



FULL LENGTH ARTICLE

Therapeutic values of chick early amniotic fluid (ceAF) that facilitates wound healing via potentiating a SASP-mediated transient senescence

Mashaal Ahmad ^{a,**,1}, Yandi Sun ^{a,1}, Xueyao Jia ^a, Jingjia Li ^a, Lihong Zhang ^a, Ze Yang ^a, Yindan Lin ^a, Xueyun Zhang ^a, Zara Ahmad Khan ^b, Jin Qian ^{c,***}, Yan Luo ^{a,*}

^a Department of Biochemistry and Cancer Institute of the Second Affiliated Hospital, Key Laboratory of Cancer Prevention and Intervention of China National MOE, Zhejiang University School of Medicine, Hangzhou, Zhejiang 310058, PR China

^b State Key Laboratory of Oncogenes and Related Genes, Institute for Personalized Medicine, School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200240, PR China

^c Zhejiang HygeianCells BioMedical Co. Ltd., Hangzhou, Zhejiang 310000, PR China

Received 30 November 2020; received in revised form 24 February 2021; accepted 12 March 2021
Available online 22 March 2021

KEYWORDS

Amniotic fluids;
Chick early embryo;
Cutaneous wound healing;
Senescence associated secretory phenotypes; (transient) Senescence

Abstract Inflammatory, proliferative and remodeling phases constitute a cutaneous wound healing program. Therapeutic applications and medication are available; however, they commonly are comprised of fortified preservatives that might prolong the healing process. Chick early amniotic fluids (ceAF) contain native therapeutic factors with balanced chemokines, cytokines and growth-related factors; their origins in principle dictate no existence of harmful agents that would otherwise hamper embryo development. Instead, they possess a spectrum of molecules driving expeditious mitotic divisions and possibly exerting other functions. Employing both *in vitro* and *in vivo* models, we examined ceAF's therapeutic potentials in wound healing and found intriguing involvement of transient senescence, known to be intimately intermingled with Senescence Associated Secretory Phenotypes (SASP) that function in addition to or in conjunction with ceAF to facilitate wound healing. In our cutaneous wound

* Corresponding author. No. 866 Yuhangtang Road, Xihu District, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang 310058, PR China.

** Corresponding author. No. 866 Yuhangtang Road, Xihu District, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang 310058, PR China.

*** Corresponding author. No. 63 Jiuhuan Road, Building 2C, 4th Floor, Jianggan District, Hangzhou, Zhejiang 310019, PR China.
E-mail addresses: 11618398@zju.edu.cn (M. Ahmad), qianjin@hygeiancells.com (J. Qian), luoyan2011@zju.edu.cn (Y. Luo).

Peer review under responsibility of Chongqing Medical University.

¹ These authors contributed equally.

healing models, a low dose of ceAF exhibited the best efficacies; however, higher doses attenuated the wound healing presumably by inducing p16 expression over a threshold. Our studies thus link an INK4/ARF locus-mediated signaling cascade to cutaneous wound healing, suggesting therapeutic potentials of ceAF exerting functions likely by driving transient senescence, expediting cellular proliferation, migration, and describing a homeostatic and balanced dosage strategy in medical intervention.

Copyright © 2021, Chongqing Medical University. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

The INK4-ARF locus comprises of tumor suppressor genes *ARF/INK4A(p16)/INK4B* that encode proteins with anti-proliferative functions exerted via RB and p53 mediated pathways.^{1,2} RB regulates the cell cycle downstream of the signaling with p16 and CDK4/CDK6, and ARF transduces a signal via MDM2, p53 and p21. Stress responses by which proliferative cells lose dividing potentials in an almost irreversible fashion are dubbed cellular senescence² exerted by diverse intra- and extra-cellular stimuli via activating the above pathways in a combinatorial manner.^{3–5}

In general, cellular senescence occurs in aging and tumor suppression; disparate biological processes as they may seem, there could be an evolutionary logic for such antagonist pleiotropy. Senescent cells mobilize a cohort of inflammatory cytokines, proteases, chemokines and growth factors known as senescence-associated secretory phenotypes (SASP),³ which can be induced upon activation of the INK4-ARF locus. This locus is epigenetically silenced in fetal, embryo and adult stem cells; however, it can be reprogrammed in differentiated cells to become hyper-responsive to mitogenic signals.⁶ Most senescent cells express p16⁷ that blocks cell cycle by inhibiting CDK4/CDK6⁸ hence making p16 a commonly recognized senescence marker.⁹ Pathologies aside, senescence also occurs in embryogenesis presumed to play roles in maintaining embryonic structures or regulating restructuring of organs.^{10,11}

In many species, embryonic amniotic fluids are a dynamic milieu that participate in, e.g., cushioning, hydrating and providing immunity to embryos. Recently, we isolated the soluble fraction (that could include embryonic stem cell-derived factors) of the chick early amniotic fluids (ceAF) of developing eggs at days 6–8 when embryos expeditiously, if not exponentially, grow, and found that ceAF comprises proliferation-stimulants that at a low-level support robust proliferation of *in vitro* cultured cells, almost as potently as fetal bovine serum (FBS; results). This characteristic and other intriguing ceAF activities beyond conventional growth factors prompted us to establish *in vitro* and *in vivo* cutaneous wound healing models to explore its therapeutic potentials and underlying mechanism(s).

We found that senescent fibroblasts and endothelial cells were enriched at wound areas a few days post skin injury and were more recruited by daubing ceAF to the wound areas, which at a low dose facilitated wound healing. The healing was likely to be mediated by SASP factors,

e.g., PDGF-B, TGF- β and VEGF known to promote myofibroblast differentiation, enhanced epithelial growth with more sebaceous glands and cellular layers. The ceAF components may function by signaling SASP factors as a marker for transient senescence that in turn promote faster cell migration and scar-less restoration by, in part, expediting cell proliferation. p16, reported to be able to enhance SASP factors release, was found to attenuate wound healing presumably because of an above-a-threshold expression level. Our studies also provided inkling that p21, rather than p16, could play roles in orchestrating a cutaneous wound healing program.

Materials and methods

Construction of Flag-1X-p16 and production of flag-tagged p16

PCR-amplified p16 cDNA was cloned into the pXJ40 vector between BamHI/XhoI sites downstream of and in-frame with a Flag-tag epitope to create the Flag-1X-p16 construct; p16 primers were listed in Table S2(B). HaCaT cells were transfected with this construct in a large scale, cell lysates made, Flag-tagged p16 immuno-enriched on M2-agarose beads, eluted with Flag peptide and quantified using BSA as a reference. The purity of p16 was validated by Coomassie Blue (CB) staining (Fig. S1).

Chick early amniotic fluid (ceAF) preparation

Fertilized chick eggs were incubated at $(38 \pm 1)^\circ\text{C}$ alongside 50% humidity, and ceAF was collected from eggs between days 6–8. After centrifugation of the samples at $2500\times g$ for 20 min, supernatants were filtered over a $0.22\ \mu\text{m}$ sterilization device (Millipore, USA) and stored in aliquots at -80°C after quick-freezing in liquid nitrogen.

Animals

Specific pathogen-free (SPF) C57BL/6 mice (all 5-week old males weighted at 20–26 g) were purchased from the SIPPR-BK Lab Animals Co. Ltd. Shanghai, China [Certificate # SCK (hu) 2013-0016]. Grouped randomly (3 mice/cage), kept in the Animal Facility, Zhejiang University (Permit #: ZJU20170013) with a 12-h day/light cycle, access to water and rodent chow. Animal procedures were approved by the institutional animal care use committee.

Surgical wound and related procedures

We strictly followed guidelines and protocols. Anesthesia (phentolamine; 8 mg/kg) was administered intraperitoneally.¹² In a stepwise manner, procedures were conducted aseptically as: a full-thickness wound (10 mm × 10 mm) was excised from the dorsum of mice after hair removal; excessive panniculus carnosus layer's contraction was controlled by silicon disks cut and adjusted to wound diameters; the disks were fixed by interrupted sutures with no dressing applied. Cocktails, in triplicates, with 0% (control), 5% or 10% ceAF and ± p16, were made in PBS/40% glycerol and sterilized by filtration. Each surgical wound was evenly/gently daubed with 250 µl of cocktail; 40% glycerol was used to stabilize proteins in, and enhance the viscosity of, the cocktails for easier topical daubing, which sustains a longer-lasting moist surface after daubing. Flag-tagged p16 or BSA at 1 ng/µl was employed when appropriate. Reduction in wound diameters were monitored daily until day 10 when animals were euthanized for histological and other analyses on excised healed/non-healed areas of wounds.

Histological procedures

Excised tissue samples were fixed in 10% formalin for minimally 5 days and processed for immunohistochemistry. Briefly, embedded in paraffin, samples were sectioned in 3 µm thickness, mounted on glass slides and, after deparaffinizing, stained with hematoxylin-eosin and Masson's trichrome. The density of inflammatory cells and blood vessels density in dermis were analyzed using M-42 system.¹³ Random fields of the tissue were observed and counted for particular sections with images taken by an optic microscope (Olympus, BX41).

Cell lines and other reagents

HaCaT cells, a gift from YU Faxing (Fudan University), were experimentally confirmed free of mycoplasma and assured to have normal morphologies. Cells were cultured in DMEM (Basal Formula with 1% penicillin-streptomycin [GIBCO-Life Technologies]) supplemented with 10% FBS (Capricorn, FSS500, Uruguay) or indicated doses of ceAF. Other reagents included anti-p16 (#80772), anti-RANTES (CCL-5) (#2989), anti-PDGF- α (#3174) antibodies and Senescence Activated β -Galactosidase Staining Kit (#9860) [Cell Signaling Technology, Danvers, MA]. Anti-p21 (#sc-6246) was purchased from Santa Cruz-Biotechnology (California, USA). Primers for qPCR were from Shanghai Generay Biotech, China.

cDNA preparation and qPCR

cDNA was prepared from isolated total RNA using a cDNA preparation kit. RNA was extracted from wounded tissue samples as well as from cell lysate. From tissues, sonication method with RNA extraction kit was used as mentioned in the protocol.¹⁴ For cells, RNase free double distilled water, 4× gDNA wiper mix and 1 µg total RNA were sequentially pipetted into a tube and incubated at 42 °C for 2 min. Then

5× Hi Script Mix II was added and the samples kept at 50 °C for 15 min and 85 °C for 5 sec before proceeding to qPCR. Information of PCR primers is in Table S2(B).

Cell viability assay

Cell viability was generally assessed in 96-well format by Cell Counting Kit-8 (CCK-8). When added into cultured cells, WCT-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)] is converted by the reducing environment of viable cells and turned into orange (WST-8-formazan) in color. Briefly, 10 µl of CCK-8 solution was added in each well of the plate and incubated in the dark for 1–4 h. The absorbance at 450 nm was measured using microplate reader. The readings were measured at 24, 48, 72, 96 and 120 h post starvation and treating cells with ceAF.

Cell scratch assay

HaCaT cells were cultured in 6-well plates to attain an ~80% confluence, and starved for 24 h. Cells were grouped in triplicates for transfection: DMEM with no ceAF, i.e., control, or 5%/10% ceAF, and with or without (+/–) the p16 plasmid. The vector plasmid was added to the samples without p16. After 24 h, scratches were drawn with a 10 µl pipette-tip. The cell migration rates at the “wounded” sites were recorded at 0, 24 and 48 h.

Western blotting

Aspirated from media, cells were later rinsed with PBS twice and lysed with HEPES lysis buffer (115 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 2.4 mM K₂HPO₄, 20 mM Hepes-KOH, pH 7.0, 1% NP40) supplemented with protease and phosphatase inhibitors. Proteins levels were normalized for SDS-PAGE loading, and protein expression further normalized with an internal control (β -actin). Signals were detected by primary antibodies followed by HRP-conjugated secondary antibodies.

SA- β -gal staining

Solutions were provided by the kit; 48 h after treatment, cells were rinsed with PBS twice and 1 ml fixation solution was added to fix the cells for 15 min at room temperature. Cells were rinsed again with PBS and 1 ml β -gal solution added to develop the enzyme activity. The plates were sealed with paraffin to prevent evaporation and kept in an incubator at 37 °C without CO₂ until color manifestation. Readings and measurements were taken after 48, 96 and 144 h post seeding/staining the cells with SA- β -Gal.

Trans-well migration assay

Cells were collected/suspended at a density of 3×10^4 cells in 200 µl serum-free medium after transfection with or without p16-expressing plasmid for 24 h, and seeded in the upper chamber. Media with indicated doses of ceAF (600 µl in total) were suspended in the lower chamber. After 24 h, chambers were separated and the cells on the upper

surface of membrane wiped off with cotton buds. Cells that invaded into the microporous membrane (8 μm in diameter) were rinsed with PBS for three times, fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet. Number of cells that trans-well-migrated was observed/counted/recorded by a microscope (Olympus BX51).

Cell cycle analysis

Cells were suspended at $1\text{--}2 \times 10^6$ cells/ml, transferred into 5 ml polystyrene tubes and washed with ice-cold PBS. After centrifugation at 1000 rpm for 5 min at 4 °C, cells were fixed by drop-wise ice-cold 80% ethanol while being mixed by vortex; the fixation continued at 4 °C for 3 h. Cells were washed twice with PBS, centrifuged at 3000 rpm for 5 min at 4 °C, and re-suspended in 200 μl of propidium iodide (Sigma–Aldrich) supplemented with 30 $\mu\text{g/ml}$ RNase A for 30 min before flow cytometry.

Statistical analyses

Numerical data were expressed as mean \pm SEM and statistical analysis was performed using Prism GraphPad (California). One-way ANOVA for ungrouped data and Two-way ANOVA for grouped and time interval depending experiments were used to reveal significance. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ were considered statistically significant. All *in vitro* experiments were independently repeated in triplicates.

Results

ceAF supports cell growth and promotes wound healing *in vitro*

We believed that ceAF comprised biologically balanced secretory growth factors, cytokines and other ligands collectively contain proliferation-stimulants and, as a precursor to exploring its therapeutic potential, we tested its ability to support the growth of cultured HaCaT cells using FBS as a control. Starting from ~20% confluence, cells were cultured in DMEM supplemented with increments of ceAF (0–20%, v/v) or 10% FBS for 48 h; 5% ceAF optimally, and almost as potently as 10% FBS, supported cell growth that roughly underwent 2 doubling times (Fig. 1A, B). Given that cultured HaCaT cells exhibited elevated p16 expression in higher doses of ceAF (20% in particular; Fig. 1B), we proposed that the lower efficacies of higher ceAF doses, as compared to 5% ceAF, for supporting cell growth (Fig. 1B) were attributable to an anti-proliferative function of p16. This suggests a manifestation of attenuating activities with higher doses of ceAF and emphasizes an importance of balanced (or optimal) dose of ceAF in its application on wound healing. We also quantified this proliferation using a CCK-8 assay to test if ceAF harbors promising components that support cell growth. As seen, 5% ceAF (v/v) turned out to be the optimum dosage for cell growth and their viability when compared with control groups (Fig. 1B).

Prompted by the above, we established an *in vitro* wound healing model utilizing HaCaT cells, representing an epidermal origin¹⁵; given fastidious nature of ceAF, we employed 5% and 10% doses in our later efforts on the presumption that one or a dose somewhere in between works optimally. Scratches were made on near-confluent monolayers and cells treated with ceAF. Agreeing with cell proliferation assays, 5% ceAF healed the scratches better than 10% ceAF over examined periods, and ectopic expression of p16 hindered the healing *in vitro* (Fig. 1C, D; also see below).

Encouraged by the above results, we examined healing capacities of ceAF on surgically-made cutaneous wounds in a murine model. Mice treated with 5% ceAF healed expeditiously, manifesting virtually scar-less skin within 10 days (Fig. 2A, B). Dermal application of naturally produced and topically administered exogenous p16 has been a practice in wound healing research, given that this small-size protein can be up-taken by G-protein coupled receptors.^{16,17} But, in our study, p16 when co-applied in a dosage employed by other practices significantly attenuated wound healing, offsetting the action facilitated by ceAF particularly at a 5% dose (Fig. 2A, B). This is consistent with results of the *in vitro* model (Fig. 1C, D).

p16 impedes wound healing *in vitro* and *in vivo*

Under stresses such as those that induce senescence, p16 protein expression is up-regulated; p16 was reported to be able to drive senescence-associated secretory phenotypes (SASP) that provoke chemokine and cytokine pathways in the inflammatory phase of wound healing precluding the proliferative phase. SASP factors exert functions transiently, but whether this transient senescence is p16-dependent remains largely unresolved. To examine whether p16 expression in conjunction with ceAF facilitates wound healing, we ectopically expressed Flag-tagged p16 in HaCaT cells supplemented with either 5% or 10% ceAF; p16 unexpectedly and markedly retarded the closure of scratched areas (Fig. 1C, D) and cellular proliferation (seen below). We had serially titrated down p16-expressing plasmid in transfection assays, and were unable to identify a dose in which ectopic p16 expression would additively or synergistically facilitate wound healing by ceAF (data not shown). In the animal models, similar patterns were observed whereby wound healing was attenuated when p16 was co-daubed with ceAF (Fig. 2A, B). Taken together, these results suggest that, in our assay systems, the signaling involving SASP factors and transient senescence is not strictly dependent on p16. Other pathway(s) such as, notably, a p21-mediated pathway may compensate for and/or play redundant roles in this program (seen below).

ceAF facilitates wound healing *in vivo* and manifests increased collagen density and thickened epidermis on healed wounds

On day 10, we observed prominent contraction on the ceAF-daubed wounds particularly with a 5% dosage, and significant re-epithelialization (Fig. 2A) [significance of 5% ceAF as compared to other groups summarized in Table S1(A)].

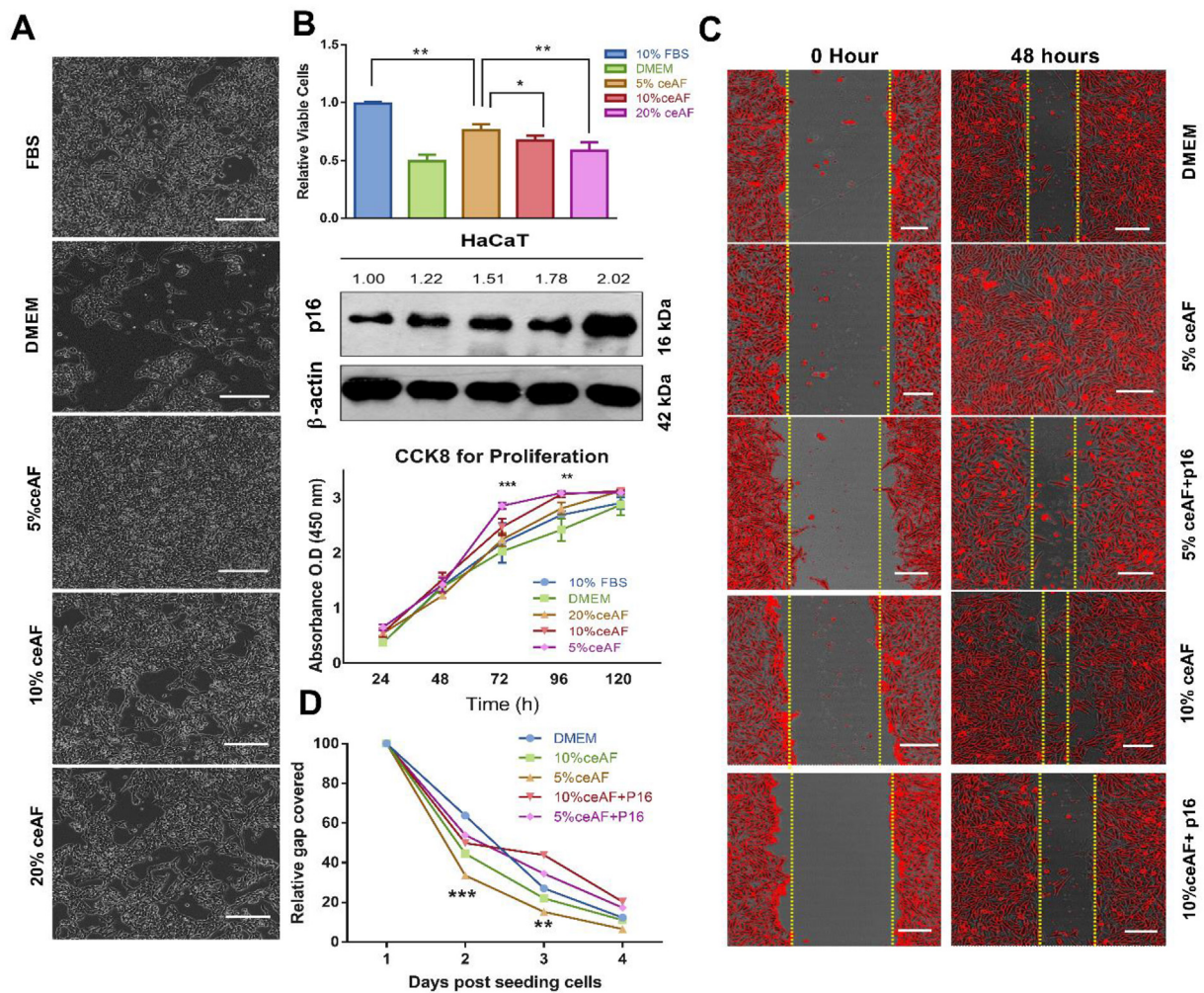


Figure 1 ceAF supports cell growth and promotes wound healing *in vitro*. (A) Snapshots of a typical cell proliferation assay with increasing ceAF concentrations (0, 5%, 10% and 20%) comparing efficacies among doses and with 10% FBS to support the growth of HaCaT cells (scale bar at 100 μ m). Images taken 48 h post treatment. (B) Quantification of relative number of viable cells post treatment with ceAF vs. FBS (upper panel); CCK-8 assay for determining the proliferation of cells using different fractions of ceAF (bottom panel); protein expression of endogenous p16 induced by higher doses of ceAF (middle panel), from left to right (10% FBS, DMEM only, 5% ceAF, 10% ceAF and 20% ceAF). (C) Wound healing scratch assay on confluent/24-h serum-starved HaCaT cells incubated with DMEM, or DMEM supplemented with 5% or 10% ceAF and $-/+$ ectopic p16 expression. Phase-contrast micrographs were taken at the wound areas measuring gap closures at 0 or 48 h post scratch formation as indicated on top (scale bar at 100 μ m). (D) Healing quantification as assessed by measuring the reduced gap distance within the specified time frames. Experiment was repeated at least three times.

However, mice treated with ceAF+p16 exhibited wounds with wide epithelial and dermal edges with the gap comprising necrotic fibrinoid, cell debris, intense inflammation and angiogenesis plus reduced granulation tissues and significant lower deposition of collagen as judged by the trichrome staining (Fig. 2B), suggesting a wound healing that was hampered by p16. Furthermore, histological (H&E) staining showed that, in the 5% ceAF group, wound sections showed a neoformative epidermal layer that was remarkably thicker (Fig. 2C), indicating dense epidermal ridges, sebaceous glands, cellular layers and more formation of primitive hair follicle structures. On the contrary, the ceAF+p16 group wounds exhibited much lower migration of endothelial cells and fibroblasts as well as significantly reduced epidermal

thickness of the skin with impaired granulation tissues (Fig. 2C), again indicating a wound healing hampered by p16.

ceAF facilitates an S to G2/M transition and cell migration that is offset by p16

To test whether the healing process facilitated by ceAF and attenuated by p16 expression or administration (Fig. 1, 2) were related to cell cycle progression, we cultured HaCaT cells with ceAF (5% or 10%) with or without ectopic p16 expression and subjected cells to FACS analyses. Cells in the 5% ceAF group exhibited highest percentile of G2/M cells, but p16 expression on top of that retarded quite some

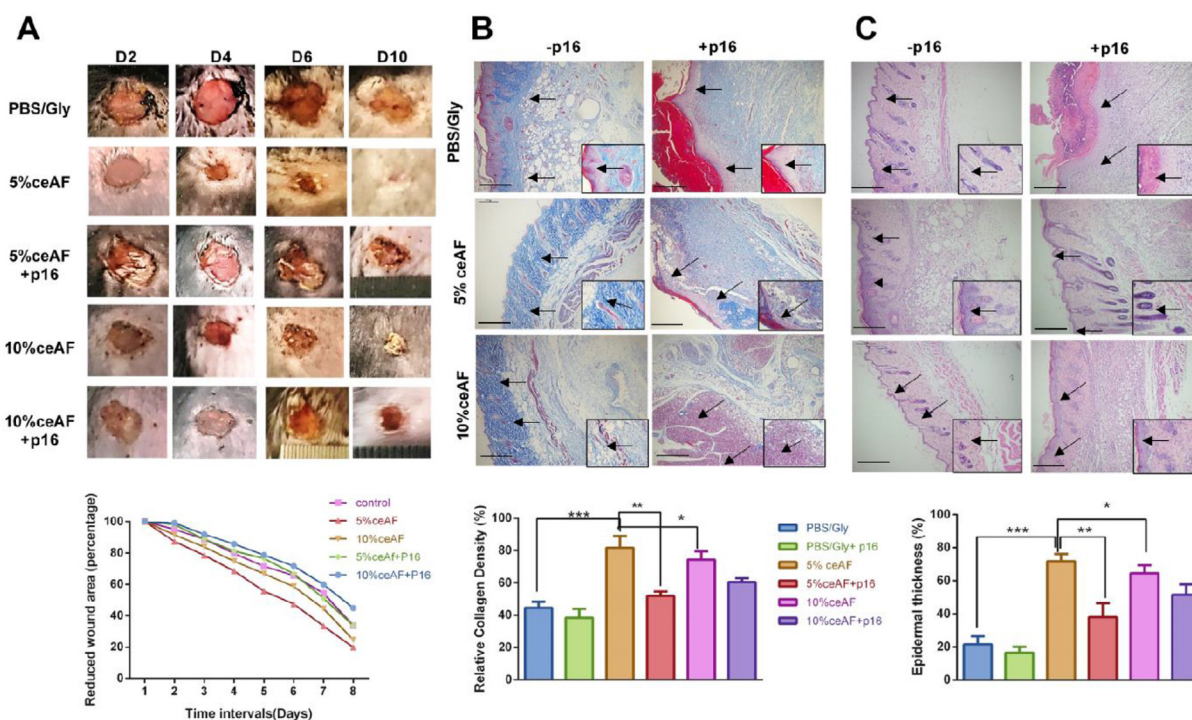


Figure 2 In an animal model, ceAF facilitates wound healing (A), increases the collagen density (B) and enlarges epidermis thickness (C). (A) Macroscopic appearance of representative groups of cutaneous wounds pre- and post-treatment with ceAF with or without recombinant p16. (PBS/Gly stands for PBS/40%, glycerol was used to reconstitute the cocktails). The pictures (upper panel) were taken on indicated days post-wounding. The graph (bottom panel) is quantification of wound closure rates that were measured on a daily basis. Wound areas determined using image analysis and expressed as the percentage of wound area immediately post-injury as described in methods ($n = 3$ mice/group). Statistical significance was evaluated with a one-way ANOVA with Bonferroni post-test. (B, C) Masson's Trichrome (B) and H&E (C) stained punch biopsy wound tissue sections of day 10 post wounding with 10-mm punch. Arrows represent relative number of collagen fibers in (B) and edges of healed/non-healed epidermis in (C). Scale bar represents 100 μm . The bottom two panels show quantifications of collagen deposition (density) [left] and epidermal thickness [right]. The 5% ceAF-treated and to a lesser extent the 10% ceAF-treated samples exhibit smaller number of inflammatory cells, more collagen deposition, thickened epidermis and a larger population of fibroblasts, indicative of facilitated/more complete wound healing. These indicators are impeded by co-application of p16; e.g., a larger population of acute inflammatory cells with inflammatory exudate on the surface, in line with a persisting/prolonged inflammatory response.

cells in the S-phase that had obvious difficulty to enter the G2 phase (Fig. 3A), suggesting that the wound-healing-promoting (by ceAF) and the wound-healing-attenuating (by p16 expression/administration) phenotypes (Fig. 1, 2) are at least in part due to their functions exerted on cell cycle progression.

Cell migration also plays important roles in wound healing^{18–20}; we performed trans-well migration assays using HaCaT cells. The 5% ceAF-treated group exhibited a high number of trans-well-passed cells; likewise, and consistent with the cell cycle results (Fig. 3A), expressing p16 on top of ceAF significantly attenuated trans-well passaging (Fig. 3B).

ceAF likely triggers transient senescence

Senescence is generally recognized to be a chronic mechanism associated with, e.g., age-related pathologies; but tissue damages such as skin wound can induce, albeit transiently, senescence dubbed transient senescence.¹⁷ This might be an evolutionarily acquired mechanism. Immune

surveillance later clears the transiently senescent cells, which manifest certain levels of Senescence Activated β -galactosidase (SA- β -gal) activity that can be detected by histochemical staining, scoring both numbers of positive cells and intensities. Thus, albeit controversial, likely SA- β -gal is a biomarker for senescent cells in both cell culture and *in vivo*.

We stained the SA- β -gal activities in experimental groups and examined the sustainability of cells that were β -gal positive in a time course. The β -gal signal accumulated in the 5% ceAF group gradually declined after 4 days, which became more evident after 8 days suggesting a transient senescence induced by ceAF; however, the p16-expressing groups accumulated more sustainable β -gal signals in that positive cells persisted at day 8, which suggests a more permanent senescence consistent with more absorbed signals (Fig. 4A, B). Because the 5% ceAF dosage exerted optimal functions (Fig. 1–3), a high likelihood is that a transient senescence as implied here plays role(s) mediating cutaneous wound healing.

To gain knowledge of potential transient senescent marker(s) as implied by the SA- β -gal staining, we carried

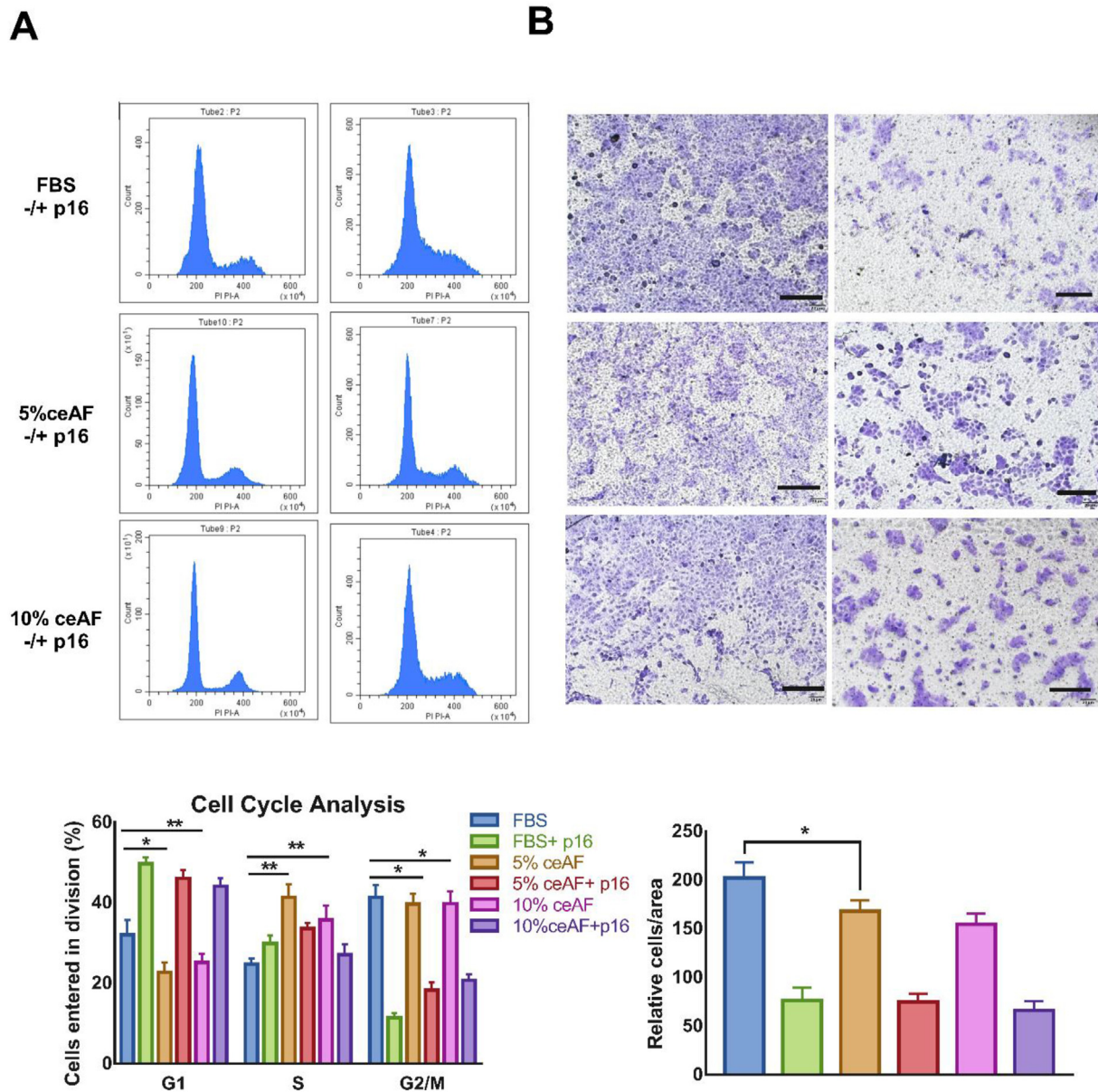


Figure 3 ceAF facilitates S to G2/M transition and cell migration that are hampered by p16. **(A)** Cell cycle profiles of HaCaT cells cultured with FBS vs. 5%/10% ceAF without or with (-/+) p16 ectopic expression analyzed post 24-h incubation. The bottom panel shows percentiles of cells at G2/M. ($n = 3$). **(B)** Trans-well migration assay for cell groups as in **(A)**. (Scale bar at 20 μm). The bottom panel shows the quantification of cells per field. The error bars represent standard deviation of three different experiments.

out immuno-blot analyses of the p16 and p21 protein expression in cells that were treated as in the β -gal assay (4 days). We found that, while the relative expression of p16 was not significantly effected by 10% FBS versus 5/10% ceAF (Fig. 4C, lanes 1,3,5; the increased signals in other lanes were due to p16 ectopic expression), the expression of p21 was induced by 5/10% ceAF (as compared with 10% FBS), which was offset by p16 overexpression (Fig. 4C 1st panel that was additionally and internally controlled by the beta-actin in 2nd panel). Thus, in our system, as far as the

transient senescence is concerned, the additional (potential) marker aside from the SA- β -gal is more-likely p21 (also seen below); however, we do not rule out a possibility that a threshold expression of p16 (e.g., the ~1.5-fold stimulation in Fig. 1B middle panel by 5% ceAF) might also be a transient senescence marker involved in an optimal wound healing program. Likewise, the p16-attenuated wound healing is presumably as a result of more permanent senescence because of an above-a-threshold expression level (e.g., >2-fold stimulation seen in Fig. 1B middle panel

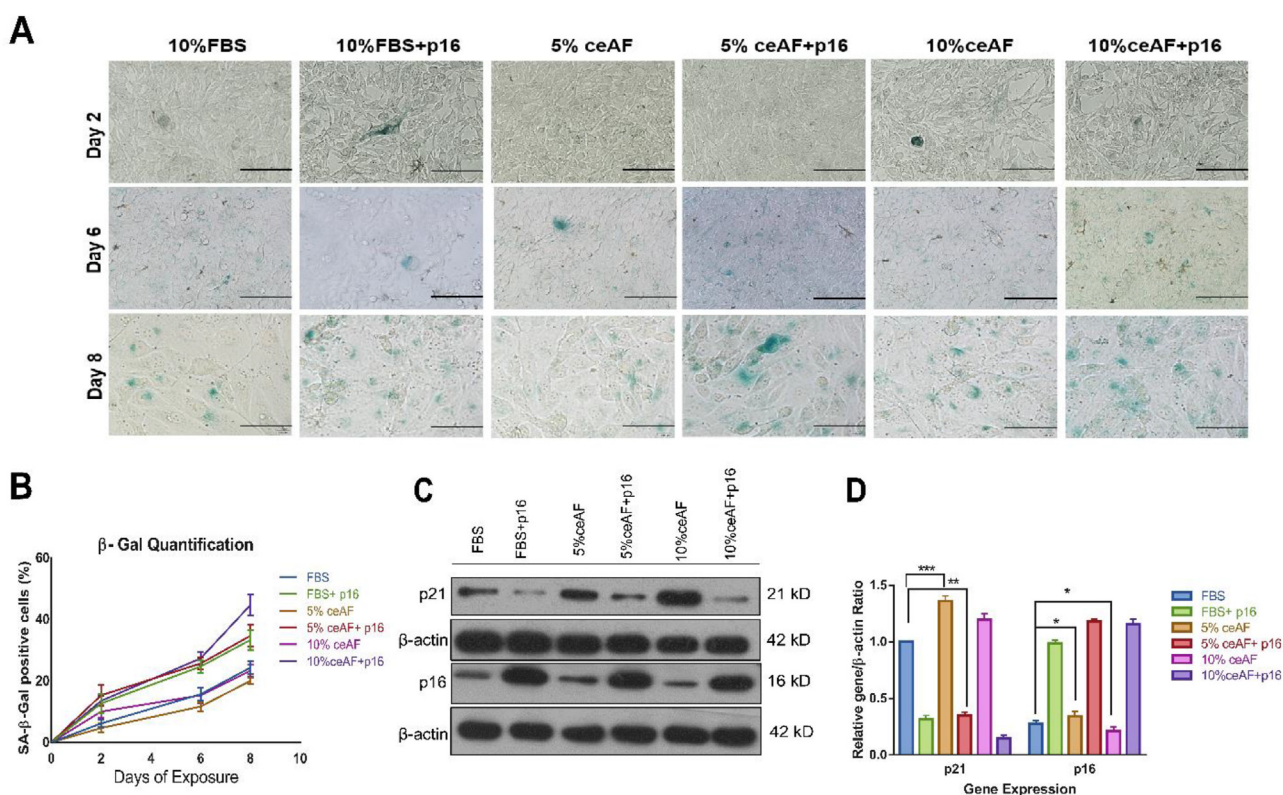


Figure 4 Transient senescence plays a significant role mediating the wound-healing effects of ceAF. Role of transient senescence was verified by observing cells in a time course. SA- β -Gal activity in HaCaT cells treated as indicated for 48 h and maintained in fresh media for 2 days. Micrographs were obtained with phase contrast microscopy. (A) Micrographs of SA- β -Gal⁺ cells in different groups as marked and taken at different time intervals (scale bar represents 20 μ m). (B) Quantification of β -gal positive cells among different groups plotted against days of exposure. (C) Immunoblots assessing the protein expression levels of p16 and p21 in HaCaT cells treated with 10% FBS versus 5/10% ceAF plus/minus p16 ectopic expression, which was doubly and internally controlled by β -actin. (D) The p21 and p16 protein expression levels in C were quantified using that of β -actin as an internal control.

by 20% ceAF), underscoring the therapeutic values with ceAF at homeostatic level(s).

Expression profiling of factors involved in cutaneous wound healing

Inflammation, proliferation and remodeling are three sequential yet overlapped phases of wound healing; upon wounding and thereafter, diverse chemokines, cytokines and growth factors are locally enriched and distantly attracted at wound sites, a signaling cascade triggered by SASP factors transiently released by transient senescent cells. They in turn sequentially or concomitantly orchestrate a healing program. For instance, involving diverse kinds of cells (e.g., epidermal and endothelial cells, fibroblasts, keratinocytes, neutrophils etc.), these SASP factors comprise a cohort of chemokines that recruit pro-inflammatory cells, including macrophages, which release additional cytokines and growth factors to facilitate wound healing.^{21,22}

We employed wounded and repaired skin tissues from animals (*in vivo* samples) and HaCaT cells (*in vitro* samples) treated under specified conditions and built an expression profile of cellular factors as afore-emphasized to be involved in tissue repair. *In vivo* samples (collected at day

10) were used to examine the expression pattern of genes encoding proteins that dictate the integrity of restored skin boundary, i.e., degree of remodeling. As seen (Fig. 5A, quantification), mRNA expression levels of three tested genes, i.e., that of keratin, keratin-10 and endothelin, were significantly up-regulated in the 5% ceAF group (to a lesser degree in the 10% ceAF group); p16 played an offset role (the control was the wound daubed with PBS/40% glycerol). In conjunction with the data shown below, we propose that ceAF plays multi-complex roles to facilitate wound healing.

We also screened an expression profile of 12 of ~25 SASP factors reported^{23,24} using HaCaT cells incubated with FBS versus ceAF, with or without ectopic p16 expression, at the mRNA (for all 12) and protein (for 3 out of 12) levels. The mRNA levels of tested SASP factors, save p16, were up-regulated significantly in cells incubated in 5% ceAF and to a lesser degree 10% ceAF (Fig. 5A, quantifications normalized against a house-keeping gene). Fig. 5B is a Heatmap that exhibits mRNA expression patterns of the 15 above-mentioned proteins. Also, as seen, p16 played offset roles on ceAF-enhanced expression of genes (Fig. 5A, B); the ostensible variations of p16 expression per se (Fig. 5A, last panel) were most-likely due to the presence or absence of the ectopic p16 gene. Of 12 tested SASP

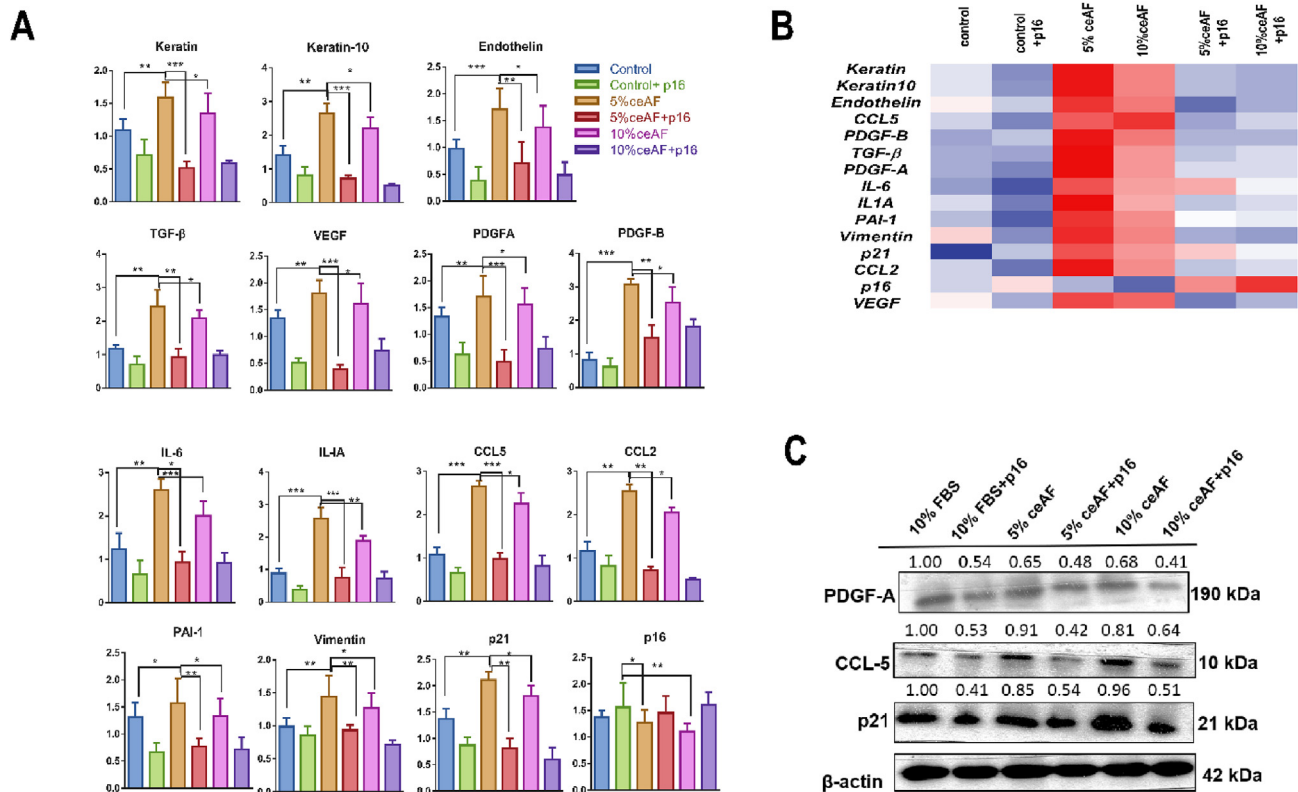


Figure 5 SASP factors mediate and contribute to facilitating the cutaneous wound healing and transient senescence. (A) Quantitative real-time PCR for expression of wound healing, tissue integrity and inflammatory markers of either wounded skin samples of mice (endothelin, keratin and Keratin-10) or HaCaT cells (other factors). The qRT-PCR analyses were carried out to determine the gene expression patterns of wound healing markers that include PAI1, p21, p16, endothelin, keratin and Keratin-10, and of SASP factors that include CCL5, PDGF-B, VEGF, PDGF- α , TGF- β , IL-1A and IL-6. The experiments were conducted in triplicates. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (B) Heatmap analyses for qRT-PCR of factors as listed in (A). (C) Western blot of 3 representative SASP factors validating the mRNA expression data in (A) and (B) by quantifying their protein expression levels. The densitometry analysis represents normalization of band intensities as compared with that of β -actin as an internal control.

factors, we selected 3 proteins that represent chemokines (CCL-5), growth factors (PDGF- α) and a cell cycle regulator/SASP-factor mediator, p21, to examine the protein expression levels (Fig. 5C; Immuno-blot). The results are consistent with that for mRNA expression (Fig. 5A, B); for p21, the protein expression patterns were also consistent with the results exhibited in Figure 4C.

Discussion

Amniotic fluids of diverse species contain critical factors for fetal development; many researchers have revealed that, in conjunction with other interventional methods and other specific properties of fetal tissues, amniotic fluids were able to facilitate wound healing.²⁵ Indeed, amniotic fluids from mice²⁶ or sheep and horses^{27,28} at varying embryonic stages, or even that of human at different trimesters,^{25,28–30} were employed to unravel their applications for diverse therapeutic interventions, e.g., fetal wound healing, corneal wounds regeneration, diabetic impaired healing of wounds. However, the precise mechanisms are still not yet adequately understood. Our, albeit preliminary, exploration on mechanisms aside, the unique

advantage of the present studies is that we employed poultry eggs for isolation of amniotic fluids, which can be produced at industrial scale, is very economic, ethical and safe, and feasible for in-depth mechanistic studies and pre-clinical testing. Further, in contrast to other studies, we used amniotic fluids at an early stage of chick embryogenesis (6–8 days post incubation) shortly after the kidney development initiates. In a developmental biology term, this is a stage at which embryos grow almost most expeditiously with ample amounts of extractable amniotic fluids yet before a drastic up-shift of the ratio, between abundance of wasteful metabolites and excretions and that of the amniotic growth factors, takes place following development of diverse organs. In fact, after kidneys develop at certain length, e.g., beyond 8 days post incubation, amniotic fluids increasingly comprise of significant amounts of urine in conjunction with declining abundance of embryonic active antigens, growth factors and potentially other active components.

Intrinsic to and secreted by developing chick embryos 6–8 days post incubation, ceAF exhibits powerful wound healing capacities as apparent in mammalian cutaneous wound healing models in which ceAF with no preservatives or fortification displays potent efficacy both *in vitro* and

in vivo. Naturally, ceAF provides nourishment and protection against environmental stresses including pathogens, and is thought to possess diverse signaling molecules at homeostatic levels to support embryogenesis. This view is especially important given a manifestation of the attenuating activities at high doses (Fig. 1B). Notably, given that the control for establishing the expression profiling with culture cells (Fig. 5A–C) was FBS, ceAF must contain components above and beyond ordinary growth factors and/or other molecules that solely support cell growth and migration as revealed in Figure 1A, 1B and 3B. Recently, occurrence of senescence and roles of SASP factors during embryogenesis³¹ and in limiting fibrosis upon tissue injury^{32,33} were reported. Thus, scar-less tissue repair might take place in embryos, supporting our postulation that ceAF comprises a set of evolutionarily conserved, at least among vertebrates, factors involved in wound healing.

Structural proteins

For normal skin physiology, the epithelial keratinization is of fundamental importance for counteracting mechanical stress and protecting against pathogenic invasion, and endothelin was suggested to be involved in anabolism of collagen and related proteins.^{34,35} In addition, endothelin is a mediator for morphogenesis in other systems including bone regeneration and skeleton formation.³⁶ Thus, elevated expression levels of keratin, keratin-10 and endothelin in the cutaneous wound healing model reflect a more completed remodeling phase.

Interleukins, chemokines and growth factors

Interleukins serve key modulators for inflammatory responses including that in skin repair, and is involved in activation, differentiation and proliferation of endothelial cells, fibroblasts, keratinocytes and leukocytes.³⁷ For instance, IL-6 is released as an early response to tissue injury to induce a signaling of pro-inflammatory cytokines from the resident macrophages and stromal cells.³⁸ In addition, IL-1A is an autocrine regulator for basal keratinocytes proliferation inside the bulge region of hair follicles and an integral agent of the epidermal stem cell population³⁹; chemokines CCL5/CCL2 can function as attractants for local and distant macrophages to be enriched at wound sites. Furthermore, growth factors such as TGF- β , VEGF, PDGFA/B can regulate direct (cell–cell) and indirect (paracrine) linkage between different cell types and cellular basement and, more relevant to this work, are involved in restoring skin boundary integrity. Therefore, these factors conceivably function at different healing stages covering the inflammatory and proliferative phases and possibly even the remodeling phase. Emphasizing important roles on wound healing, the expression of these factors is quite prominently up-regulated in ceAF-treated cells and offset by p16 (Fig. 5).

PAI-1 and vimentin

Plasminogen activator inhibitor type 1 (PAI-1) is expressed on the surface of keratinocytes, its activation enhances

cellular proliferation.²¹ PAI-1 limits plasmin generation to sustain cell migration and proliferation, a key indicator for re-epithelialization of keratinocytes.⁴⁰ The intermediate filament protein vimentin plays significant role for the “epithelial to mesenchymal transition (EMT)”, and studies reveal that vimentin participates in a number of cellular processes including migration and invasion of cells, cell adhesion, cytoskeletal rearrangements, signaling, plasticity and regulation of cell morphology.^{41,42} These proteins in principle are largely involved in the remodeling phase of a wound healing program.

Cell cycle/senescence regulators

Both p16 and p21 are proteins known to regulate cell cycle and senescence^{43,44}; in our assay systems and as opposed to studies by others, p16 is unlikely playing a role in establishing a transient senescence that readies wounds to undergo healing. From all criteria, p16 is actually an offsetting factor for therapeutic values of ceAF. Our data (Fig. 4, 5) support that p21 might play compensatory role(s) by itself or in conjunction with other factors, e.g., a non-redundant function afforded by another component of the INK4-ARF locus, INK4B.

Conclusion

Conceivably, as in many a signaling cascade, signals from ceAF, however chick-early-embryo-derived, obviously can be received and interpreted by cells in the wound and surrounding areas of a murine model to facilitate wound healing; a potentially involved transient senescence is probably marked by upregulated p21 expression rather than that of p16 as conventionally thought. A few challenges, e.g., efforts to rid of the attenuating activities (Fig. 1B) and enrich fastidious active components by biochemical fractionation and reconstitution, remain. Despite these challenges, however, endeavors on exploring ceAF potentials constitute a worthy goal both for basic research and therapeutic development.

Author contributions

AM, YS, JQ and YL jointly conceived the project; YL guided AM and YS to design and carry out the experiments, during which XJ, JL, LZ, ZY, YDL, XZ and KZA provided technical help and were involved in bioinformatics and statistical analyses; AM and YL co-wrote the paper, in which YS and JQ helped in editing and figures.

Conflict of interests

JQ is a lead applicant of a series of patents on therapeutic potentials of ceAF on diverse medical conditions including cutaneous wound. JQ and YL are shareholders of Zhejiang HygeianCells BioMedical Co. Ltd. Authors otherwise declare no any other conflict of interest with anyone.

Acknowledgements

Zhejiang Hygeian Cells BioMedical Co. Ltd., Hangzhou, China provided ceAF, other materials and financially supported in part, the project. AM was a recipient of a PhD scholarship from Zhejiang University (Sept. 2016 to Mar. 2020). The help from Jessica Hennacy (of Princeton University) in English polishing is gratefully appreciated.

Abbreviations

| | |
|--------------|--|
| ceAF | chick early amniotic fluid |
| SASP | senescence associated secretory phenotypes |
| RB | retinoblastoma |
| CDK4/6 | cyclin dependent kinase 4/6 |
| PDGF | platelet derived growth factor |
| TGF- β | transforming growth factor- β |
| VEGF | vascular endothelial growth factor |
| IL | interleukin |
| CCL-5/2 | chemokine C–C motif-5/2 |
| PAI-1 | plasminogen activator inhibitor type 1 |

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2021.03.003>.

References

- Sherr CJ. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol.* 2001;2(10):731–737.
- Ross CH. *Serial Cultivation of Human Diploid Cells in the Lab (1958–1961) by Leonard Hayflick and Paul S. Moorhead.* Embryo Project Encyclopedia; 2017.
- Burkhardt DL, Sage J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer.* 2008;8(9):671–682.
- Vousden KH, Prives C. Blinded by the light: the growing complexity of p53. *Cell.* 2009;137(3):413–431.
- McCarthy N. Self-renewal migrates onwards. *Nat Rev Cancer.* 2010;10:6–7.
- Sherr CJ. Ink4-Arf locus in cancer and aging. *Wiley Interdiscipl Rev Dev Biol.* 2012;1(5):731–741.
- Ohtani N. Deciphering the mechanism for induction of senescence-associated secretory phenotype (SASP) and its role in ageing and cancer development. *J Biochem.* 2019;166(4):289–295.
- Mohan A, Asakura A. CDK inhibitors for muscle stem cell differentiation and self-renewal. *J Phys Fit Sports Med.* 2017;6(2):65–74.
- Kim KM, Noh JH, Bodogai M, et al. SCAMP4 enhances the senescent cell secretome. *Genes Dev.* 2018;32(13–14):909–914.
- Storer M, Keyes WM. Detection of senescence markers during mammalian embryonic development. *Methods Mol Biol.* 2017;1534:199–210.
- Ritschka B, Storer M, Mas A, et al. The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration. *Genes Dev.* 2017;31(2):172–183.
- Coppé JP, Patil CK, Rodier F, et al. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol.* 2008;6(12):2853–2868.
- Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc Natl Acad Sci U S A.* 2001;98(21):12072–12077.
- Brown RAM, Epis MR, Horsham JL, Kabir TD, Richardson KL, Leedman PJ. Total RNA extraction from tissues for microRNA and target gene expression analysis: not all kits are created equal. *BMC Biotechnol.* 2018;18(1):16.
- Collado M, Gil J, Efeyan A, et al. Tumour biology: senescence in premalignant tumours. *Nature.* 2005;436(7051):642.
- Santos-Otte P, Leysen H, van Gestel J, Hendrickx JO, Martin B, Maudsley S. G protein-coupled receptor systems and their role in cellular senescence. *Comput Struct Biotechnol J.* 2019;17:1265–1277.
- Natarajan E, Omobono JD 2nd, Jones JC, Rheinwald JG, et al. Co-expression of p16INK4A and laminin 5 by keratinocytes: a wound-healing response coupling hypermotility with growth arrest that goes awry during epithelial neoplastic progression. *J Invest Dermatol Symp Proc.* 2005;10(2):72–85.
- Dimri GP, Lee X, Basile G, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc Natl Acad Sci U S A.* 1995;92(20):9363–9367.
- Itahana K, Campisi J, Dimri GP. Methods to detect biomarkers of cellular senescence. In: *Biological Aging.* Springer; 2007:21–31.
- Demaria M, Ohtani N, Youssef SA, et al. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev Cell.* 2014;31(6):722–733.
- Gao L, Chao L, Chao J. A novel signaling pathway of tissue kallikrein in promoting keratinocyte migration: activation of proteinase-activated receptor 1 and epidermal growth factor receptor. *Exp Cell Res.* 2010;316(3):376–389.
- Calenic B, Greabu M, Caruntu C, Tanase C, Battino M. Oral keratinocyte stem/progenitor cells: specific markers, molecular signaling pathways and potential uses. *Periodontol 2000.* 2015;69(1):68–82.
- Wilkinson HN, Hardman MJ. Wound senescence: a functional link between diabetes and ageing? *Exp Dermatol.* 2021;30(1):68–73.
- Faget DV, Ren Q, Stewart SA. Unmasking senescence: context-dependent effects of SASP in cancer. *Nat Rev Cancer.* 2019;19(8):439–453.
- Chrissouli S, Pratsinis H, Velissariou V, Anastasiou A, Kletsas D. Human amniotic fluid stimulates the proliferation of human fetal and adult skin fibroblasts: the roles of bFGF and PDGF and of the ERK and Akt signaling pathways. *Wound Repair Regen.* 2010;18(6):643–654.
- Takigawa T, Shiota K. Amniotic fluid induces rapid epithelialization in the experimentally ruptured fetal mouse palate—implications for fetal wound healing. *Int J Dev Biol.* 2007;51(1):67–77.
- Longaker MT, Adzick NS, Hall JL, et al. Studies in fetal wound healing, VII. Fetal wound healing may be modulated by hyaluronic acid stimulating activity in amniotic fluid. *J Pediatr Surg.* 1990;25(4):430–433.
- Castro-Combs J, Noguera G, Cano M, et al. Corneal wound healing is modulated by topical application of amniotic fluid in an ex vivo organ culture model. *Exp Eye Res.* 2008;87(1):56–63.
- Nyman E, Huss F, Nyman T, Junker J, Kratz G. Hyaluronic acid, an important factor in the wound healing properties of amniotic fluid: in vitro studies of re-epithelialisation in human skin wounds. *J Plast Surg Hand Surg.* 2013;47(2):89–92.
- Bazrafshan A, Owji M, Yazdani M, Varedi M. Activation of mitosis and angiogenesis in diabetes-impaired wound healing by processed human amniotic fluid. *J Surg Res.* 2014;188(2):545–552.
- Lundqvist EN, Egelrud T. Biologically active, alternatively processed interleukin-1 β in psoriatic scales. *Eur J Immunol.* 1997;27(9):2165–2171.

32. Hou J, Kim S. Possible role of ginsenoside Rb1 in skin wound healing via regulating senescent skin dermal fibroblast. *Biochem Biophys Res Commun*. 2018;499(2):381–388.
33. Blokland KEC, Waters DW, Schuliga M, et al. Senescence of IPF lung fibroblasts disrupt alveolar epithelial cell proliferation and promote migration in wound healing. *Pharmaceutics*. 2020;12(4):389.
34. Couteaudier M, Trapp-Fragnet L, Auger N, et al. Derivation of keratinocytes from chicken embryonic stem cells: establishment and characterization of differentiated proliferative cell populations. *Stem Cell Res*. 2015;14(2):224–237.
35. Rees PA, Greaves NS, Baguneid M, Bayat A. Chemokines in wound healing and as potential therapeutic targets for reducing cutaneous scarring. *Adv Wound Care*. 2015;4(11):687–703.
36. Tatrai A, Foster S, Lakatos P, Shankar G, Stern PH. Endothelin-1 actions on resorption, collagen and noncollagen protein synthesis, and phosphatidylinositol turnover in bone organ cultures. *Endocrinology*. 1992;131(2):603–607.
37. Kishimoto T. The biology of interleukin-6. *Blood*. 1989;74(1):1–10.
38. Angele MK, Knöferl MW, Ayala A, et al. Trauma-hemorrhage delays wound healing potentially by increasing pro-inflammatory cytokines at the wound site. *Surgery*. 1999;126(2):279–285.
39. Houle S, Papez MD, Ferazzini M, Hollenberg MD, Vergnolle N. Neutrophils and the kallikrein–kinin system in proteinase-activated receptor 4-mediated inflammation in rodents. *Br J Pharmacol*. 2005;146(5):670–678.
40. Xue M, Campbell D, Sambrook PN, Fukudome K, Jackson CJ. Endothelial protein C receptor and protease-activated receptor-1 mediate induction of a wound-healing phenotype in human keratinocytes by activated protein C. *J Invest Dermatol*. 2005;125(6):1279–1285.
41. Savagner P. Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays*. 2001;23(10):912–923.
42. Dave JM, Bayless KJ. Vimentin as an integral regulator of cell adhesion and endothelial sprouting. *Microcirculation*. 2014;21(4):333–344.
43. Karimian A, Ahmadi Y, Yousefi B. Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair*. 2016;42:63–71.
44. Lukas J, Parry D, Aagaard L, et al. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature*. 1995;375(6531):503–506.