Reversibly immortalized keratinocytes (iKera) facilitate re-epithelialization and skin wound healing: Potential applications in cell-based skin tissue engineering

Jiamin Zhong\textsuperscript{a,1}, Hao Wang\textsuperscript{a,b,1}, Ke Yang\textsuperscript{b,c}, Huifeng Wang\textsuperscript{d}, Chongwen Duan\textsuperscript{d}, Na Ni\textsuperscript{a,b}, Liqin An\textsuperscript{a}, Yetao Luo\textsuperscript{a}, Piao Zhao\textsuperscript{a,b}, Yannian Gou\textsuperscript{a}, Shiyan Sheng\textsuperscript{a}, Deyao Shi\textsuperscript{b,e}, Connie Chen\textsuperscript{b}, William Wagstaff\textsuperscript{b}, Bryce Hendren-Santiago\textsuperscript{b}, Rex C. Haydon\textsuperscript{b}, Hue H. Luu\textsuperscript{b}, Russell R. Reid\textsuperscript{b,f,g}, Sherwin H. Ho\textsuperscript{b}, Guillermo A. Ameer\textsuperscript{d,g,h}, Le Shen\textsuperscript{b,i}, Tong-Chuan He\textsuperscript{b,h,g}, Jiaming Fan\textsuperscript{a,b,1}

\begin{itemize}
  \item \textsuperscript{a} Ministry of Education Key Laboratory of Diagnostic Medicine, And Department of Clinical Biochemistry, School of Laboratory Medicine, Chongqing Medical University, Chongqing, 400016, China
  \item \textsuperscript{b} Molecular Oncology Laboratory, Department of Orthopaedic Surgery and Rehabilitation Medicine, The University of Chicago Medical Center, Chicago, IL, 60637, USA
  \item \textsuperscript{c} The Pediatric Research Institute, The Children’s Hospital of Chongqing Medical University, Chongqing, 400014, China
  \item \textsuperscript{d} Biomedical Engineering Department, Northwestern University, Evanston, IL, 60208, USA
  \item \textsuperscript{e} Department of Orthopaedics, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
  \item \textsuperscript{f} Department of Surgery, The University of Chicago Medical Center, Chicago, IL, 60637, USA
  \item \textsuperscript{g} Center for Advanced Regenerative Engineering (CARE), Evanston, IL, 60208, USA
  \item \textsuperscript{h} Department of Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL, 60616, USA
  \item \textsuperscript{1} These authors contributed equally to the work.
\end{itemize}

\section*{ARTICLE INFO}

Keywords:
- Keratinocytes
- Skin tissue engineering
- Reversible immortalization
- SV40 large T antigen
- PPCN
- Skin wound healing

\section*{ABSTRACT}

Skin injury is repaired through a multi-phase wound healing process of tissue granulation and re-epithelialization. Any failure in the healing process may lead to chronic non-healing wounds or abnormal scar formation. Although significant progress has been made in developing novel scaffolds and/or cell-based therapeutic strategies to promote wound healing, effective management of large chronic skin wounds remains a clinical challenge. Keratinocytes are critical to re-epithelialization and wound healing. Here, we investigated whether exogenous keratinocytes, in combination with a citrate-based scaffold, enhanced skin wound healing. We first established reversibly immortalized mouse keratinocytes (iKera), and confirmed that the iKera cells expressed keratinocyte markers, and were responsive to UVB treatment, and were non-tumorigenic. In a proof-of-principle experiment, we demonstrated that iKera cells embedded in citrate-based scaffold PPCN provided more effective re-epithelialization and cutaneous wound healing than that of either PPCN or iKera cells alone, in a mouse skin wound model. Thus, these results demonstrate that iKera cells may serve as a valuable skin epithelial source when, combining with appropriate biocompatible scaffolds, to investigate cutaneous wound healing and skin regeneration.

\section*{1. Introduction}

Skin is the largest organ of the body, accounting for about 15\% of the human body by mass. The primary functions of skin are to serve as a protective barrier against biological and chemical agents, to moderate temperature, and to retain body fluids [1–3]. To fulfill these functions, skin is composed of several cell types, such as keratinocytes, fibroblasts, and melanocytes, which are organized into three layers: epidermis,
polycaprolactone (PCL), and hydrogels and extracellular matrix (ECM), have been investigated for skin wound healing and/or skin regeneration [7]. Numerous scaffold materials, especially hydrogels and extra cellular matrix (ECM), have been investigated for skin wound healing and/or skin regeneration [7–9]. It has been recently reported that extracellular citrate can elevate cell energy and metabolic status, which should be beneficial for the survival and proliferation of the cells embedded in the scaffolds [10–12]. One interesting citrate-based degradable polymer gel is poly (polyethylene glycol citrate-co-N-isopropylacrylamide) (PPCN), which is thermoresponsive and exerts intrinsic antioxidant and anti-inflammation activities [13]. Significant efforts have been devoted to the development of stem cell-based therapeutic strategies, such as the possible use of embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and various sources of mesenchymal stem cells (MSCs), to treat wounds, although the use of these progenitor cells may be limited due to the concerns about their availability and/or tumorigenicity [1,8,9]. Thus, more practical sources of skin cells need to be explored for effective wound treatment. Here we focused on keratinocytes because they make up the first barrier of the skin and play a critical role in the re-epithelialization process [2]. Nonetheless, significant progress has been made in developing novel therapeutic approaches for wound treatment, as well as advanced and regenerative dressings to promote wound closure within a clinically relevant timeframe, through the use of novel scaffolds and/or cell-based therapeutic strategies [1].

Significant efforts have been devoted to the development of stem cell-based therapies, such as the possible use of embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and various sources of mesenchymal stem cells (MSCs), to treat wounds, although the use of these progenitor cells may be limited due to the concerns about their availability and/or tumorigenicity [1,8,9]. Thus, more practical sources of skin cells need to be explored for effective wound treatment. Here we focused on keratinocytes because they make up the first barrier of the skin and play a critical role in the re-epithelialization process [2]. However, adult keratinocytes are difficult to isolate and maintain for long-term culture [14].

To overcome the limited life span of culturing primary keratinocytes, we established the reversibly immortalized mouse keratinocytes (iKera) cells by stably expressing SV40 large T antigen (SV40 T), which can be reversed by the FLP recombinase or silencing SV40 T. The iKera cells expressed keratinocyte markers and were responsive to UVB-induced inhibition of cell proliferation. While retaining long-term proliferative activity in vitro, the iKera cells were non-tumorigenic. In a proof-of-concept experiment, we demonstrated that the citrate-based scaffold PPCN embedded with iKera cells provided more effective cutaneous wound healing and re-epithelialization than that of either PPCN or iKera cells alone in a mouse skin wound model. Therefore, our results demonstrate that the iKera cells may serve as a valuable epithelial cell source, in combination with the appropriate biocompatible scaffolds, for cutaneous wound healing and skin tissue engineering.

2. Materials and methods

2.1. Synthesis and characterization of the citrate-based polymer PPCN

The citrate-based polymer poly (polyethylene glycol citrate-co-N-isopropylacrylamide) (PPCN) was synthesized by employing a simple two-step method as previously reported [13]. First, poly (polyethylene glycol citrate) acrylate prepolymer (PPCac) was prepared by the
For the recovery of primary dermal fibroblasts, the full thickness skin was minced into small pieces, directly seeded onto cell culture dishes, and cultured at 37 °C in 5 % CO₂ in high glucose complete DMEM supplied with 10 % FBS. The medium was changed every 24 h to remove non-adherent cells. The adherent cells were passage once reaching subconfluence (designated as P0, 1, 2, and 3 to reflect the number of passages) for in vitro assays.

2.4. Establishment of the reversibly immortalized keratinocyte cell line (iKera)

The isolated mouse primary keratinocytes (less than 3 passages) were used to generate the immortalized keratinocytes (iKera). Briefly, the retroviral vector SSR#41 that expresses SV40 T antigen (SV40 T) flanked with FRT sites was co-transfected with pCLAmpho packaging vector into HEK-293 cells to produce packaged retrovirus as described [26–35]. Subconfluent mouse primary keratinocytes were infected with the packaged SSR#41 retrovirus. The infected keratinocytes were selected with hygromycin B (0.3 mg/mL, Invitrogen) for 7–10 days, and replated every 3 days. The resultant immortalized keratinocyte cell line was designated as iKera, which has been passaged for more than 30 generations.

2.5. RNA isolation and touchdown-quantitative real-time PCR (TqPCR)

Total RNA was isolated by using the TRIzol Reagent (Invitrogen, China), and subjected to reverse transcription using hexamer and M-MulV reverse transcriptase (New England Biolabs, Ipswich, MA). The cDNA products were used as PCR templates. Gene-specific PCR primers were designed by using Primer3 program (Table S1). TqPCR was carried out by using 2x SYBR Green qPCR Master Mix (Bimake, Shanghai, China) on the CFX-Connect unit (Bio-Rad Laboratories, Hercules, CA) as described [36–39]. All TqPCR reactions were done in triplicate. Gapdh was used as a reference gene. Quantification of gene expression was carried out by using the 2^−ΔΔCq method as described [38,40–42].

2.6. WST-1 cell proliferation assay

Exponentially growing cells were seeded in 96-well plates (2000 cells/well). At the indicated time points, the Premixed WST-1 Reagent (Clontech, Mountain View, CA) was added, followed by incubating at 37 °C for 120 min and reading absorbance at 450 nm using a microplate reader (Biotek, EON, USA) as described [43–46].

2.7. Crystal violet cell viability assay

Exponentially growing cells were seeded in 24-well plates (6000 cells/well). At the indicated time points, the cells were gently washed with PBS and stained with 0.5 % crystal violet/formalin solution for 10 min. The stained cells were washed with tap water and air-dried for scanning. For quantitative analysis, the stained cells were dissolved in 10 % acetic acid and measured for absorbance at 592 nm as described [47–51].

2.8. Differentiation of iKera cells induced by all-trans retinoic acid (ATRA)

Exponentially growing iKera cells were seeded in 60 mm dishes (1 × 10⁶ cells) and treated with 1 μM ATRA or DMSO. Total RNA was isolated and TqPCR was used to test the expression of genes related to keratinocyte differentiation at days 3 and 5.

2.9. Tumorigenicity analysis of the iKera cells in athymic nude mice

The use and care of the athymic nude mice were approved by the Research Ethics and Regulations Committee of Chongqing Medical University, Chongqing, China. The animals were obtained from and housed in the Experimental Animal Research Center of Chongqing Medical University. The iKera-Fluc cells were stably transduced with the firefly luciferase-expressing retroviral vector pSEB-Fluc as described [31,52,53]. Exponentially growing iKera-Fluc cells were resuspended and injected subcutaneously into the flanks of athymic nude mice (6-week old, male, 2 × 10⁶ cells per injection, and 4 sites per mouse). Potential subcutaneous mass growth was assessed at 3, 7 and 14 days after implantation by whole body bioluminescence imaging using the IVIS Lumina Series III In Vivo Imaging System (PerkinElmer, Waltham, MA). The acquired data were quantitatively analyzed by the Living Image Software (PerkinElmer).

2.10. Construction and generation of recombinant adeno-viruses

All recombinant adeno-viruses used in this study were generated by using the AdEasy technology [54–56]. The adeno-viral vector Ad-FLP was constructed and characterized in our previous studies [49,50,57,58]. To construct the adeno-viral vector expressing sRNAs targeting SV40 T, we employed our previously developed pSOS-related FAMSi system, in which siRNA expression is driven by the converging human U6 and H1 promoters as described [17,19,59,60]. Three siRNA sites targeting SV40 T were designed by using Invitrogen’s BLOCK-IT RNAi Designer and/or Dharmacon Horizon Discovery’s siDESIGN programs (Table S2). The oligo cassettes for the three siRNAs were subcloned into a single adeno-viral shuttle vector to generate recombinant adeno-viral vector and subsequently the adeno-virus Adsi-LTA in 293pTP or RAPA cells. Adsi-LTA also co-expresses the RFP marker gene. Adeno-viral vector expresses RFP (Ad-RFP) or GFP (Ad-GFP) alone was used as a mock adeno-virus control [40,61]. For all adeno-viral infections, poly-brene (8 μg/mL) was added to enhance infection efficiency as reported [62,63].

2.11. Transmission electron microscope (TEM) analysis

Approximately 2 × 10⁶ cells were collected by centrifugation at 1,200 rpm for 10 min. The cell pellets were fixed with 2.5 % glutaraldehyde and stored at 4 °C. The cell pellets were washed with 0.1 M sodium cacodylate and further fixed with 4 % osmium tetroxide for 1 h, followed by serial dehydration in ascending concentrations of acetone (from 35 % to 100 %). The samples were infiltrated with a mixture of acetone and resin at the ratio of 1:1 for 1 h, 1:2 for 2 h and 100 % resin overnight as reported [64]. Finally, the samples were polymerized at 60 °C for 36 h prior sectioning to prepare the 90 nm thick sections using the ultra-microtome Leica EM UC7 (Leica, Germany). The ultrathin sections were stained with lead citrate/uranyl acetate, and analyzed under a transmission electron microscope (Hitachi 7500, Japan).

2.12. Immunofluorescence (IF) staining

The IF staining was carried out as previously reported [24,25,27,65,66]. Briefly, cells seeded and treated in chamber slides were fixed with 4 % paraformaldehyde for 15 min at RT, treated with 0.5 % Triton X-100 for 20 min, and blocked with 5 % goat serum (1:10 dilution) for scanning. For quantitative analysis, the stained cells were dissolved in 10 % acetic acid and measured for absorbance at 592 nm as described [47–51].
2.13. Preparation of conditioned medium (CM) and treatment of immortalized keratinocytes (iKera), primary dermal fibroblasts (Fib) or immortalized melanocytes (iMCs)

Conditioned medium (CM) was prepared as previously described [67,68]. Briefly, $2 \times 10^6$ iKera cells were plated in 10 cm$^2$ cell culture dishes. 293 cells were set up in the same fashion as the control conditioned medium. The supernatants were collected from the cultured iKera cells (iKera-CM) or 293 cells (293-CM) at 48 h, and filtered for culturing the primary dermal fibroblasts (Fib) or melanocytes (iMCs). Subconfluent Fib cells and iMCs were treated with iKera-CM or 293-CM for the indicated time points for further analysis. $2 \times 10^6$ Fib cells were plated in 10 cm$^2$ cell culture dishes. 293 cells were set up in the same fashion as the control conditioned medium. The supernatants were collected from the cultured Fib cells (Fib-CM) or 293 cells (293-CM) at 48 h, and filtered for culturing the iKera cells. Subconfluent iKera cells were treated with Fib-CM or 293-CM for the indicated time points for further analysis.

2.14. Transwell co-culture of iKera and iMCs and melanin secretion assay

Melanin production was assessed qualitatively and quantitatively. Briefly, $5 \times 10^5$ iMCs per well were seeded in 6-well plates, while $5 \times 10^5$ iKera or 293 cells/well were seeded in 0.4 μm transwell inserts for co-culturing experiments. Melanin secretion was analyzed under bright field microscope at indicated time points as described [22]. For quantitative analysis, the iMCs from iKera or 293 co-culture were collected for visual examination and lysed with 1 M NaOH for 5 min, followed by absorbance determination at 470–490 nm as described [69].

2.15. Mouse cutaneous wound healing model and PPCN-iKera based therapy

The experimental mouse skin wound healing model was established as described [70]. The use and care of animals, and the experimental procedures were approved by the Research Ethics and Regulations Committee of Chongqing Medical University. Briefly, 12 male 6–8-week-old athymic nude mice were divided into four groups. After the animals were anesthetized, the dorsal skin of each mouse was disinfected with 70% alcohol; and 1-cm diameter full-thickness cutaneous wound was created at the dorsum part of each mouse. In the Blank (sham) group, the wounds were treated with 100 μL PBS/mouse as a control. In the iKera group, the wounds were treated with $2 \times 10^6$ iKera cells in 100 μL PBS/mouse. In the PPCN group, the wounds were treated with $100 \mu$L PPCN/mouse. In the PPCN + iKera group, the wounds were treated with $2 \times 10^6$ iKera cells in 100 μL PPCN/mouse. All wounds were dressed with sterile gauze. The mice were given antibiotics orally in drinking water. Wound sizes were measured at days 0, 3, 6 and 9, respectively. The average areas of wound openness in each group at the indicated time points were calculated by using the Image-Pro Plus software (Media Cybernetic, Rockville, USA).

2.16. H & E staining, Masson’s trichrome staining and sirius red staining

The skin wound healing samples (i.e., 1-cm diameter from the center of the cutaneous wound) were retrieved from athymic nude mice at the endpoint day 9. The samples were fixed, paraffin-embedded, and sectioned along maximum diameter. Serial sections were deparaffinized, rehydrated, and subjected to H & E staining, Masson’s trichrome staining (Masson’s Trichrome Stain Kit, G1340, Solarbio, China), and Sirius red staining (Picro Sirus Red solution, G1471, Solarbio, China) as described [71,72].

2.17. Immunohistochemical (IHC) staining

IHC staining protocol was carried out by using the IHC staining (SP Kit, SP-9001, ZSGB-Bio, China) as described [73,74]. Briefly, the sections were deparaffinized and subjected to immunostaining with the primary antibodies against Krt10 (1:50 dilution; Bimake; Cat# AS266), Krt15 (1:50 dilution; Bimake; Cat# AS627), Involucrin (1:50 dilution; Bimake; Cat# AS788), SV40 T (1.50 dilution; Santa Cruz; Cat# sc-147). The Biotin labeled goat anti-mouse IgG (SP Kit, SP-9000, ZSGB-Bio, China) or goat anti-rabbit IgG (SP Kit, SP-9001, ZSGB-Bio, China) were used to visualize the presence of the proteins of interest. Hematoxylin was used to stain the nuclei. Sections incubated without primary antibodies were used negative controls. The results were recorded under a bright field microscope (Leica DM4B/Nikon 80i).

2.18. Statistical analysis

All experiments were performed at least three times and/or repeated three batches of independent experiments. Data were analyzed using GraphPad Prism 7 and presented as the mean ± standard deviations (SD). Statistical significance was determined by one-way analysis of variance and the student’s t-test for the comparisons between groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Reversibly immortalized mouse keratinocytes (iKera cells) can be effectively established via the stable expression of SV40 T (SV40 T)

Skin consists of the epidermis, dermis, hair follicle and sebaceous gland; and the epidermis is the outermost layer of skin and consists of 15–20 layers of keratinocytes [2,3]. To establish a constant source of keratinocytes for scaffold-based skin wound healing studies, we sought to establish a reversibly immortalized keratinocyte line. We first isolated the mouse primary keratinocytes (designated as Kera cells) from newborn mice as described in methods (Fig. 1A, a to f). As SV40 large T antigen (SV40 T) is one of the most commonly used immortalization genes [27,50,75], the retroviral vector SSR#41, which expresses a hygromycin B-resistance gene and SV40 T flankeld with FRT sites, was used to generate reversibly immortalized keratinocyte line, designated as iKera cells (Fig. 1B). The Kera (0 P0) and iKera cells were morphologically similar in vitro (Fig. 1C). The expression of SV40 T in iKera cells was verified by TqPCR (Fig. S1A) and IF staining (Fig. 1D, and the negative control was shown in Fig. S1B) in comparison with that in Kera cells. Unlike Kera cells, the iKera cells could be passaged and maintained high proliferative activity for at least 30 generations at the time when this work was reported. We further demonstrated that iKera cells exhibited higher proliferation rate and cell viability, compared with Kera cells (P1) in WST-1 assay (Fig. 1E) and crystal violet cell viability assay (Fig. 1F). These results indicate that the SV40 T-mediated immortalization strategy was effective to establish the iKera cells as a long-term keratinocyte line.

As shown in Fig. S1C, keratinocytes can be divided into five layers from inside to outside: basal layer, spinous layer, granular layer, hyaline layer and stratum corneum layer [2]. To test the mature and differentiation potential of the iKera cells, we treated the iKera cells with 1 μM ATRA for 3 and 5 days respectively, and found that the characteristic genes related to basal layer (Krt5, Krt14 and Krt15), spinous layer (Krt1 Krt10 and IVL), granular layer (IVL and LOR) and stratum corneum layer (LOR) were highly expressed, compared with that of the control group by qPCR (Fig. S1D), suggesting that the iKera cells may retain the mature and differentiation potential while maintaining long-term proliferative activity.

To test whether the immortalization phenotype of the iKera cells was reversible, we employed two strategies: 1) using a recombinant adeno-virus overexpressing FLP recombinase (namely Ad-FLP) and 2) using a recombinant adenovirus expressing siRNAs to silence SV40 T (namely Adsi-LTA). The iKera cells could be effectively transduced by these adenoviruses, along with control Ad-GFP or Ad-RFP virus (Fig. 1G). The
TqPCR analysis indicates that SV40 T expression was significantly reduced in the iKera cells transduced with Ad-FLP or Adsi-LTA, compared with that treated with the control viruses (Fig. 1H). The removal of SV40 T by Ad-FLP or silencing SV40 T by Adsi-LTA was shown to significantly decrease the proliferation rate of the iKera cells after 48 h as assessed by WST-1 assay (Fig. 1I). Both qualitative and quantitative crystal violet cell viability assays further confirmed that the removal of SV40 T antigen or silencing SV40 T expression in the iKera cells led to a significant decrease in cell viability and proliferation (Fig. 1J). Even though SV40 T was likely partially removed or silenced in the above assays, these results strongly suggest that the SV40 T-mediated immortalization phenotype of the iKera cells may be reversible.
The peeled keratin cambium tissues; (e) the cultured keratin cambium tissues after being minced into small pieces; and (f) the recovered and proliferating primary keratinocytes (Kera). (B) The schematic representation of SV40 T-mediated retroviral vector SSR#41. The SV40 T gene is flanked with the FRT sites, allowing the removal of SV40 T upon the expression of FLP recombinase. (C) The morphologic comparison of Kera (P0) and iKera cells under phase contrast microscope. (D) Expression of SV40 T: Immunofluorescence (IF) staining was used to assess the expression and localization of SV40 T in Kera (P0) and iKera cells. The nuclei were stained with DAPI. Representative results are shown. (E) Cell proliferation assay. WST-1 was used to assess the cell proliferation of Kera (P1) and iKera cells at 0, 24, 48, 72 and 96 h, respectively. **p < 0.01, Kera cells vs iKera cells. (F) Cell viability assay. Crystal violet staining assay and quantitative analysis were used to assess the viability and proliferation of Kera (P1) and iKera cells at days 0, 1, 2, 3, 4, 5 and 6. ***p < 0.01, Kera cells vs iKera cells. (G) Subconfluent iKera cells were infected with Ad-FLP, Ad-GFP, Adsi-LTA and Ad-RFP, respectively. The fluorescence signals were recorded at 36 h after infection. (H) Removal and knockdown of SV40 T expression in iKera cells. The iKera cells were infected with Ad-FLP, Ad-GFP, Adsi-LTA or Ad-RFP for 48 h. Total RNA was isolated for qPCR analysis of the expression of SV40 T in iKera cells. **p < 0.05, Ad-FLP group vs Ad-GFP group, ***p < 0.01, Adsi-LTA group vs Ad-RFP group. (I) Effect of SV40 T removal and silencing on cell proliferation of iKera cells. Subconfluent iKera cells were infected with Ad-FLP, Ad-GFP, Adsi-LTA, or Ad-RFP. WST-1 was used to assess the cells proliferation at 0 h, 24 h, 48 h, 72 h and 96 h. **p < 0.01, Ad-FLP group vs Ad-GFP group, Adsi-LTA treated group vs Ad-RFP treated group. (J) Cell viability upon the removal or silencing of SV40 T in iKera cells. Subconfluent iKera cells were infected with Ad-FLP, Ad-GFP, Adsi-LTA or Ad-RFP. Crystal violet cell viability assay and quantitative analysis were used to assess cell viability and proliferation of the iKera cells. **p < 0.01, ***p < 0.005, Ad-FLP group vs Ad-GFP group, or Adsi-LTA group vs Ad-RFP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. The iKera cells retain the ultrastructure of keratin filaments, express characteristic markers of keratinocytes and are responsive to UVB irradiation-induced inhibition of cell proliferation

We first compared the ultrastructure features between primary Kera and the iKera cells. As profilaggrin is synthesized at the granular layer and stored in keratohyalin granules, and proteolyzed and broken down into many filaggrin monomers when granular cells differentiate to corneocytes, leading to the disappearance of the nucleus and cell organelles, but retention of keratin filaments, a prominent characteristic of differentiated cells, the ultrastructure analysis revealed that abundant keratin filaments were presented in both Kera and iKera cells (Fig. 2A).

We next conducted qPCR analysis and determined the expression of the well-established keratinocyte markers [77,78]. We found that the expression of most of these marker genes, especially Tgf-α, Tgf-β, Krt1, Krt5, Krt14 and Krt15, was readily detected in both Kera and iKera cells (Fig. S1E). Immunofluorescence staining assay further confirmed the expression of Krt10, Krt14 and Krt15 in the Kera and iKera cells (Fig. 2B). Collectively, these results indicate the iKera cells possess structural and marker expression characteristics of keratinocytes.

We analyzed the effect of UVB irradiation on iKera cell proliferation. When subconfluent iKera cells were treated with different doses of UVB irradiation, we found that cell proliferation was inhibited by UVB in a dose-dependent and time-dependent fashion (Fig. S1F). We found that treatment with 225 μW/cm² UVB began to suppress the proliferation of iKera cells at 48 h (Fig. 2C). It has been reported that in response to DAMPs (damage-associated molecular patterns) released by host cells during UVB irradiation or wounding, keratinocytes produce a variety of pro-inflammatory cytokines or chemokines [4,77]. We found that the expression of most pro-inflammatory cytokine or chemokine genes related to radiation, such as Egr, Csf2, Csf3, Cxc10, Cxc15 and IL-1α, was significantly induced upon 225 μW/cm² UVB treatment in both Kera and iKera cells at 48 h (Fig. 2D). It has been reported that in response to DAMPs (damage-associated molecular patterns) released by host cells during UVB irradiation or wounding, keratinocytes produce a variety of pro-inflammatory cytokines or chemokines [4,77]. We found that the expression of most pro-inflammatory cytokine or chemokine genes related to radiation, such as Egr, Csf2, Csf3, Cxc10, Cxc15 and IL-1α, was significantly induced upon 225 μW/cm² UVB treatment in both Kera and iKera cells at 48 h (Fig. 2D). Meanwhile, the WST-1 assay confirmed that UVB treatment significantly inhibited the cell proliferation and viability of the iKera cells from 24 h to 72 h (Fig. 2E), and the crystal violet staining confirmed that UVB treatment significantly inhibited the cell viability of the iKera cells from Day 2-5 (Fig. 2F) [4,77]. Collectively, these results demonstrated that the iKera cells, were highly similar to primary keratinocytes at ultrastructure level and in marker expression, and were sensitive and responsive to UVB irradiation treatment.

3.3. The iKera cells promote melanogenesis and the proliferation of melanocytes and dermal fibroblasts

As it has been reported that keratinocytes can stimulate the proliferation and melanogenesis of melanocytes [79], we found that, compared with that of the control conditioned medium, the conditioned medium prepared from iKera cells significantly enhanced cell proliferation and viability of the melanocyte IMCs as assessed by WST-1 assay (Fig. 3A), and crystal violet assay (Fig. 3B). Co-culture of the IMCs and iKera cells was shown to promote the secretion and production of melanin of IMCs, compared with the control cells, both qualitatively (Fig. 3C) and quantitatively (Fig. 3D). Furthermore, co-culture with the iKera cells significantly impacted the expression of the genes related to synthesis and secretion of melanin, such as Mmp1, Mmp14 (Fig. 3E).

During cutaneous wound healing process, the activated keratinocytes produce numerous signaling molecules such as IFN-γ, TGF-β, while dermal fibroblasts (Fib) synthesize collagen, and fibronectin, and act on both dermal melanocytes and dermal fibroblasts to repair wounds [2,5], allowing the migration of dermal fibroblasts along the fibrin network and the wound edges and the initiation of re-epithelialization from the wound edges [80]. We found that conditioned medium prepared from iKera cells promoted the cell proliferation of primary dermal fibroblasts (Fib), or vice versa, as assessed by WST-1 assay (Fig. 3F), or crystal violet cell viability assay (Fig. 3G). Since it has been reported that keratinocytes can promote dermal fibroblasts to synthesize and secrete growth factors and cytokines [81], we found that iKera conditioned medium impacted the expression of growth factor and cytokine genes, such as Egr1, Il1r1, Mmp1, Csf3, Kgf, and Csf2 in the dermal fibroblasts (Fib) as assessed by qPCR analysis (Fig. 3H). Collectively, these results demonstrate that the iKera cells possess the similar biological characteristics of primary keratinocytes.

As there is a likelihood that immortalized cells may acquire tumorigenic potential [82], we sought to test if the iKera cells had a tendency to form tumors in vivo. The iKera cells were first stably labeled with firefly luciferase (namely iKera-FLuc) with retroviral vector, and injected subcutaneously into the flanks of athymic nude mice. Through whole body bioluminescence imaging using Xenogen IVIS 200, the bioluminescence signal was readily detected at day 3 after injection, but significantly decreased at day 7, and completely disappeared at day 14 after injection (Fig. 3I). The animals were monitored for an extended period, and no detectable subcutaneous masses were observed for up to six weeks, indicating that the iKera cells were not tumorigenic in vivo. Collectively, the above in vitro and in vivo results demonstrate that the iKera cells maintain long-term proliferative activity while retaining the biological characteristics of primary keratinocytes, suggesting that the iKera cells may be used as a valuable source of keratinocytes for optimizing scaffold-based skin wound healing strategy or skin tissue engineering studies.
Fig. 2. The ultrastructural and functional characteristics of iKera cells. (A) The ultrastructure of the iKera and primary Kera cells. TEM was used to assess the ultrastructure of the Kera and iKera cells. The keratin filaments are indicated by red arrows. Representative results are shown. (B) Expression of keratinocyte markers in the iKera cells. IF was used to assess the expression and localization of Krt10, Krt14 and Krt15 in Kera and iKera cells. The nuclei were counter-stained with DAPI. Representative results are shown. NC, negative control (minus primary antibody or control IgG). (C) The iKera cells are sensitive to UVB. The iKera cells were treated with 225 μw/cm² UVB for 48 h and observed under light microscope at 48 h. Representative results are shown. (D) UVB-induced gene expression in the Kera and iKera cells. Subconfluent Kera (P1) and iKera cells were treated with 225 μw/cm² UVB for 48 h. Total RNA was isolated for TqPCR analysis to assess the expression of the irradiation responsive genes. **p < 0.01, UVB treated group vs Blank group. (E) Effect of UVB radiation on cell proliferation of the iKera cells. Subconfluent iKera cells were seeded in 96-well plates and treated with 225 μw/cm² UVB. WST-1 was used to assess the cells proliferation at 0 h, 24 h, 48 h and 72 h. **p < 0.01, UVB treated group vs Blank group. (F) Effect of UVB radiation on cell viability of the iKera cells. Subconfluent iKera cells were seeded in 24-well plates and treated with 225 μw/cm² UVB. Crystal violet cell viability assay and quantitative analysis were used to assess cell viability and proliferation of the iKera cells at days 0, 1, 2, 3, 4, and 5. **p < 0.01, UVB treated group vs Blank group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
J. Zhong et al.

Bioactive Materials 9 (2022) 523–540

A

B

C

D

E

F

G

H

I

530

(caption on next page)
3.4. The characteristics of the citrate-based scaffold PPCN

The citrate-based polymer gel PPCN ([polyethylene glycol citrate-co-N-isopropylacrylamide]) has a hierarchical architecture of micropores and can accommodate live cells in vivo [13]. The synthesis of PPCN was previously described [13] and is shown in Fig. 4A. The newly synthesized PPCN was further characterized. Specifically, the proton nuclear magnetic resonance (1H NMR) was used to confirm the chemical structure of PPCN. The composition of PPCN is verified by 1H NMR (600 MHz, DMSO-d6, ppm): 3.91 (s, 1H), 3.81 (t, 2H), 3.73 (s, 4H), 3.66 (t, 2H), 2.53–2.68 (q, 4H), 2.03–2.14 (d, 1H), 1.47–1.74 (t, 2H), 1.17 (s, 6H) (Fig. 4B). PPCN were further characterized by FT-IR to verify its structure. The absorption of O–H stretching was observed at 3280 cm⁻¹. The peaks displayed from 2873 to 2971 cm⁻¹ were corresponding to symmetric and asymmetric stretching vibration of C–H bond. The C=O stretching of both ester and carboxylate groups are located at 1639 and 1544 cm⁻¹. The peaks located at 1455 and 1387 cm⁻¹ were C–O–H vibration and C–H bending. The strong absorption bands of C–O stretching vibration were shown at 1102 cm⁻¹. The wavenumber ranged from 400 to 4000 cm⁻¹ with the resolution of 4 cm⁻¹ and scans of 32 (Fig. 4C). These results validated the chemical structure of PPCN used in the study.

The rheological properties of PPCN were analyzed by TA Discovery HR 20 hybrid rheometer. The temperature ramp rheological curves revealed that at low temperature (< −29 °C), the loss modulus (G′′) was higher than storage modulus (G′), indicating a liquid-like material. Above 29 °C, both G′ and G′′ dramatically increase. After a crossover of G′ and G′′, the storage modulus was higher than loss modulus, indicating the hydrogel formation. The crossover of moduli is corresponding to lower critical solution temperature (LCST) of PPCN, which is 29.74 °C (Fig. 4D). The thermoresponsiveness of PPCN was further shown in Fig. 4E, i.e., in liquid form under 26 °C and transient to solid form above 30 °C. When iKera cells were transduced with Ad-GFP adenovirus and mixed with gelatin-containing PPCN to form 3-D culture in vitro (Fig. 4F, panel a), GFP signal from the entrapped iKera cells lasted at least up to 10 days (Fig. 4F, panel b), supporting the excellent biocompatibility of PPCN.

3.5. The iKera cells facilitate citrate-based scaffold PPCN-promoted cutaneous wound healing in vivo

We previously used PPCN gel as a vehicle for the in vivo delivery of various types of mesenchymal stem cells for bone regeneration studies [15,19,23,29,49,72,83,84]. Here, we tested whether PPCN could serve both as a scaffold and vehicle for delivering iKera cells to promote skin wound healing in a mouse model. The experimental mouse skin wound healing model was established as described [70]. The animals were divided into four groups: Blank, PPCN, iKera cells, and iKera + PPCN (Fig. 5A, panels a–d). While the skin wounds failed to close completely at day 9, the wounds were almost completely closed in other three groups at day 9, and the combination of iKera and PPCN provided the fastest closure of the cutaneous wounds in the mouse model (Fig. 5A panels bc vs. d & Fig. 5B). These results indicate that while the iKera cell alone or PPCN scaffold alone may promote cutaneous wound healing to certain extents, PPCN loaded with the iKera cells can effectively facilitate skin wound healing in the mouse model.

3.6. The combination of iKera cells and PPCN scaffold yields optimal histologic restoration and re-epithelialization during skin wound healing

Severe acute skin loss injury usually leads to serious host response involved in tissue granulation and re-epithelialization, which is characterized by a rapid proliferation of fibroblasts to deposit randomly oriented collagen fibers to fill the tissue defect, and the migration of keratinocytes and contraction of myofibroblasts to restore the barrier, resulting in a fibrotic scar [7,8,85]. The fibrotic scar is a disorganized and flawed tissue with limited or no native skin functions such as sensation and elasticity. Thus, the histologic features of skin wound healing are important criteria to assess the restoration of native skin functions.

In order to accurately and quantitatively assess the wound healing efficiency and functional restoration, the retrieved samples were carefully processed, embedded and sectioned as illustrated in Fig. 6A. H & E stain analysis revealed that, at both lower and higher magnifications, the wounding area was partially covered with a thin layer of disorganized fibrotic tissue in the blank control group (Fig. 6B-a), whereas the wounding area was almost completely covered with a thick layer of disorganized fibrotic tissue in the PPCN group (Fig. 6B-b). While the addition of the iKera cells partially improved the re-epithelialization of the wounding area in the iKera group (Fig. 6B-c), the combination of iKera cells and PPCN scaffold yielded rather well-organized full thickness skin features and re-epithelialization of the wounding area, compared with that of the normal skin tissue (Fig. 6B-d & e). It is noteworthy that a significant restoration of skin accessory structures in the dermis region of the wound areas was observed in both the iKera cell group and the iKera + PPCN group (Fig. 6B).

Masson’s trichrome is a commonly-used specialty staining method to distinguish cells from surrounding connective tissue. Masson staining showed that a lot of collagen fibers (stained in blue) generated at the wound areas was observed in both the iKera cell group and the iKera + PPCN group (Fig. 6C).
group and the iKera + PPCN group (Fig. 6C).

Similarly, Sirius red staining revealed that significant amounts of collagen deposition in the reticular dermis region were observed in all groups, compared with that in the normal control skin dermis (Fig. 6D), indicating the existence of active wound repair process. Collectively, the histologic analysis results demonstrate that, even though the PPCN alone or the iKera cells alone was shown to promote wound closure by forming disorganized multi-layered structures, only did the combination of the iKera and PPCN group show the effective restoration of the well-organized full thickness skin-like structures.

We also examined the expression of keratinocyte differentiation markers. IHC staining analysis indicated that the expression of keratinocyte markers Krt15, Krt10 and Involucrin was readily detected in all groups (Fig. 6E), compared with that of the negative controls (Fig. S2), indicating the existence of active wound repair process, and/or interact with dermal keratinocytes. Thus, the iKera cells should have an acceptable biosafety concern. Mesenchymal stem cells (MSCs) are somatic progenitor cells that can self-renew and differentiate into multiple cell types including keratinocytes, although it remains a challenge to effectively direct iPSCs to differentiate into various skin cells including keratinocytes [87]. Furthermore, possible tumorigenic potential of iPSCs remains a concern. Mesenchymal stem cells (MSCs) are somatic progenitor cells that can self-renew and differentiate into multiple cell types including skin cells [88]. It has been reported that MSCs induced wound healing with little immunoreactivity in the host after local transplantation or systemic administration [1,7,8]. While MSCs are most commonly derived from adult bone marrow, they can also be isolated from many other tissues such as adipose tissue, umbilical cord blood, or peripheral blood [1,7,8]. Since undifferentiated MSCs can produce cytokines and exert immunomodulatory functions, MSCs have long been studied for their beneficial effects on wound healing [1,7]. However, the overall efficacy of MSC-based therapies is often difficult to assess due to the phenotypic variation of MSCs used. The isolation process of MSCs is time-consuming, and yet limited numbers of autologous MSCs can be harvested for large wounds. Furthermore, it remains a challenge to drive MSCs to differentiate into skin cells with high efficiency, and thus these progenitor cells have limited use in point-of-care settings.

In this study, we demonstrated that the reversibly immortalized keratinocytes iKera can serve as a valuable epithelial cell source for cutaneous wound healing and skin regeneration. Interestingly, a commercially available point-of-care product known as ReCell Autologous Cell Harvesting Device is an autologous skin cell suspension spray for cutaneous wound healing and skin regeneration. Nonetheless, numerous efforts have been devoted to explore the potential use of stem cells to improve the rate and quality of wound healing and/or skin regeneration [1,8].

4.2. Keratinocyte availability may be essential to the quality of skin wound healing

Normal wound healing is a dynamic multi-phase process that requires coordinated interactions between growth factors and various cells [1,8]. As the major cellular component of the epidermis, keratinocytes are epithelial cells of ectodermal origin and responsible for the production of the major extracellular protein keratin of the epidermis, thus playing a critical role in re-epithelialization [1,8]. Thus, keratinocytes play an essential role in cutaneous wound healing. Nonetheless, numerous efforts have been devoted to explore the potential use of stem cells to improve the rate and quality of wound healing and/or skin regeneration [1,8].
system, which uses non-cultured autologous skin cells harvested from a patient [7]. The harvested skin cells were suspended in solution and sprayed on the wound, allowing the cells to adhere to the target tissue surface [89]. Such cell suspension was shown to predominantly contain keratinocytes (~64 %) and dermal fibroblasts (~30 %), with a small population of melanocytes (~3.5 %) [89]. A comparative clinical study of ReCell and autologous split-thickness skin grafting (STSG) in the treatment of acute burns revealed that ReCell was as effective as STSG, while requiring almost 40 times less donor tissue, as well as less pain at the ReCell donor sites and improved appearance relative to STSG [7,90]. It is conceivable that reversibly immortalized or transiently immortalized keratinocytes, along with dermal fibroblasts and melanocytes, may

![Fig. 5. The iKera cells entrapped in the citrate-based scaffold PPCN promote skin wound healing. (A) The effects of the iKera cells and/or PPCN gel on skin wound healing. The 1-cm diameter full-thickness skin wound was created at the dorsum part of each mouse, and the mice were divided into four groups: Blank (a; 100 μL sterile PBS), PPCN (b; 100 μL PPCN), iKera cells (c; 2 × 10^6 of iKera cells in 100 μL PBS), and iKera + PPCN (d; 2 × 10^6 iKera cells in 100 μL PPCN, mixed at 20 °C). (B) Quantitative measurement of skin wound healing rate. The opening areas of skin wounds were measured and calculated at days 0, 3, 6 and 9. **p < 0.01, *p < 0.05, the PPCN group, the iKera group, or the iKera + PPCN group compared with that of the Blank group at respective time points; ##p < 0.01, the iKera + PPCN group compared with that of the PPCN group at day 9; ^^^ p < 0.01, the iKera + PPCN group compared with that of the iKera group at day 9.](image-url)
serve a valuable allogeneic or xenogeneic cell source for wound repair if potential recipient immune response can be mitigated.

4.3. Citrate-based scaffold may provide metabonegenic advantage for cell-based therapy to treat chronic skin wounds

Numerous scaffold materials, especially hydrogels, have been investigated for skin wound healing and/or skin regeneration [7]. It is conceivable that biomaterial scaffolds such as the extracellular matrix (ECM) or individual components of the ECM including collagen, laminin, or hyaluronan, are more appealing for skin wound healing since they are more responsive to and optimized for the physiologic and biomechanical requirements of wound tissue [8,9].

In this study, we focused on the citrate-based, biodegradable, and thermo-responsive polymer gel PPCN [13]. Citrate is normally an integral component in native bone and stored in bone matrix, which can be released during bone resorption. Citrate is also a key intermediate metabolite in the tricarboxylic acid (TCA) cycle, and thus plays crucial regulatory roles in maintaining cell energy homeostasis [10–12]. In fact, it was reported that extracellular citrate uptake through citrate transporter SLC13a5 supported osteogenic differentiation via regulation of energy-producing metabolic pathways, leading to elevated cell energy status that fuels the high metabolic demands of hMSCs osteodifferentiation [12]. We have recently demonstrated that PPCN-based scaffolds effectively supported bone formation from BMP9-stimulated MSCs [18,51,72,91]. Interestingly, the incorporation of copper metal organic framework nanoparticles (HKUST-1 NPs) within the antioxidant thermo-responsive citrate-based PPCN hydrogel induced angiogenesis, collagen deposition, and re-epithelialization during wound healing in diabetic mice [92]. Thus, it is conceivable that citrate-based scaffold materials may provide metabologenic advantage of cell-based therapies or tissue engineering to treat chronic wound healing.

5. Conclusion

In this study, we established the reversibly immortalized...
keratinocytes (iKera) from mouse epidermis tissues by stably expressing SV40 large T antigen (SV40 T), which is flanked with FRT sites. The immortalization phenotype of the iKera cells could be effectively reversed by the FLP recombinase or silencing SV40 T. The iKera cells exhibited typical characteristics of keratinocytes by expressing keratinocyte markers, and were highly responsive to UVB-induced inhibition of cell proliferation, as well as ATRA-induced differentiation. While retaining long-term proliferative activity in culture, the iKera cells were non-tumorigenic in xenograft tumor formation assay. Using the iKera cells, we demonstrated that the citrate-based scaffold PPCN embedded with iKera cells provided more effective cutaneous wound healing and re-epithelialization than that of either PPCN or iKera cells alone, in a mouse skin wound model. Therefore, our results demonstrate that the reversibly immortalized keratinocyte iKera cells can serve as a valuable epithelial cell source, in combination with the biocompatible scaffolds, for skin tissue engineering to treat skin burn injury, skin radiation injury, pigmentation, scar hyperplasia and other skin related disorders.

Authors’ contributions

JF, TCH, JZ, Hao-W and KY conceived the project and oversaw the study. JZ, Hao-W, KY, LA, YL PZ and YG performed most of the experiments. Huifeng-W, CD, and GAA synthesized and characterized the PPCN. SS, NN, DS, FH, QL, CC, WW, BHS, and GAA provided technical supports and/or research resources/reagents. JZ, Hao-W, JF, TCH, LS, and YG collected and analyzed the collected data. JF, TCH, SHH, RRR, RCH, and HHL secured funding supports. JF, JZ, TCH, RCH, HHL, RRR, SHH, and LS drafted the manuscript. All authors reviewed and revised the manuscript, and approved the final version for submission.

Declaration of competing interest

All the authors declare that they have no potential conflicts of interest.

Acknowledgments

The reported study was supported in part by research grants from the 2019 Chongqing Support Program for Entrepreneurship and Innovation (No. cxc2019113) (JF), the 2019 Science and Technology Research Plan Project of Chongqing Education Commission (KJQN201900410) (JF), the 2019 Youth Innovative Talent Training Program of Chongqing Education Commission (No. CY200409) (JF), the 2019 Funding for Postdoctoral Research (Chongqing Human Resources and Social Security Bureau No.298) (JF) and the National Key Research and Development Program of China (2016YFC1000803). RRR, TCH and GAA were partially funded by the National Institutes of Health (DE030480). WW was supported by the Medical Scientist Training Program of the National Institutes of Health (T32 GM07281). This project was also supported in part by The University of Chicago Cancer Center Support Grant (P30CA14599) and the National Center for Advancing Translational Sciences of the National Institutes of Health through Grant Number UL1 TR000430. TCH was also supported by the Mabel Green Myers Research Endowment Fund, The University of Chicago Orthopaedics Alumni Fund, and The University of Chicago SHOCK Fund. Funding sources were not involved in the study design; in the collection, analysis and/or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2021.07.022.

Abbreviations

iKera mouse immortalized keratinocytes
Kera mouse primary keratinocytes
iKera-FLuc iKera cells stably expressing firefly luciferase
P0 Passage 0
SV40 T SV40 large T antigen
TqPCR touchdown quantitative real-time PCR
LSCM laser scanning confocal microscopy
IF immunofluorescence
ATRA all-trans retinoic acid
Ad-FLP Adenoviral vector expressing FLP recombinase
Ad-GFP Adenoviral vector expressing green fluorescent protein (GFP)
Ad-siLTA Adenoviral vector expressing siRNAs that silence SV40 T
Ad-RFP Adenoviral vector expressing red fluorescent protein (RFP)
TEM Transmission electron microscope
iMCs immortalized mouse melanocytes
Fib fibroblasts
PPCN poly (polyethyleneglycol citrate-co-N-isopropylacrylamide)

References

[1] E. Coalition, E. Bishop, W. Liu, Y. Feng, M. Spezia, B. Liu, Y. Shen, D. Wu, S. Du, A. J. Li, Z. Ye, L. Zhao, D. Cao, A. Li, O. Hagag, A. Deng, W. Liu, M. Li, R.C. Haydon, L. Shi, A. Athiviraham, M.J. Lee, J.M. Wolf, G.A. Ameer, T.C. He, R.R. Reid, Stem cell therapy for chronic skin wounds in the era of personalized medicine: from bench to bedside, Genes Dis 6 (2019) 342–358. https://10.1016/j.gendis.2019.09.008.

[2] E.A. Gantwerker, D.B. Horn, Skin: histology and physiology of wound healing, Facial Plast Surg Clin North Am 19 (2011) 441–453. https://10.1016/j.fsc.2011.06.009.

[3] M. Boer, E. Ducknik, R. Malezlska, M. Marchlewicz, Structural and biophysical characteristics of human skin in maintaining proper epidermal barrier function, Postepy Dermatol Alergol 33 (2016) 1-5. https://10.5131/pola.2015.48037.

[4] D. Mohania, S. Chandel, P. Kumar, V. Verma, K. Digvijay, D. Tripathi, K. Choudhury, S.K. Mitten, D. Shah, Ultraviolet radiation: skin defense-damage mechanism, Adv. Exp. Med. Biol. 996 (2017) 71–87. https://10.1007/978-3-319-56017-5_7.

[5] R.B. Idrus, M.A. Rameli, K.C. Low, J.X. Law, K.H. Chua, M.B. Latiff, A.B. Saim, Full-thickness skin wound healing using autologous keratinocytes and dermal fibroblasts with fibrin: layered versus single-layered substitute, Adv. Skin Wound Care 27 (2014) 171–180. https://10.1097/01.ASW.0000445199.26874.9d.

[6] M. Rodrigues, N. Kosaric, C.A. Bonham, C.G. Gurtner, Wound healing: a cellular perspective, Physiol. Rev. 99 (2019) 665–706. https://10.1152/physrev.00067.2017.

[7] J.R. Yu, J. Navarro, J.C. Coburn, B. Mahadik, J. Molnar, J.R Holmes, A.J. Nam, J. P. Fisher, Current and future perspectives on skin tissue engineering: key features of biomedical research, translational assessment, and clinical application, Adv. Healthc Mater 8 (2019), e1801471. https://10.1002/adhm.201801471.

[8] A. Nourian Dekaordi, F. Mirahmadi Babaheydari, M. Chehelgerdi, S. Raeisi, Therapeutic strategies, Stem Cell Res. Ther. 10 (2019) 111. https://10.1186/s13287-019-1213-2.

[9] B.C. Dash, Z. Xu, L. Lin, A. Koo, S. Ndon, F. Berthiaume, A. Dardik, H. Hsia, Stem cells and engineered scaffolds for regenerative wound healing, Bioengineering (Basel). 5 (2018). https://10.3390/bioengineering5010023.

[10] C. Ma, E. Gerhard, D. Lu, J. Yang, Citrate chemistry and biology for biomaterials design, Biomaterials 178 (2018) 383–400. https://10.1016/j.biomaterials.2018.05.003.

[11] C. Ma, M.L. Kuzma, X. Bai, J. Yang, Biomaterial-based metabolic regulation in regenerative engineering, Adv. Sci. 6 (2019) 1900819. https://10.1515/advs-201900819.

[12] C. Ma, X. Tian, J.P. Kim, D. Xie, X. Ao, D. Shan, Q. Lin, M.R. Hudock, X. Bai, J. Yang, Citrate-based materials fuel human stem cells by metabologenic regulation, Proc. Natl. Acad. Sci. U. S. A. 115 (2018) E17417-E17450. https://10.1073/pnas.1813000115.

[13] J. Yang, R. van Lith, K. Balcer, R.A. Hoshi, G.A. Ameer, A thermoresponsive biodegradable polymer with intrinsic antioxidant properties, Biomacromolecules 15 (2014) 3942–3952. https://10.1021/bm5010094.

[14] U. Lichti, J. Anders, S.H. Yuspa, Isolation and short-term culture of primary keratinocytes, hair follicle populations and dermal cells from newborn mice and keratinocytes from adult mice for in vitro analysis and for grafting to immunodeficient mice, Nat. Protoc. 3 (2008) 799–810. https://10.1038/nprot.2008.850.

[15] J. Ye, J. Wang, Y. Zhu, Q. Wei, X. Wang, J. Yang, S. Tang, H. Liu, J. Fan, F. Zhang, E.M. Farina, M.K. Mohammed, Y. Zou, D. Song, J. Liao, J. Huang, D. Guo, M. Lu, F. Liu, J. Liu, L. Li, C. Ma, X. Hu, R.C. Haydon, M.J. Lee, R.R. Reid, G.A. Ameer, L. Yang, T.C. He, A thermoresponsive polydiolcitrate-gelatin scaffold and delivery system mediates effective bone formation from BMP9-transduced mesenchymal
cells (ADSCs) contain multiple microRNAs and promote the migration and invasion of tumor cells, Cells 8 (2019) 298.

26. J. Zhang, L. Zhao, Z. Zeng, B. Feng, B. Zhang, Y. Yang, Z. Lei, Y. Zou, H. Feng, J. Fan, Transcriptional regulation by the 14 types of bone morphogenetic proteins (BMPs) in lineage-restricted mouse melanocyte progenitors, J. Investigative Dermatology 139 (2019) 2255–2267.

27. Y. Shu, K. Wu, Z. Zeng, S. Huang, X. Ji, C. Yuan, L. Zhang, W. Liu, B. Huang, Y. Lei, Y. Shu, Y. Zhu, C. Duan, J. Zhong et al., Bone morphogenetic protein-9 (BMP9) induces effective bone formation from reversibly immortalized multipotent adipose-derived mesenchymal stem cells, Am. J. Transl. Res. 8 (2016) 371–381.

28. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

29. Y. Shu, K. Wu, Z. Zeng, B. Feng, B. Zhang, Y. Yang, Z. Lei, Y. Zou, H. Feng, J. Fan, Transcriptional regulation by the 14 types of bone morphogenetic proteins (BMPs) in lineage-restricted mouse melanocyte progenitors, J. Investigative Dermatology 139 (2019) 2255–2267.

30. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

31. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

32. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

33. J. Fan, Q. Wei, L. Zhao, Y. Song, D. Dong, X. Chang, M. Xu, H. Xu, Q. Lu, L. Chen, L. Li, Y. Yu, Y. Zou, Z. Zeng, R. Zeng, S. Yang, Y. Xu, Y. Shu, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

34. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

35. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

36. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

37. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.

38. Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, R.R. Reid, M.J. Lee, W. Huang, J.M. Wolf, T.C. He, Y. Zou, Highly expressed BMP9/DF2 in postnatal mouse liver and lungs may account for its pleiotropic effects on stem cell differentiation, angiogenesis, tumor growth and metabolism, Genes Dis 7 (2020) 298–306. https://10.1039/c8gd01411c.
H. Qi, R.C. Haydon, H.H. Lui, T.C. He, L. Yang. Antibiotic monomer synergizes with EGFR inhibitors and oxaliplatin to suppress the proliferation of human ovarian carcinoma cells. Cancer Biol. Ther. 9 (2008) 1576–1584. https://10.4161/cbt.9.14.17599.

[45] Y. Xu, Y. Liu, F. Zeng, R. He, Z. Yang, S. Yan, Z. Shen, Y. Shi, C. Zhao, X. Wu, J. Lei, W. Zhang, C. Yang, K. Wu, Y. Wu, L. An, S. Huang, X. Ji, C. Gong, C. Yuan, L. Zhang, Y. Feng, B. Huang, W. Liu, B. Zhang, Z. Dai, X. Wang, B. Liu, R. C. Haydon, H.H. Lui, T.C. He, L. Chen, Nestin-positive cells exhibit potent antitumor activity and synergizes with sorafenib in human renal cell cancer cells. Cell. Physiol. Biochem. 48 (2019) 957–971. https://10.1159/000490140.

[46] Z. Liao, L. Huang, J. Zhang, J. Fan, F. He, X. Zhan, H. Wang, Q. Liu, D. Shi, N. Ni, W. Wagner, M. Pakvasa, F. Xu, A.B. Tucker, C. Chen, R.R. Reid, R.C. Haydon, H.H. Lui, L. Shen, H. Qi, T.C. He. The inhibition of BFA treatment sensitizes chemoresistant human ovarian cancer cells to paclitaxel-induced cytotoxicity and tumor growth inhibition. J. Transl. Res. 3 (2014) 100499.

[47] F. Zhang, Y. Li, H. Zhang, E. Huang, L. Gao, W. Luo, Q. Wei, J. Fan, D. Song, J. Jiao, Y. Zou, F. Liu, J. Liu, J. Huang, D. Guo, C. Ma, H. Xu, L. Li, Q. Chen, X. Yu, Z. Zhang, T. Wu, H.H. Lui, R.C. Haydon, J. Song, T.C. He. P. Ji, Anthemicel lentilis extracts elicits cisplatin’s effect on suppressing cell proliferation and promotes differentiation of head and neck squamous cell carcinoma (HNSCC). Oncotarget 8 (2017) 12968–12982. https://10.18632/oncotarget.22915.

[48] O. Cao, Y. Lei, Z. Ye, L. Zhao, H. Wang, J. Zhang, J. Fei, H. Huang, D. Shi, Q. Liu, N. Ni, M. Pakvasa, W. Wagner, X. Zhao, K. Fu, A.B. Tucker, C. Chen, R.R. Reid, R. C. Haydon, H.H. Lui, T.C. He. L. Ziao, Blockade of IGFR/IGF-1R signaling axis with soluble IGF-1R mutants suppresses the cell proliferation and tumor growth of human osteosarcoma. Am. J. Cancer Res. 10 (2020) 3248–3266.

[49] X. Hu, L. Li, X. Yu, B. Zhan, Z. Zeng, Y. Shen, Q. Zhao, X. Wu, J. Lei, Y. Li, W. Zhang, C. Yang, K. Wu, Y. Wu, L. An, S. Huang, X. Ji, C. Gong, C. Yuan, L. Zhang, W. Liu, B. Huang, Y. Feng, B. Zhang, R. C. Haydon, H.H. Lui, R.R. Reid, M.J. Lee, J.M. Wolf, Z. Yu, T.C. He. CRISPR/Cas9-mediated reversibly immunomodulated mouse tumor stem cells (BMSCs) retain multipotent features of mesenchymal stem cells (MSCs). Oncotarget 8 (2017) 11847–11865. https://10.18632/oncotarget.22915.

[50] N. Wang, W. Zhang, J. Cui, H. Huang, Z. Chen, R. Li, N. Wu, X. Chen, S. Wen, Z. Jiao, L. Yin, L. Jiang, J. Song, Z. Cao, W. Zhao, B. Liu, J. Shi, D. Zeng, W. Miao, H.H. Lui, R.C. Haydon, L.L. Shi, H. Liang, T.C. He. The piggybax transposon-mediated expression of SV40 T antigen efficiently immortalizes mouse embryonic fibroblasts (MEFs). PloS One 9 (2014), e97316.

[51] R. Li, W. Zhan, Z. Yan, W. Liu, J. Fan, Y. Feng, Z. Deng, C. Ocao, R.C. Haydon, H. H. Lui, Z.L. Deng, T.C. He, Y. Zou, Long non-coding RNA (LncRNA) HOTAIR regulates BMP9-induced osteogenic differentiation by targeting the proliferation of mesenchymal stem cells (MSCs). Aging (N Y) 13 (2021) 4199–4214. https://10.18632/aging.202384.

[52] W. Luo, L. Zhang, B. Huang, H. Zhang, Y. Zhang, F. Zhang, P. Liang, Q. Chen, Q. Cheng, D. Tan, Y. Jiao, S. Zhao, R.C. Haydon, R.R. Reid, H.H. Lui, M. J. Lee, M. El Daflawy, P. H. T. C. He, G. Liou, BMP-induced osteogenic/odontogenic differentiation of mouse tooth germ mesenchymal cells (TGMCs) requires Wnt/beta-catenin signalling activity, J. Cell. Mol. Med. 25 (2021) 2666–2678. https://10.1111/jcmm.15329.

[53] J. Liao, Q. Wei, Y. Zou, J. Fan, D. Song, J. Cui, W. Zhang, Y. Zou, C. Shi, M. Xu, X. Hu, X. Qu, L. Chen, X. Yu, Z. Zhang, C. Wang, C. Zeng, B. Zhang, S. Yan, T. Wu, X. Wu, Y. Shi, J. Lei, Y. Li, H.H. Lui, M.J. Lee, R.R. Reid, G.A. Amer, J.M. Wolf, T. C. He, W. Huang, X. Meng, Z. Zhang, R. C. Haydon, L.L. Shi, H. Liang, T. C. He, Wnt/beta-catenin promoting the osteogenesis-angiogenesis coupling process in mesenchymal stem cells (MSCs), Cell. Physiol. Biochem. 41 (2017) 1905–1923. https://10.1159/000479145.

[54] J. Liu, D.L. Deng, X. Luo, N. Tang, W.X. Song, J. Chen, K.A. Sharoff, H.H. Lui, R. C. Haydon, K.W. Kinzler, B. Vogelstein, T.C. He, A protocol for rapid generation of recombinant adenosine viruses using the AdEasy system, Nat. Protoc. 2 (2007) 1236–1247. https://10.1038/nprot.2007.152.

[55] B. Breyer, W. Jiang, H. Cheng, L. Zhou, R. Paul, T. Feng, T.C. He, Adenovirus vector-mediated gene transfer for human gene therapy, Curr. Gene Ther. 1 (2001) 149–162. https://10.1562/1552562013486689.

[56] C.S. Lee, E.S. Bishop, R. Zhang, X. Yu, E.M. Farina, S. Yan, C. Zhao, Z. Zheng, Y. Sun, X. Wu, J. Lei, Y. Li, W. Zhang, C. Yang, K. Wu, Y. Wu, S. He, A. Athirivaharam, M.J. Lee, J.M. Wolf, R.R. Reid, T.C. He, Adenovirus-mediated gene delivery: potential applications for gene and cell-based therapies in the new era of personalized medicine, Genes Dis 4 (2017) 43–63. https://10.7150/iemip.16295.

[57] Q. Peng, B. Chen, B. Huang, Y. Zhu, Y. Ju, Y. Luo, G. Zuo, J. Liu, L. Zhou, Q. Shi, Y. Weng, A. Huang, T.C. He, J. Fan, Bone morphogenetic protein 4 (BMP4) alleviates hepatic steatosis by increasing hepatic lipid turnover and inhibiting the mTORC1 signaling in alloxan-induced hepatocytes, Aging (N Y) 11 (2019) 11520–11540. https://10.18632/aging.102552.

[58] S.K. Denduluri, B. Scott, J.D. Lamplot, L. Yin, Y. Zhang, J. Ye, J. Wang, Q. Wei, M.K. Mohammed, R.C. Haydon, R.W. Kang, T.C. He, A. Athirivaharam, S. Hu, L. Shi, Immunized mouse achilles tenocytes demonstrate long-term potent anticancer activity and synergizes with sorafenib in human renal cell cancer cells, Cell. Physiol. Biochem. 48 (2019) 957–971. https://10.1159/000490140.

[59] Z. Liao, L. Huang, J. Zhang, J. Fan, F. He, X. Zhan, H. Wang, Q. Liu, D. Shi, N. Ni, W. Wagner, M. Pakvasa, F. Xu, A.B. Tucker, C. Chen, R.R. Reid, R.C. Haydon, H.H. Lui, L. Shen, H. Qi, T.C. He. The inhibition of BFA treatment sensitizes chemoresistant human ovarian cancer cells to paclitaxel-induced cytotoxicity and tumor growth inhibition. J. Transl. Res. 3 (2014) 100499.
culture, J. Dermatol. Sci. 71 (2013) 45–57. https://10.1016/j.jdermsci.2013.03.012.

[80] H.D. Zomer, A.G. Trentin, Skin wound healing in humans and mice: challenges in translational research, J. Dermatol. Sci. 90 (2018) 3–12. https://10.1016/j.jdermsci.2017.12.009.

[81] S. Werner, T. Krieg, H. Smola, Keratinocyte-fibroblast interactions in wound healing, J. Invest. Dermatol. 127 (2007) 998–1006. https://10.1097/j.id.0b013e31805078f0.

[82] M.I. Maqsood, M.M. Matin, A.R. Bahrami, M.M. Ghasroldasht, Immortality of cell lines: challenges and advantages of establishment, Cell Biol. Int. 37 (2013) 1038–1045. https://10.1002/cbin.10137.

[83] D. Song, F. Zhang, R.R. Reid, J. Ye, Q. Wei, J. Liao, Y. Zou, J. Fan, C. Ma, X. Hu, X. Qu, L. Chen, L. Li, Y. Yu, X. Yu, Z. Zhang, C. Zhao, Z. Zeng, Z. Zhang, S. Yan, T. Wu, X. Wu, Y. Shu, J. Lei, Y. Li, W. Zhang, J. Wang, M.J. Lee, J.M. Wolf, D. Huang, T.C. He, BMP9 induces osteogenesis and adipogenesis in the immortalized human cranial suture progenitors from the patent sutures of craniosynostosis patients, J. Cell Mol. Med. 21 (2017) 2782–2795. https://10.1111/jcmm.13193.

[84] J. Wang, J. Liao, F. Zhang, D. Song, M. Lu, J. Liu, Q. Wei, S. Tang, H. Liu, J. Fan, Y. Zou, D. Guo, J. Huang, F. Liu, C. Ma, X. Hu, L. Li, X. Qu, L. Chen, Y. Weng, M. J. Lee, T.C. He, R.R. Reid, J. Zhang, NELL-like molecule-1 (Nell1) is regulated by bone morphogenetic protein 9 (BMP9) and potentiates BMP9-induced osteogenic differentiation at the expense of adipogenesis in mesenchymal stem cells, Cell. Physiol. Biochem. 41 (2017) 484–500. https://10.1159/000456885.

[85] V.W. Wong, G.C. Gurtner, M.T. Longaker, Wound healing: a paradigm for regeneration, Mayo Clin. Proc. 88 (2013) 1022–1031. https://10.1016/j.mayocp.2013.04.012.

[86] C. Coraux, C. Hilmi, M. Rouleau, A. Spadafora, J. Himrasky, J.P. Ortonne, C. Dani, D. Aberdeen, Reconstituted skin from murine embryonic stem cells, Curr. Biol. 13 (2003) 849–853. https://10.1016/s0960-9822(03)00296-3.

[87] M. Ohyama, H. Okano, Promise of human induced pluripotent stem cells in skin regeneration and investigation, J. Invest. Dermatol. 134 (2014) 605–609. https://10.1038/jid.2013.376.

[88] D.S. Krause, N.D. Theise, M.I. Collector, O. Henegariu, S. Hwang, R. Gardner, S. Neutzel, S.J. Shanks, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell, Cell 105 (2001) 369–377. https://10.1002/cbic.10132.

[89] F.M. Wood, N. Giles, A. Stevenson, S. Rea, M. Fear, Characterisation of the cell suspension harvested from the dermal epidermal junction using a ReCell(R) kit, Burns 38 (2012) 44–51. https://10.1016/j.burns.2011.03.001.

[90] J.H. Holmes Iv, J.A. Molnar, J.E. Carter, J. Hwang, B.A. Cairns, B.T. King, D. J. Smith, C.W. Cruse, K.N. Foster, M.D. Peck, R. Stodd, M.J. Feldman, M.H. Jordan, D.W. Mozingo, D.G. Greenhalgh, T.L. Palmieri, J.A. Griswold, S. Dissanaike, W. H. Hickerson, A comparative study of the ReCell(R) Device and autologous split-thickness meshed skin graft in the treatment of acute burn injuries, J. Burn Care Res. 39 (2018) 694–702. https://10.1093/jbcr/iry029.

[91] C.S. Lee, E.S. Bishop, Z. Dumanian, C. Zhao, D. Song, F. Zhang, Y. Zhu, G.A. Ameer, T.C. He, R.R. Reid, Bone morphogenetic protein-9-stimulated adipocyte-derived mesenchymal progenitors entrapped in a thermoresponsive nanocomposite scaffold facilitate cranial defect repair, J. Craniofac. Surg. 30 (2019) 1915–1919. https://10.1097/SCS.0000000000005465.

[92] J. Xiao, S. Chen, J. Yi, H. Zhang, C.A. Ameer, A cooperative copper metal-organic framework-hydrogel system improves wound healing in diabetes, Adv. Funct. Mater. 27 (2017). https://10.1002/adfm.201604872.