Evaluation of the effects of human dental pulp stem cells on the biological phenotype of hypertrophic keloid fibroblasts

Ling-ling Fu

UKE: Universitätsklinikum Hamburg-Eppendorf

Ming Yan

UKE: Universitätsklinikum Hamburg-Eppendorf

Ola A. Nada

UKE: Universitätsklinikum Hamburg-Eppendorf

Li-ming Chen

guiyang hospital of stomatology

Martin Gosau

UKE: Universitätsklinikum Hamburg-Eppendorf

Ralf Smeets

UKE: Universitätsklinikum Hamburg-Eppendorf

hongchao feng (✉ hongchaofeng@hotmail.com)

Guiyang hospital of stomatology

Reinhard E. Friedrich

UKE: Universitätsklinikum Hamburg-Eppendorf

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Abstract

Background Despite numerous existing treatments for keloids, the responses in the clinic have been disappointing due to either low efficacy or side effects. Numerous studies dealing with preclinical and clinical trials have been published about effective therapies for fibrotic diseases using mesenchymal stem cells, However, no research has yet been reported to scientifically investigate the effect of HDPSCs on the treatment of keloids.

The objective is to provide an experimental basis to provide a novel proposal for the application of stem cells in the treatment of keloids.

Methods HNFs and HKFs were cultured alone and in combination with DPSCs using a trans-well cell-contact-independent cell culture system. The effects of DPSCs on HKFs were tested using CCK-8 assay, Live/dead staining assay, quantitative polymerase chain reaction, Western blot

Results DPSCs don't inhibited HKFs and HNFs proliferation and induce apoptosis, but inhibited migration. Furthermore, DPSCs significantly decreased expression of profibrotic genes, transforming growth factor- β 1 and transforming growth factor- β 2. DPSCs suppressed the ECM synthesis in HKFs, as indicated by decreased expression of collagen I and fibronectin and low levels of hydroxyproline in cell culture supernatant

Conclusions 1) Co-culture of DPSCs inhibits the migration of HKFs but does not affect the proliferation and apoptosis of HKFs. 2) DPSCs co-culture inhibits the expression of pro-fibrotic genes and promotes the expression of anti-fibrotic genes in HKFs. 3) DPSCs co-culture inhibits the synthesis of extracellular matrix by HKFs.

Introduction

During wound healing, a dynamic balance of synthesis and degradation of collagen usually results in either a physiological or a pathological scar. A pathological scar can be described as a fibroproliferative disorder disease caused by hyperproliferation of fibroblasts and excessive synthesis of the extracellular matrix (ECM) in the process of dermis wound healing following skin trauma or serious burns[1-3]. Moreover, pathological scars can be classified into two types: hypertrophic scar and keloid. The former is predominantly localized above the original wound region, with a reddish or pinkish appearance, which can sometimes be pruritic. Furthermore, a hypertrophic scar usually regresses after years to form a matured scar[4, 5]. Whereas, the situation is strikingly different when the latter is involved. Although a keloid is a non-malignant disease, keloids almost always overgrow onto the surrounding skin, where they can often lead to malignant manifestations such as pain, ulceration, secondary infection, active angiogenesis, and even carcinogenesis[6]. Such symptoms not only impact the quality of life, but they can also be highly unsightly, contributing further to psychological disturbances and corresponding social distress.

Despite numerous existing treatments for keloids, such as surgical removal, hormonal therapy, laser treatment, or radiotherapy, as well as interventional sclerotherapy, the responses in the clinic have been disappointing due to either low efficacy or side effects, or a combination of both[7]. In order to address the aforementioned existing and urgent clinical need, new and more efficient chemotherapeutic strategies are in order.

Numerous studies dealing with preclinical and clinical trials have been published about effective therapies for fibrotic diseases using mesenchymal stem cells, including the following: bone marrow derived mesenchymal stem cells (BMSCs)[8], adipose derived mesenchymal stem cells (ADSCs)[9]. Human dental pulp stem cells (HDPSCs) are a type of adult stem cell that possess self-renewal, self-replication and multi-differentiation properties, where they can differentiate into a variety of mesodermal tissue cells, such as chondrocytes, osteoblasts, cardiomyocytes, as well as adipocytes[10]. However, no research has yet been reported to scientifically investigate the effect of HDPSCs on the treatment of keloids.

The objective is to provide an experimental basis to provide a novel proposal for the application of stem cells in the treatment of keloids. A co-culture method was set up to investigate the influence and mechanism of dental pulp stem cells on keloid fibroblast properties, such as cell proliferation, migration, collagen synthesis, invasion, and apoptosis.

Materials And Methods

Cell Isolation and Cultures Isolation of human dental pulp stem cells was performed using the explant growth method. Extracted teeth were obtained from patients (aged 18–25 years) with informed consent Guiyang hospital of stomatology (Guiyang, China). The retrieved dental pulp tissue was cut with sterilized scissors into small pieces and the tissue pieces were then plated onto 100-mm cell culture dishes in DMEM containing 10% FBS at 37 °C and 5% CO₂. Cells were further grown until confluence was reached. Human dental pulp stem cells were obtained by a limiting dilution method, a method previously established by our group[11]. Only passages 3–5 of dental pulp stem cells were used in this study.

Seven tissue samples (0.5cm³) of keloids and seven corresponding normal skin tissue samples were used as a reference. All tissue samples were taken from the maxillofacial area of patients in the same hospital with informed consent. The samples were classified into the 2 groups (keloid and normal skin) based on clinical and pathological diagnosis. Furthermore, pathological characteristics were diagnosed upon staining with Hematoxylin and Eosin (H&E) with the following findings: 1. the dermal layer of the skin is seen to be markedly thickened; 2. disappearance of skin appendages; 3. collagen fibers begin to coarse into thick bundles. Finally, fibroblasts from keloid and normal skin isolation were also performed using the explant growth methodology at passages 3-5.

Flow cytometry was used to detect the surface markers of HDPSCs

HDPSCs at passage 3 were trypsinized by 0.25% trypsin, washed twice with PBS, and resuspended at a concentration of 1×10^9 /L. 1 mL of the cell suspension was collected into flow cytometry tubes with 5 μ l of each of the following anti-fluorescein isothiocyanate-conjugated antibodies: CD29 (cat. no. 14-6688-82; Thermo Fisher Scientific), CD73 (cat. no. ab156756; Abcam), CD90 (cat. no. ab139364; Abcam), CD34 (cat. no. 560941; BD Biosciences), and CD45 (cat. no. MCA2035S; Bio-Rad Laboratories). Cells were incubated at 4°C for 1 hour in the dark, and the cell surface markers were then analyzed by flow cytometry.

Transwell Co-culture systems Cells were grown in 6-well plates with an inner chamber pore size of 0.4 μ m. Culture media was composed of DMEM with the following constituents; 10% FBS, 1% glutamine and 1% penicillin/streptomycin. The co-culture models were prepared as displayed in figure 3 where in one model, human normal fibroblasts were cultured in the lower layer with a lack of cells in the upper layer (Figure 3b). In the second model, human normal fibroblasts were seeded in the lower chamber and co-cultured with dental pulp cells in the upper chamber (Figure 3c). Furthermore, the 3rd model was set up with human keloid fibroblasts cultured in the lower layer, together with a lack of cells in the upper layer (Figure 3d). Finally, the 4th model was set up with human keloid fibroblasts seeded in the lower chamber with a co-culture of dental pulp cells in the upper chamber. (Figure 3d).

Cell morphology Cells were photographed under an inverted microscope (ECLIPSE Ts2-FL, Nikon, Japan) at days 1, 3, 5 and respectively.

Cell proliferation In this study, cellular proliferation was analyzed using the CCK8 assay (Dojindo, Kumamoto, Japan) according to manufacturer's instructions. After removal of the supernatant, 660 μ L of CCK8 solution (60 μ L CCK8:600 μ L medium) was added to each well and incubated in the incubator for 2 hours at days 1, 3, 5 and 7. Then, a 100- μ L volume of the supernatant was separated by centrifugation and transferred into a fresh 96-well plate. Readings at wavelengths of 450 nm were recorded using a Fluostar Omega plate reader, and a standard curve was then plotted against the readings of the standards.

Collagen synthesis detection Both normal and keloid fibroblasts were each individually seeded into 6-well plates at a concentration of 5×10^5 cells per well. Supernatants were retrieved from 6-well plates after 48 hours and handled in accordance with the Hydroxyproline Assay Kit (MAK008; Sigma Aldrich). 1.5 ml of the supernatant was harvested and resuspended in 0.05 ml of digestion solution at 37 °C for 3 hours. Then, 0.5 ml solution A was transferred into the mixture for 10 minutes and 0.5 ml solution B was added and allowed to react for 5 minutes, followed by the addition of 1 ml solution C at 60 °C for 15 minutes. Absorbance was then read at a wavelength of 550 nm. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline (0–100 mg/ml).

Wound scratch assay to detect the cell migration ability Normal and keloid fibroblasts were seeded individually into 6-well plates at a concentration of 5×10^5 cells per well. When the cells reached 90% confluency, 200ul sterile pipette tips were used to scratch the bottoms of the 6-well plate culture wells. After rinsing out the floating cells by PBS, cells were starved for 24 hours in serum-free medium. In the

test group, trans well chambers were seeded with a concentration of 5×10^5 of dental pulp stem cells. Images of the migration of fibroblasts were recorded using a light microscope camera after scratching at 0 and 24 hours. Finally, the rate of cellular migration was quantified using image J software (NIH software).

Live and Dead staining Live and dead cells were assessed using LIVE/DEAD® staining kits (Cat# L3224; Molecular Probes) according to the manufacturer's instructions, where 500 μ l of staining solution was added to each well after the chamber was removed at days 1, 3, 5 and 7, the cells were then incubated in the dark at room temperature for 10 minutes, and then photographed with the employment of immunofluorescence microscopy.

Western blot Cells were fixed with 4% paraformaldehyde, permeated with 0.1% Triton X-100 (Sigma-Aldrich, cat no. X100), and blocked with 2% Bovine Serum Albumin (BSA). Following Li-Cor Odyssey solution blocking, the cells were incubated with a primary antibody overnight at 4 °C in a humidified chamber. Cells were then washed with ice-cold TBST (Tris Buffered Saline, pH 7.4, 0.1% Tween-20) $2 \times 200 \mu$ L before adding the secondary antibody. Fluorescent secondary antibodies were then added and immunoblots were thereafter imaged with a two-channel (at 700 plus 800 nm) IR fluorescent Odyssey CLx imaging system (LI-COR®). Results were quantified using image J software.

Real-time quantitative PCR (RT-qPCR) Cells were seeded in six-well plates at a density of 5×10^5 cells/well and cultured in complete medium for 24 hours. The medium was discarded and the cells were washed 3 times with phosphate buffered saline (PBS). Thereafter, cells were harvested using a cell scraper after the addition of a lysis buffer. RNA was consequently extracted through the following steps: lysing spirochetes in Trizol reagent (Life Technologies, Cat No. 15596-026), followed by extracting RNA in trichloromethane, and then precipitating it in isopropanol, and finally resuspending it in RNase-free water. The RNA concentration and purity levels were determined using a Nanodrop2000 Spectrophotometer (Thermo Fischer Scientific). Total RNA (2.5 μ g) was subjected to cDNA synthesis using a qScript cDNA SuperMix (Quanta BioSciences) through the following consequent cycles: firstly at 25 °C for 5 minutes, followed by 42 °C for 30 minutes and finally at 85 °C for 5 minutes. Real-time PCR was performed to determine the mRNA levels of TGF- β 1, fibrinogen, α -SMA and GAPDH using SYBR Green Master MIX (ABI, USA). For relative mRNA expression, the $2^{-\Delta\Delta Cq}$ method in which ΔCq = each corresponding Cq value - minimum Cq value was calculated.

Statistical analyses All the experiments were repeated 3 times in duplicate wells. Significant differences were defined as $p < 0.05$. All statistical analyses were performed using Graph Pad Prism software (Graph pad Prism, RRID:SCR_002798) version 7.0a.

Results

Identification of DPSCs Cell surface markers were detected using flow cytometry to determine the mesenchymal origin of the DPSCs. The following markers were positively expressed at the respective

percentages; CD29 at 99.57%, CD73 at 99.72%, and CD90 at 99.62%, whereas the hemopoietic stem cell marker, CD34 was minimally expressed at a percentage of 0.2%. See figure 2.

Effects of DPSCs on the proliferation of human normal fibroblasts (HNFs) and human keloid fibroblasts (HKFs) Compared to the HKFs cultured alone, the CCK-8 assay showed that no difference in the proliferation of HKFs was observed in the DPSCs/HKFs co-culture group at days 1,3 ,5,and 7 ($P \geq 0.05$). Moreover, as a positive control, there was also no detected difference in the proliferation of HNFs in the DPSCs/HNFs co-culture group at days 1,3 ,5,and 7 ($P \geq 0.05$). However, the proliferation rate of HKFs was much higher than that of HNFs on the 3rd day (Fig. 4).

HDPSCs did not influence the apoptosis of HNFs and HKFs Our results indicate that cellular apoptosis of HNFs and HKFs was not affected by HDPSCs. As shown in Figure 5, with respect to cell apoptosis, both HKFs and HNFs were not affected through their coculture with HDPSCs ($P > 0.05$). This result further validates the cell proliferation results.

The effects of HDPSCs on the migration of HKFs and HNFs The wound scratch assays showed that there was only a difference in the cell migration ability in the co-culture group of the human keloid fibroblast after 24 hours of culture ($P < 0.05$). As a positive control, cellular migration of the HNFs remained uninfluenced ($P \geq 0.05$). See figure 6.

Inhibited expression of fibrosis-associated gene phenotype and protein expression in HKFs and KNFs

Compared with solely cultured cells, and with the exception of the expression levels of CTGF in HNFs, HNFs were observed to be unaffected ($P \geq 0.05$), whereas the expression levels of TGF- β 1, and TGF- β 2 in HKFs and KNFs at the mRNA and protein levels were significantly inhibited when co-cultured with the HDPSCs. ($P < 0.05$). These findings indicate that both the transcriptional and posttranslational levels were inhibited.

See figure 7.

DPSCs inhibits extracellular matrix synthesis of HKFs and HNFs

Compared with solely cultured cells, and with the exception of the expression levels of collagen I in HNFs, HNFs were observed to be unaffected ($P \geq 0.05$), whereas the expression levels of collagen I and α -SMA in HKFs and KNFs at the mRNA and protein levels were significantly inhibited when co-cultured with the HDPSCs. ($P < 0.05$). These findings indicate that both the transcriptional and posttranslational levels were inhibited. Figure 8 A, B, C, D, F.

There was a significant decrease in the resultant hydroxyproline concentrations of the HKFs co-culture group ($P < 0.05$), whereas no significant changes were detected in the other three groups. ($P \geq 0.05$). Figure 7.E

Discussion

Wound repair is a complex process that often leads to the formation of scars following traumatic skin injuries. This process is associated with the functions of various cells such as fibroblasts, endothelial cells, macrophages, and lymphocytes[12-14], among which the biological behavior of fibroblasts is considered to be a key factor in the scar formation process. Numerous studies have shown that fibroblasts, which happen to be the main constituents of keloid tissue have the ability to over proliferate and are accompanied by incomplete apoptosis, together with abnormal synthesis of collagen, which overall results in the continuous proliferation of keloid tissue[15, 16]. Therefore, the inhibition of fibroblast proliferation and induction of apoptosis in keloid tissues can majorly reduce keloid tissue proliferation and thereby delay disease progression, which is important for the improvement and treatment of keloid scars. Therefore, understanding the biology of keloid fibroblasts is important for the treatment of keloids.

Mesenchymal stem cells (MSCs) are an important member of the stem cell family, which have been used to treat scar formation-related diseases such as pulmonary fibrosis. Moreover, great progress has been made where they have been also used to inhibit cardiac scar formation through the secretion of various cytokines. Studies have shown that stem cells have been used in animal models and in a few clinical trials for the regeneration of diseased organs. Furthermore, stem cells have been shown to improve tissue repair by secreting interleukins such as interleukin 6, interleukin 8, interleukin 10 and other proteins that are suitable for inducing tissue regeneration [17, 18]. Recent studies have shown that adipose-derived stem cells are able to inhibit mRNA expression levels of COL1A1, transforming growth factor β 1, connective tissue growth factor and ACTA2 in renal fibrosis tissues, thereby playing a therapeutic role in renal fibrosis[19]. Whether or not dental pulp stem cells can play a role in the repair of skin scars by inhibiting the proliferation and migration of keloid fibroblasts through the secretion of cytokines has not been clearly reported so far.

Dental pulp stem cells, human keloid fibroblasts and human normal skin fibroblasts were isolated by tissue explant method. The molecular mechanisms of action of dental pulp stem cells on the proliferation, migration and apoptosis of keloid fibroblasts were investigated by trans well co-culture. The histology of keloid scars is highlighted by the secretion and deposition of large amounts of extracellular collagen[20]. Furthermore, both skin keloids and fibrous tumors are pathologically fibrous connective tissue lesions with large extracellular collagen deposition[21]. In this study, we also found that the co-culture of hDPSCs inhibited the migration of HKFs and HNFs, but did not inhibit neither the proliferation of HKFS and HFS nor did it induce apoptosis of HKFs and HNFs. Our experimental data is similar to the results of a recent study reporting that conditioned medium and cell lysates of human-derived WJ-MSCs inhibits the migration of human KFs[22]. In comparison with this report, we used human HDPSCs, which have the advantages of being easily accessible with minimal patient harm, and the fact that they can be used autologously. However, literature reporting opposing results also exist [23], in which conditioned medium of human WJ-MSCs could promote KFs proliferation in a paracrine manner through a trans well indirect co-culture treatment system. We hypothesize that the reasons for these different results are attributed to the different types of MSCs used, the different cell treatment cultures and different assays, all of which impact the following biological behaviors of keloid fibroblasts; migration, proliferation, and collagen secretion, in addition to apoptosis involved in wound repair after skin injury.

Therefore, by inhibiting the migration and proliferation of keloid fibroblasts, keloid formation is also consequently inhibited[24]. Moreover, it has been shown that the inhibition of matrix metalloproteinase expression in keloid fibroblasts can inhibit the migration ability of keloid fibroblasts and therefore function as a keloid treatment aid [29]. Furthermore, similar findings have been reported where that inhibition of mTOR protein expression in keloid fibroblasts can also inhibit the migratory ability of keloid fibroblasts and thereby further inhibiting the development of keloids [30]. Similarly, and in consistence with the aforementioned studies, the results of the present study revealed that dental pulp stem cells are also able to inhibit the migration of keloid fibroblasts in vitro, thereby suggesting that dental pulp-derived stem cells can promote wound healing by inhibiting scar formation.

The expression of anti-fibrotic and pro-fibrotic genes is closely related to the pathogenesis of fibrotic diseases, which in another way confirms that hyperplastic keloids and keloids are classified as fibrotic diseases. TGF- β 1 and TGF- β 2 overexpression is an important cause for excessive scar proliferation and fibrosis, and studies have shown that targeted reduction of TGF- β 1 and TGF- β 2 expression in hyperplastic keloids and keloids can inhibit scar proliferation and achieve clinical therapeutic effects[25, 26]. Therefore, TGF- β 1 and TGF- β 2 have also become one of the targets of numerous studies that tackle the treatment of hyperplastic keloids and keloids. Moreover, CTGF is a marker protein of fibrotic diseases in which it promotes both the proliferation of fibroblasts as well as the secretion and deposition of extracellular matrix proteins, such as collagen I and fibronectin[27, 28]. In the current study, it was found that after 48 hours of DPSC co-culture with HKFs, both gene and protein expression of TGF- β 1, TGF- β 2, and CTGF were significantly reduced in HKFs. This is in accordance with studies that have shown MSCs being capable of secreting cytokines in order to alter some biological phenotypes of fibroblasts, such as fibrotic and proliferative phenotypes, through paracrine functions[29, 30]. Increasing evidence thereby suggests that the paracrine function of MSCs is an important potential mechanism for their cellular therapeutic function. Thereupon, after being injected into the body, MSCs can, on the one hand, inhabit the area of tissue damage through the processes of chemotaxis, proliferation, and eventually differentiation by evolving into the cell type required for the secretion of extracellular matrix that would be needed to repair the damage in the recipient area; on the other hand, MSCs upon entering the body, can exhibit a paracrine function, which entails the secretion of cytokines and nutrient-active substances required for repairing the damage, and inducing the body's self-generated cells to repair the tissue damage.

A very distinctive feature of proliferative keloid and keloid tissues is the excessive deposition of extracellular matrix[31]. There are two main mechanisms that can lead to an excessive extracellular matrix deposition, one being, an increase in the extracellular matrix synthesis, and the other being a decrease in the extracellular matrix degradation. Compared to normal skin, the amount of collagen synthesis in hyperplastic keloid scars is three times higher, while keloid scars can reach up to 20 times higher[4]. In our experiments, it was further observed that due to the co-culture with DPSCs, both HSFs and HKFs displayed a reduction in extracellular matrix synthesis, as shown by the reduced expression of collagen type I, α -SMA, and hydroxyproline. Moreover, in the extracellular matrix of scar tissue, collagen type I is normally the predominantly present collagen type. Furthermore, the detection of hydroxyproline

content in cell culture media is recognized as a reliable indicator of the ability of fibroblasts to synthesize collagen. Relevantly, it was previously reported that both bone marrow MSCs and dermal MSCs were able to inhibit collagen synthesis and the expression of α -SMA in keloid fibroblasts [27].

Finally, our findings are consistent with previous studies by showing that dental pulp stem cells were able to inhibit keloid fibroblast collagen synthesis in vitro. Furthermore, this study revealed the molecular mechanism by which dental pulp stem cells inhibit the proliferation and migration of keloid fibroblasts, and whether or not dental pulp stem cells play a regulatory role by secreting cytokines. Nevertheless, the specifically secreted cytokines that influence the biological behavior of keloid fibroblasts need to be further investigated by subsequent experiments.

Conclusion

- 1) Co-culture of DPSCs inhibits the migration of HKFs but does not affect the proliferation and apoptosis of HKFs.
- 2) DPSCs co-culture inhibits the expression of pro-fibrotic genes and promotes the expression of anti-fibrotic genes in HKFs.
- 3) DPSCs co-culture inhibits the synthesis of extracellular matrix by HKFs.

Abbreviations

HDPSCs: human dental pulp stem cells, HKFs: human keloid fibroblasts, HNFs: human normal fibroblasts

Declarations

Acknowledgements: Not applicable.

Declaration of Conflict of Interest: The authors declare that they have no conflicting interests.

Ethics approval and consent to participate

Administrative permissions were acquired by our team to access the data used in our research. The study protocol was approved by the Guiyang Hospital of Stomatology that approved the study. Accordingly, all teeth were coded with number and all personal identification of the patients were removed. All parent or guardian of participants provided written informed consent for using their teeth which otherwise would have been discarded as waste.

Consent to publish

Not applicable.

Availability of data and materials

The data are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MY: conceived the study, supervised the experiments and drafted the manuscript.

ON: conceived the study, supervised the experiments and drafted the manuscript.

LF: data evaluation, manuscript preparation.

LC: data evaluation, manuscript preparation.

MG: analyzed the data and revised the manuscript.

REF: analyzed the data and revised the manuscript.

RS: performed the data collection.

HF: conceived the study, designed the data evaluation, manuscript preparation.

References

- [1] K.A. Bielefeld, S. Amini-Nik, B.A. Alman, Cutaneous wound healing: recruiting developmental pathways for regeneration, *Cell Mol Life Sci* 70(12) (2013) 2059-81.
- [2] S. McGinty, W.J. Siddiqui, *Keloid*, StatPearls, Treasure Island (FL), 2020.
- [3] L.J. van den Broek, G.C. Limandjaja, F.B. Niessen, S. Gibbs, Human hypertrophic and keloid scar models: principles, limitations and future challenges from a tissue engineering perspective, *Exp Dermatol* 23(6) (2014) 382-6.
- [4] S. Ud-Din, A. Bayat, New insights on keloids, hypertrophic scars, and striae, *Dermatol Clin* 32(2) (2014) 193-209.
- [5] A. Armour, P.G. Scott, E.E. Tredget, Cellular and molecular pathology of HTS: basis for treatment, *Wound Repair Regen* 15 Suppl 1 (2007) S6-17.

- [6] J.P. Andrews, J. Marttala, E. Macarak, J. Rosenbloom, J. Uitto, Keloids: The paradigm of skin fibrosis - Pathomechanisms and treatment, *Matrix Biol* 51 (2016) 37-46.
- [7] B. Berman, A. Maderal, B. Raphael, Keloids and Hypertrophic Scars: Pathophysiology, Classification, and Treatment, *Dermatol Surg* 43 Suppl 1 (2017) S3-S18.
- [8] F. Fang, R.L. Huang, Y. Zheng, M. Liu, R. Huo, Bone marrow derived mesenchymal stem cells inhibit the proliferative and profibrotic phenotype of hypertrophic scar fibroblasts and keloid fibroblasts through paracrine signaling, *J Dermatol Sci* 83(2) (2016) 95-105.
- [9] F. Xie, L. Teng, J. Xu, J. Lu, C. Zhang, L. Yang, X. Ma, M. Zhao, Adipose-derived mesenchymal stem cells inhibit cell proliferation and migration and suppress extracellular matrix synthesis in hypertrophic-scar and keloid fibroblasts, *Exp Ther Med* 21(2) (2021) 139.
- [10] T.W. Tsutsui, Dental Pulp Stem Cells: Advances to Applications, *Stem Cells Cloning* 13 (2020) 33-42.
- [11] Y. Zhao, Y. Zheng, W. Eichhorn, J. Klatt, A. Henningsen, L. Kluwe, R.E. Friedrich, M. Gosau, R. Smeets, Enriching Stem/Progenitor Cells from Dental Pulp Cells by Low-density Culturing, *In Vivo* 33(1) (2019) 23-29.
- [12] J. Zhang, Z. Liu, W. Cao, L. Chen, X. Xiong, S. Qin, Z. Zhang, X. Li, C.A. Hu, Amentoflavone inhibits angiogenesis of endothelial cells and stimulates apoptosis in hypertrophic scar fibroblasts, *Burns* 40(5) (2014) 922-9.
- [13] L. He, A.G. Marneros, Macrophages are essential for the early wound healing response and the formation of a fibrovascular scar, *Am J Pathol* 182(6) (2013) 2407-17.
- [14] P. Bernabei, L. Rigamonti, S. Ariotti, M. Stella, C. Castagnoli, F. Novelli, Functional analysis of T lymphocytes infiltrating the dermis and epidermis of post-burn hypertrophic scar tissues, *Burns* 25(1) (1999) 43-8.
- [15] Y.F. Gong, X.M. Zhang, F. Liu, Z.Z. Wang, X.F. Deng, Y. Jiao, X.J. Li, X.Y. Huang, Inhibitory effect of recombinant human endostatin on the proliferation of hypertrophic scar fibroblasts in a rabbit ear model, *Eur J Pharmacol* 791 (2016) 647-654.
- [16] H. Wang, Z. Chen, X. Li, Y. Tang, X. Li, S. Zhang, L. Ma, X. Huang, TSG-6 treatment promoted apoptosis in human fibroblasts of pathological scar, *Cell Mol Biol (Noisy-le-grand)* 62(6) (2016) 33-7.
- [17] J.Q. Cao, Y.Y. Liang, Y.Q. Li, H.L. Zhang, Y.L. Zhu, J. Geng, L.Q. Yang, S.W. Feng, J. Yang, J. Kong, C. Zhang, Adipose-derived stem cells enhance myogenic differentiation in the mdx mouse model of muscular dystrophy via paracrine signaling, *Neural Regen Res* 11(10) (2016) 1638-1643.
- [18] H.J. Harn, S.Z. Lin, S.H. Hung, Y.M. Subeq, Y.S. Li, W.S. Syu, D.C. Ding, R.P. Lee, D.K. Hsieh, P.C. Lin, T.W. Chiou, Adipose-derived stem cells can abrogate chemical-induced liver fibrosis and facilitate recovery

of liver function, *Cell Transplant* 21(12) (2012) 2753-64.

[19] C. Zhang, T. Wang, L. Zhang, P. Chen, S. Tang, A. Chen, M. Li, G. Peng, H. Gao, H. Weng, H. Zhang, S. Li, J. Chen, L. Chen, X. Chen, Combination of lyophilized adipose-derived stem cell concentrated conditioned medium and polysaccharide hydrogel in the inhibition of hypertrophic scarring, *Stem Cell Res Ther* 12(1) (2021) 23.

[20] J. Zhao, B. Shu, L. Chen, J. Tang, L. Zhang, J. Xie, X. Liu, Y. Xu, S. Qi, Prostaglandin E2 inhibits collagen synthesis in dermal fibroblasts and prevents hypertrophic scar formation in vivo, *Exp Dermatol* 25(8) (2016) 604-10.

[21] S. Tejiram, J. Zhang, T.E. Travis, B.C. Carney, A. Alkhalil, L.T. Moffatt, L.S. Johnson, J.W. Shupp, Compression therapy affects collagen type balance in hypertrophic scar, *J Surg Res* 201(2) (2016) 299-305.

[22] S.Y. Song, J.E. Jung, Y.R. Jeon, K.C. Tark, D.H. Lew, Determination of adipose-derived stem cell application on photo-aged fibroblasts, based on paracrine function, *Cytotherapy* 13(3) (2011) 378-84.

[23] G.A. Abusin, R.F. Abu-Arja, R.D. Gingrich, M.D. Silverman, G.K. Zamba, A.J. Schlueter, An algorithm for utilizing peripheral blood CD34 count as a predictor of the need for plerixafor in autologous stem cell mobilization–cost-effectiveness analysis, *J Clin Apher* 28(4) (2013) 293-300.

[24] Q. Chun, W. ZhiYong, S. Fei, W. XiQiao, Dynamic biological changes in fibroblasts during hypertrophic scar formation and regression, *Int Wound J* 13(2) (2016) 257-62.

[25] Z.B. Kryger, M. Sisco, N.K. Roy, L. Lu, D. Rosenberg, T.A. Mustoe, Temporal expression of the transforming growth factor-Beta pathway in the rabbit ear model of wound healing and scarring, *J Am Coll Surg* 205(1) (2007) 78-88.

[26] W. Chen, X. Fu, S. Ge, T. Sun, G. Zhou, D. Jiang, Z. Sheng, Ontogeny of expression of transforming growth factor-beta and its receptors and their possible relationship with scarless healing in human fetal skin, *Wound Repair Regen* 13(1) (2005) 68-75.

[27] C.H. Lee, B. Shah, E.K. Moioli, J.J. Mao, CTGF directs fibroblast differentiation from human mesenchymal stem/stromal cells and defines connective tissue healing in a rodent injury model, *J Clin Invest* 120(9) (2010) 3340-9.

[28] D. Abraham, Connective tissue growth factor: growth factor, matricellular organizer, fibrotic biomarker or molecular target for anti-fibrotic therapy in SSc?, *Rheumatology (Oxford)* 47 Suppl 5 (2008) v8-9.

[29] K.J. Ashcroft, F. Syed, A. Bayat, Site-specific keloid fibroblasts alter the behaviour of normal skin and normal scar fibroblasts through paracrine signalling, *PLoS One* 8(12) (2013) e75600.

[30] N. Murao, K. Seino, T. Hayashi, M. Ikeda, E. Funayama, H. Furukawa, Y. Yamamoto, A. Oyama, Treg-enriched CD4⁺ T cells attenuate collagen synthesis in keloid fibroblasts, *Exp Dermatol* 23(4) (2014) 266-71.

[31] A. Mukhopadhyay, M.Y. Wong, S.Y. Chan, D.V. Do, A. Khoo, C.T. Ong, H.H. Cheong, I.J. Lim, T.T. Phan, Syndecan-2 and decorin: proteoglycans with a difference—implications in keloid pathogenesis, *J Trauma* 68(4) (2010) 999-1008.

Figures

Figure 1

Figure 1

Gross and hematoxylin and eosin (H&E) staining image of normal skin, matured scar and keloid. (A) Normal skin. (B) Hematoxylin and eosin (H&E) staining of normal skin. (C) Matured scar. (D) Hematoxylin and eosin (H&E) staining of matured scar. (E) Keloid. (F) Hematoxylin and eosin (H&E) staining of keloid.

Figure 2

Figure 2

Flow cytometric results of passage 3 dental pulp stem cells Figure Note: Cells expressed mesenchymal surface markers CD29 (99.57%, A), CD73 (99.72%, B), and CD90 (99.62%, C), while hardly expressing the hematopoietic stem cell surface marker CD34 (0.20%, D).

Figure 3

Study design. A: A schematic design of the Trans well co-culture model was established using Trans well chambers with a 0.4- μm pore size which allows for the passage of chemical and biochemical molecules. B-E:4 combinations of the inducing and differentiating cells.

Figure 4

A: The cell counts (proliferation) were analyzed using CCK-8 kits (Dojindo) on days 1, 3, 5, and 7. B: The morphology of the fibroblasts were determined on days 1, 3, 5, and 7 by direct observation with a light microscope. HNF: human normal fibroblasts, HKF: human keloid fibroblasts, DPSCs: dental pulp stem cells.

Figure 5

Apoptosis rate of HNFs and HKFs in mono and co-cultures. DPSCs does not induce apoptosis in HNFs or HKFs. A: Vital cells stain green with Calsein-AM, while dead cells stain red with propidium iodide on days 1, 3, and 5. B: The Quantification of cell apoptosis/necrosis using the percentage of PI-positive cells / AM-positive cells. (mean $\pm$ SD, $n=5$, Student t-test, $P<0.05$).

Figure 6

Cellular migration in scratch area A: Pictures were taken at 0 hour and 24 hours, a magnification of $\times 40$ was used. B: The number of the cells migrating into the scratch area were counted by Image J. Significant differences were only detected in the co-culture group of the human keloid fibroblast after 24 hours ($P < 0.05$).

Figure 7

DPSCs attenuated the pro-fibrotic phenotype of HKFs and HNFs. after 48 hours of culture, cells were subjected to real-time PCR and western blot. (A-C) Quantification of (A) CTGF, (B) TGF-β₁, and (C) TGF-β₂ gene expression, normalized to GAPDH expression (D-F). Quantification of (D) CTGF, (E) TGF-β₁, and (F) TGF-β₂ protein levels, normalized to β-tubulin expression. (G) Gels were analyzed by immunoblotting using the indicated antiserum.

Figure 8

DPSCs inhibits extracellular matrix synthesis of HKFs and HNFs after 48 hours of culture and then subjected to real-time PCR and western blot. (A-B) Quantification of (A) Collagen I, and (B) α -SMA gene expression, normalized to GAPDH expression. (C-D) Quantification of (C) Collagen I, and (D) α -SMA protein levels, normalized to β -tubulin expression levels (E) Cell culture supernatants were collected and tested for hydroxyproline content. (F) Gels were analyzed by immunoblotting using the indicated antiserum.