearly all confirmed to be fibroblasts at the third generation of primary culture (Fig. 5B).

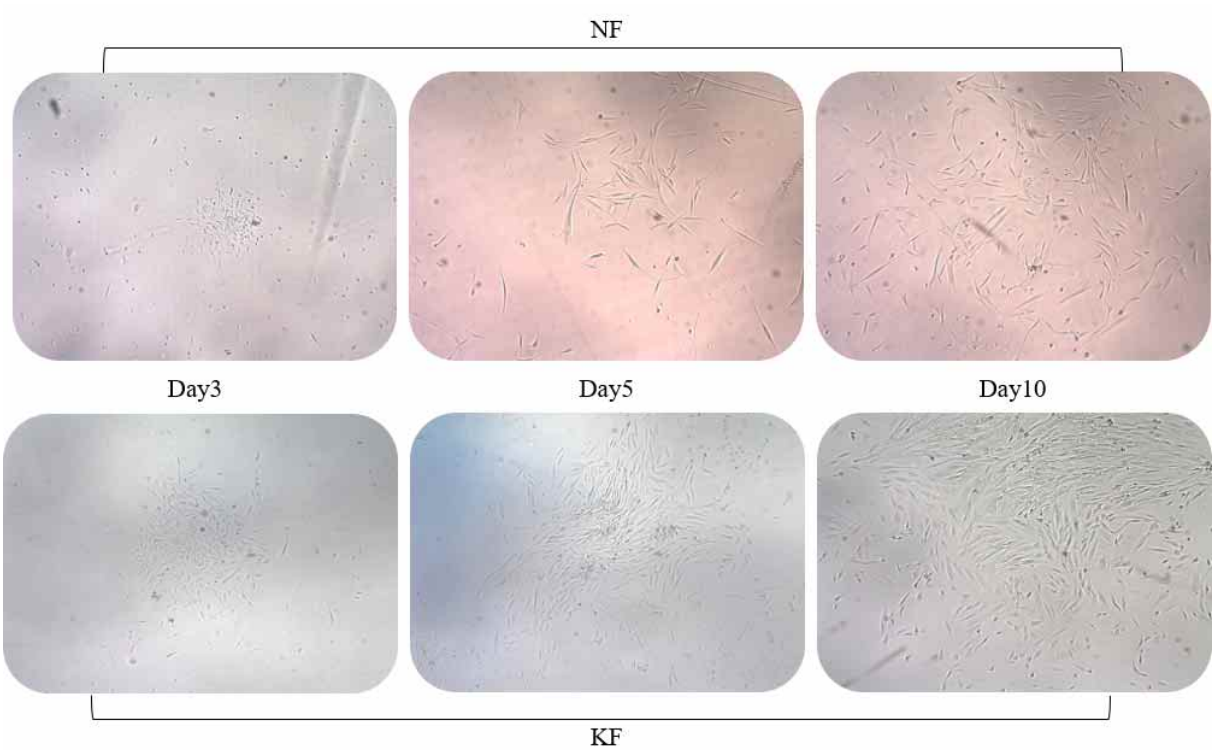

Fig. 3. Cell growth of NF and KF in collagenase digestion method. Normal skin and keloid tissue fragments were cultivated via collagenase digestion method after surgical excision. Cell adherence and growth can be observed under the microscope during the time course.

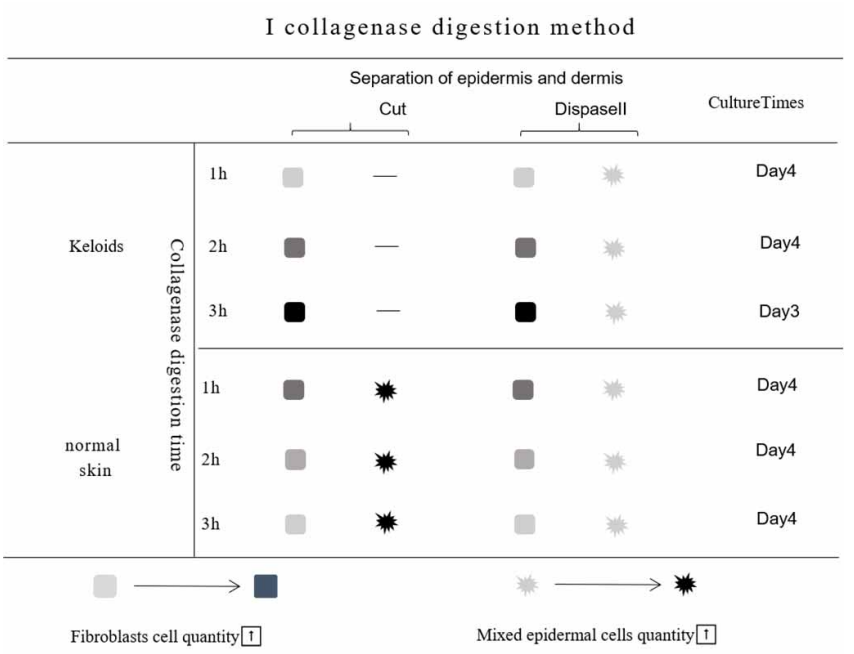

Fig. 4. Epidermis removal methods and collagenase digestion time can affect the primary fibroblast cultivation results. To generate the best result, different methods of epidermis removal and lengths of collagenase treatment are required for keloids and normal skins.

Keloid-related gene expression. When KF, KSF, and NF were cultured to thefifth passage, the mRNA expression levels of α-SMA, Col1, and Col3, which were shown to be involved in the keloid development, were examined. The RT-PCR results show that the expression levels of α-SMA, Col1 and Col3 were up-regulated in KF ($P<0.01$) and down-regulated in KSF ($P<0.01$) compared to NF (Fig. 6A). The IF staining result further confirmed that the protein expression levels of α-SMA in three primary fibroblasts were KF>NF>KSF (Fig. 6B).

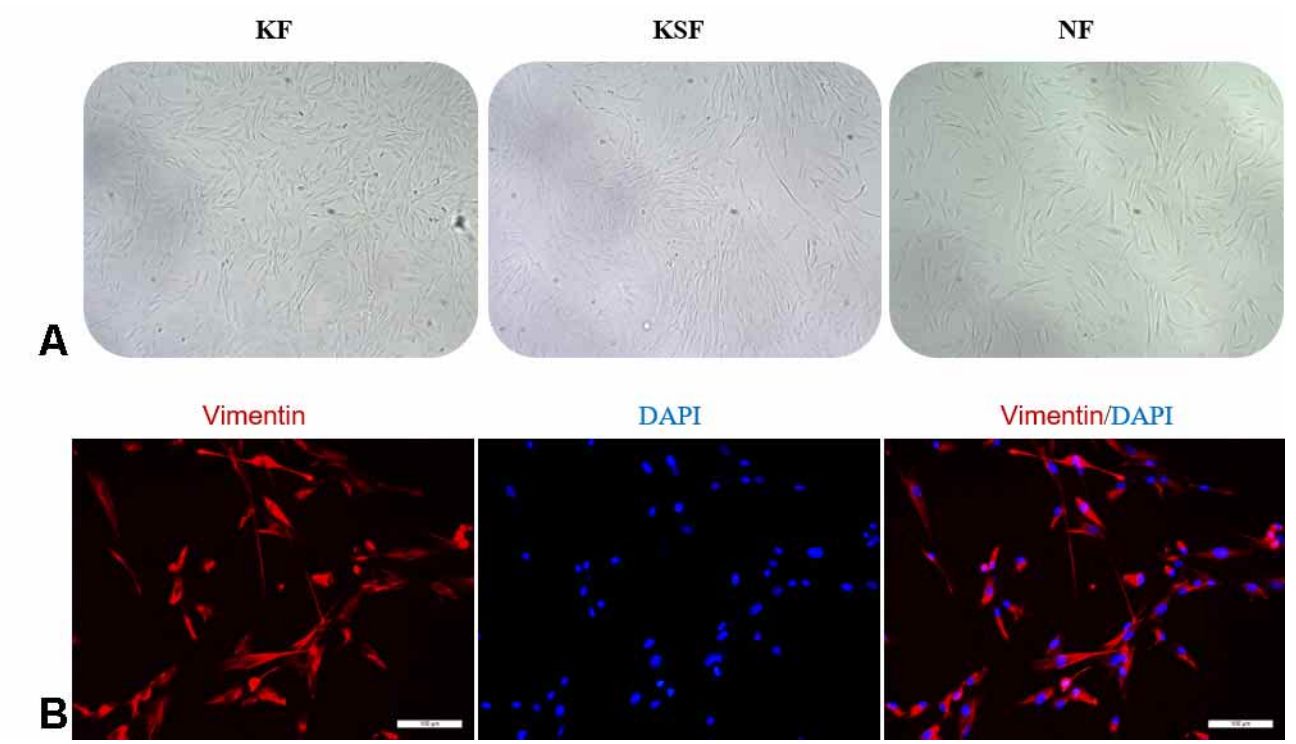

Fig. 5. Primary fibroblast morphology and fibroblast purity assessment. KF, KSF, and NF differ morphologically from each other (A). Each cell culture was stained with DAPI (blue) and rabbit anti-vimentin and second antibody AF594 goat anti-rabbit IgG (red). Images of KF cells are shown in (B). Most of the cells were shown to be positive in both, indicating that the cultivated cells were nearly all fibroblasts.

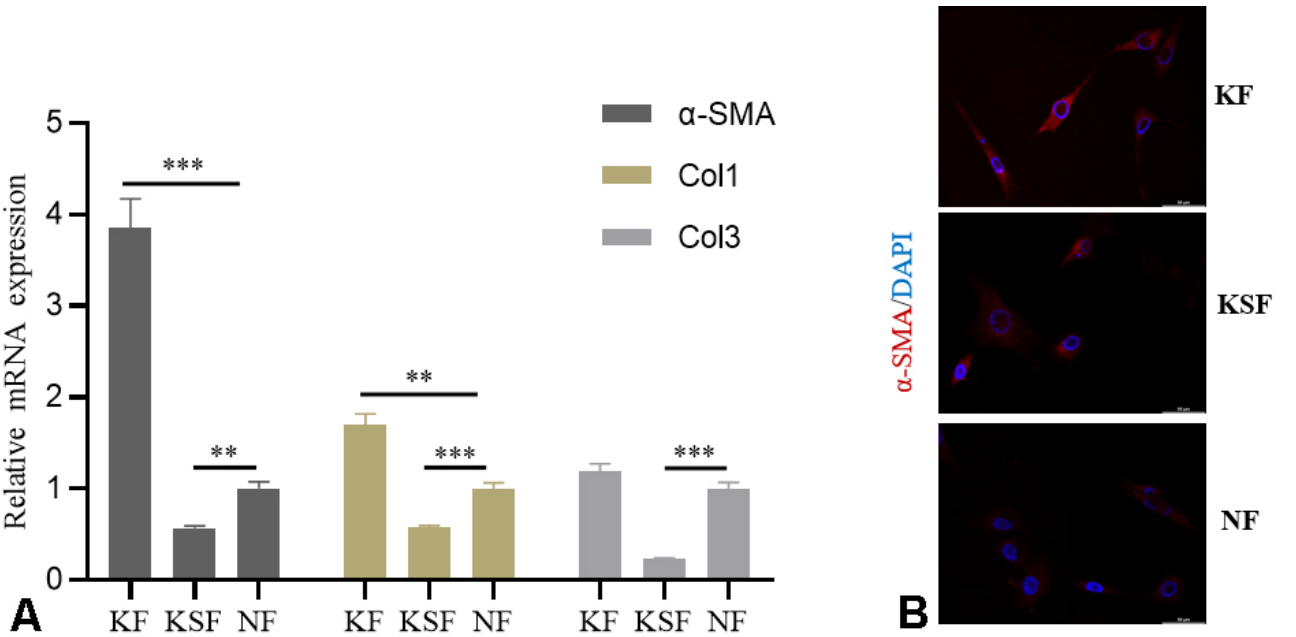

Fig. 6. Genes involved in keloid formation were shown to be up-regulated in KF but down-regulated in KSF compared to NF. The mRNA expression levels of all three genes were tested via RT-PCR (A), and the α-SMA protein level was confirmed via IF staining with DAPI (blue) and rabbit anti-α-SMA and second antibody AF594 goat anti-rabbit IgG (red) (B).

DISCUSSION

The treatment of keloids has always been a conundrum for cosmetic surgery. Due to the lack of proper animal models, cell models are the major tools for keloid etiopathogenesis studies. Currently, inducing specific cell types via cytokine TGF-β1 or siRNA knockdown based on the in vitro primary keloids or normal skins fibroblast culture is the most commonly used method (Cui *et al*., 2020; Xu *et al*., 2020). Therefore, validating the in vitro primary KF or NF cultivation is the key for further studies. Primary fibroblasts share many features with in vivo fibroblasts and can keep the characteristics for a few passages. However, the replication of primary fibroblasts has a limit, so a new primary fibroblast culture must be set up frequently. The methods commonly used to culture primary fibroblasts include tissue explant and enzyme digest (Villegas Díaz *et al*., 2018; Lago & Puzzi, 2019; Katayama *et al*.) which may also vary in process details for different labs. On top of that, factors like bacteria or epidermal cell contamination and over-digestion can easily result in unsuccessful cultures during cultivation (Künzel *et al*., 2019). Furthermore, there still must be an assessment to verify the quality of the cultivated cells and determine how much they can relate to the in vivo environment. All the factors mentioned above could add to the difficulty of beginners finding a suitable method for primary keloid fibroblast culture. Here, keloid fragments and normal skin fragments were collected from young patients aged from 18 to 30 to ensure the activity of the fibroblasts. All samples were cultured using both the tissue explant and enzyme digestion methods to generate primary fibroblasts, so a thorough comparison can be conducted between the two methods and across the three tissue types.

It was shown in this study that it was best to process samples within 4 h after excision to minimize the chance of contamination. When the storage time exceeds 4 h, the possibility of bacterial contamination increased significantly in the tissue explant method. Although the low level of bacteria did not affect the cell dissociation from the fragments, it was aggravated when cells were sub-cultured. Keloids are highly fibrotic and have thickened epidermis and dermis (Limandjaja *et al*., 2018; Potter *et al*., 2019) so the epidermis must be cut off as much as possible to avoid the contamination of epidermal cells, and it is necessary to extend the collagenase digestion time to 3 h. Normal skin tissues are thinner in both epidermis and dermis, so the tissue explant method can be more suitable when the size of the fragments is large, and 30-90 min collagenase digestion can be used when the fragments are smaller. Though the epidermis of normal skin is thinner, the separation and removal

still must be thorough to minimize the epidermal cell contamination. If epidermal cells are observed in the tissue explant method, they can be eliminated by removing the tissue fragment, scraping off the area with epidermal cells, and replacing fresh growth medium.

To validate the fibroblasts cultivated in this study, the purity of the cells was first evaluated. The purity of the third passage of the primary cells was shown to be close to 100 % in all tissues, and the few epidermal cells could be eliminated during the following sub-culturing. This suggested that the fifth passage of the cells would be in good condition to be used in further studies (Baranyi *et al*., 2019). The α-SMA, Col1, and Col3 mRNA levels in KF, KSF, and NF were next determined using RT-PCR. α-SMA, Col1, and Col3, shown to participate in keloid formation, were considered as markers for the fibrosis level of tissues and were used in the evaluation of collagen accumulation and epithelial-mesenchymal transition (Tredget *et al*., 1997; Sarrazy *et al*., 2011; Zhu *et al*., 2016; Limandjaja *et al*.; Tan *et al*., 2019). It was demonstrated that the mRNA expression levels of these genes were up-regulated in KF, which explains the keloid fibrosis level. The high expression of α-SMA in KF was further confirmed on the protein level using IF staining, suggesting that the cultivated primary fibroblasts could resemble in vivo fibroblasts and could be used as a reliable in vitro cell model in further keloid studies.

In conclusion, both tissue explant and collagenase digestion methods can successfully generate high quality and purity primary NF and KF. During the process, a few procedures must be fine-tuned according to the tissue type to produce the best results. An improved cell model for keloid study can be built based on these methods by adding cytokines or using the 3D cell culture technique to reconstruct the in vivo environment.

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QIN, H.; LIU, R.; NIE, W.; LI, M.; YANG, L.; ZHOU, C.; ZHANG, L. & ZHANG, G. Comparación de los métodos de cultivo de fibroblastos queloides primarios y las características de los fibroblastos cultivados a partir de queloides, tejidos circundantes queloides y tejidos cutáneos normales. *Int. J. Morphol., 39(1)*:302-310, 2021.

RESUMEN: La identificación de un cultivo de fibroblastos queloides primarios, siempre ha sido una medida fundamental para estudiar los mecanismos de la enfermedad queloide. La calidad del cultivo de células primarias puede afectar directamente los resultados de otros experimentos. Este estudio se realizó para investigar las condiciones óptimas de crecimiento, incluido el tiempo óptimo de almacenamiento y el tiempo de tratamiento con colagenasa, para modelos de cultivo celular *in vitro* y los métodos adecuados para la separación epidermis-dermis en diferentes tejidos. Se recogieron de los pacientes tejidos queloides, tejidos circundantes queloides y tejidos cutáneos normales, para cultivo primario de fibroblastos. Se implementaron y compararon dos métodos, explante de tejido y digestión con colagenasa. Los niveles de expresión de los genes relacionados con queloides α-SMA, Col1 y Col3 se evaluaron en células cultivadas usando ambos métodos, para verificar las cualidades de las células primarias. Se realizó un análisis comparativo entre los dos métodos y entre los tres tejidos diferentes utilizados. La contaminación de bacterias y lípidos se minimizó inmediatamente después de que se procesaron las muestras. Se requirieron varios métodos de eliminación de la epidermis y diferentes tiempos de digestión con colagenasa en los tejidos para generar resultados óptimos. Los resultados de la PCR en tiempo real mostraron que los niveles de expresión de ARNm de genes relacionados con queloides en fibroblastos cultivados se correlacionaban con su perfil de expresión *in vivo*, como se informó en estudios anteriores. Los resultados de este studio indicaron varios puntos clave en el cultivo de fibroblastos queloides primarios y han demostrado la correlación en la expresión génica entre fibroblastos queloides *in vivo* y fibroblastos queloides primarios *in vitro*.

PALABRAS CLAVE: Queloide, piel normal, fibroblastos, cultivo de células primarias, α-SMA

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