

Transient Senescence Induces Wound Healing Through SASP Factors: Therapeutic Potentials of Chick Early Amniotic Fluid (ceAF)

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Research

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Abstract

Background

Inflammatory, proliferative and remodelling phases constitute a cutaneous wound healing program. Therapeutic applications/medication are available; however, they commonly comprise 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.

Methods

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.

Results

In our in vitro and in vivo cutaneous wound healing models, a low dose of ceAF exhibited the best efficacies; however, higher doses attenuated the wound healing, presumably by inducing p16 expression.

Conclusion

Our studies link an INK4/ARF locus-mediated signalling to cutaneous wound healing, implicate the therapeutic potentials of ceAF exerting functions likely by driving transient senescence and expediting cell proliferation, and conceptualize a homeostatic and/or balanced dosage strategy in medical intervention.

Background

The Graphical Abstract shows the INK4-ARF locus comprising tumour suppressor genes ARF/INK4A(p16)/INK4B that encode proteins with anti-proliferative functions exerted via RB and p53(1, 2). Respectively, RB regulates cell cycle downstream of the signalling 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 senescence (2) exerted by diverse intra- and extra-cellular stimuli through activating the above pathways in a combinatorial manner (3-5)

Generally, cellular senescence occurs in aging and tumour suppression; disparate biological processes as they seem, there could be an evolutionary logic for such antagonist pleiotropy. Senescent cells can mobilize a cohort of inflammatory cytokines, proteases, chemokines and growth factors as 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 foetal, embryo and adult stem cells; however, in differentiated cells, it can be reprogrammed to become hyper-responsive to mitogenic signals (6). Most senescent cells express p16 (7) that blocks cell cycle by inhibiting CDK4 and CDK6 (8) thus a commonly recognized marker (9). Pathologies aside, senescence also occurs in embryogenesis presumed to play roles in regulating embryonic structures (10, 11).

In many species, embryonic amniotic fluids are a dynamic milieu that participates in, e.g., cushioning, hydrating and providing immunity to embryos. Recently, we isolated embryonic stem cells in 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 foetal bovine serum (FBS; results). This characteristic and other biological activities of ceAF 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).

Wound healing is an evolutionarily conserved, complex, multicellular process that, in skin, aims at barrier restoration. This process involves the coordinated efforts of several cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. The migration, infiltration, proliferation, and differentiation of these cells will culminate in an inflammatory response, the formation of new tissue and ultimately wound closure. This complex process is executed and regulated by an equally complex signaling network involving numerous growth factors, cytokines and chemokines. Of particular importance is the epidermal growth factor (EGF) family, transforming growth factor beta (TGF- β) family, fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family, and tumor necrosis factor- α family. Currently, patients are treated by three growth factors: PDGF-BB, bFGF, and GM-CSF. Only PDGF-BB has successfully completed randomized clinical trials in the United States. With gene therapy now in clinical trial and the discovery of biodegradable polymers, fibrin mesh, and human collagen serving as potential delivery systems other growth factors may soon be available to patients. This review will focus on the specific roles of these growth factors and cytokines during the wound healing process.

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family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), connective tissue growth factor (CTGF), interleukin (IL) family, and tumor necrosis factor- α family. Currently, patients are treated by three growth factors: PDGF-BB, bFGF, and GM-CSF. Only PDGF-BB has successfully completed randomized clinical trials in the United States. With gene therapy now in clinical trial and the discovery of biodegradable polymers, fibrin mesh, and human collagen serving as potential delivery systems other growth factors may soon be available to patients. This review will focus on the specific roles of these growth factors and cytokines during the wound healing process.

Major findings and significance

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 mediated by SASP factors, e.g., PDGF-B, TGF- β and VEGF known to promote myofibroblast differentiation and epithelial growth with much-induced sebaceous glands and cellular layers. The ceAF components may function by signalling SASP factors as a marker for transient senescence that in turn promotes 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 due to an above-a-threshold expression level. Our studies also provide an inkling that p21, rather than p16, plays roles in orchestrating a cutaneous wound healing program.

Results And Discussion

ceAF supports cell growth and promotes wound healing in vitro

Given that ceAF is a source of embryonic stem cells, we believed that ceAF comprised biologically balanced secretory growth factors, cytokines and other ligands collectively dubbed proliferation-stimulants and, as a prelude to exploring its therapeutic potential, tested its ability to support the growth of cultured HeLa cells using FBS as a control. Starting from ~20% confluence, HeLa cells were cultured in DMEM supplemented with increments of ceAF (0-20%, v/v) or 10% FBS for 48 hours; 5% ceAF optimally, and nearly as potently as 10% FBS, supported cell growth that roughly underwent 2 doubling times (Figure 1A/1B). Given that cultured HeLa cells exhibited elevated p16 expression in higher doses of ceAF (20% in particular; Figure 1B bottom panel), we proposed that the lower efficacies of higher ceAF doses, as compared to 5% ceAF, for supporting cell growth (Figure 1B upper panel) were attributable to an anti-proliferative activity 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.

Prompted by the above, we established an in vitro wound healing model utilizing HaCaT cells, representing an epidermis origin (12); 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 within 24 hours; ectopically expressing p16 hindered the healing in vitro (Figure 1C/1D; also see below).

Encouraged by the above in vitro results, we examined the healing capacities of ceAF on surgically-made cutaneous wounds in a murine model. Mice treated with 5% ceAF healed most expeditiously, manifesting virtually scar-less skin in 10 days (Figure 2A/2B). Dermal application of naturally produced/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 (13, 14). In our hands, p16 when co-applied within a dosage employed by other practices significantly attenuated the wound healing process, which served an offset for facilitated wound healing brought about by ceAF particularly at a 5% dose (Figure 2A/2B). This is consistent with results of the in vitro model (Figure 1C/1D).

p16 impedes wound healing in vitro and in vivo

Under stresses such as those that induce senescence, p16 expression is up-regulated; p16 was reported to be able to drive senescence-associated secretory phenotypes (SASP) that provoke chemokine/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 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 (Figure 1C/1D) and cellular proliferation (see 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 (Figure 2A/2B). Taken together, these results suggest that, at least in our assay systems, the signalling involving SASP factors and transient senescence might not be 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 (see 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 (Figure 2A) [significance of 5% ceAF as compared to other groups summarized in Table S1(A)]. 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 (Figure 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 (Figure 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 (Figure 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 (Figures 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 cells in the S-phase that had obvious difficulty to enter the G2 phase (Figure 3A), suggesting that the wound-healing-promoting (by ceAF) and the wound-healing-attenuating (by p16 expression/administration) phenotypes (Figures 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 (15-17) ; 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 (Figure 3A), expressing p16 on top of ceAF significantly attenuated trans-well passaging (Figure 3B).

ceAF 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 histochemically, scoring both numbers of positive cells and intensities. Thus, SA- β -gal is a good biomarker for senescent cells in both cell culture and in vivo.

We examined SA- β -gal expression patterns in HaCaT cells incubated in control vs. ceAF-supplemented media with or without a p16 ectopic expression (Figure 4A). The number of SA- β -gal⁺ cells was highest in the 5% ceAF group (Figure 4B; upper panel), but more intense SA- β -gal⁺ signals were observed in cells ectopically expressing p16 (Figure 4B; bottom panel); both criteria were deemed statistically significant.

Given that 5% ceAF exerted optimal functions on wound healing and expediting cell division (Figures 1-3), we propose that a proper (homeostatic) SA- β -gal expression level indicates a transient senescence that facilitates wound healing, while a p16-intensified SA- β -gal activity may represent senescence at a more permanent scale that is above a threshold and, based on our earlier data, hampers wound healing and blocks cell cycle.

Expression profiling of factors involved in cutaneous wound healing

Inflammation, proliferation and remodelling 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 signalling 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) and acting paracrinally, these factors comprise a host of chemokines that recruit pro-inflammatory cells including macrophages, which in turn release (additional) cytokines/growth factors to facilitate wound healing (18, 19).

We employed wounded/repared 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 aforementioned 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 remodelling. As seen (Figure 4C, quantification; Figure 4D, Heatmap analyses), 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 group here 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 established an expression profile of 12 of ~25 SASP factors reported (20, 21) using HaCaT cells incubated with FBS vs. ceAF, with or without ectopic p16 expression. Of the tested SASP factors, the expression of all, save p16, were up-regulated significantly in cells incubated in 5% ceAF and to a lesser degree 10% ceAF (Figure 4C, quantifications normalized against a house-keeping gene; Figure 4D, Heatmap analyses); matter-of-factly, p16 played offset roles on ceAF-enhanced expression of tested genes, and the ostensible variations of p16 expression per se (Figure 4C; last panel) were most-likely due to the presence/absence of an ectopic p16 gene.

Physiological interpretation of a ceAF-mediated expression profile

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 (22, 23). In addition, endothelin is a mediator for morphogenesis in other systems including bone regeneration and skeleton formation (24). Thus, elevated expression levels of keratin, keratin-10 and endothelin in the cutaneous wound healing model reflect a more completed remodelling 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(25). For instance, IL-6 is released as an early response to tissue injury to induce a signalling of pro-inflammatory cytokines from the resident macrophages and stromal cells (26). 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 population (27); 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 remodelling 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 (Figure 4C-D).

PAI-1 and vimentin: Plasminogen activator inhibitor type 1 (PAI-1) is expressed on the surface of keratinocytes, its activation enhances cellular proliferation (18). PAI-1 limits plasmin generation to sustain cell migration and proliferation, a key indicator for re-epithelialization of keratinocytes (28). 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, signalling, plasticity and regulation of cell morphology (29, 30). These proteins in principle are largely involved in the remodelling phase of a wound healing program.

Cell cycle/senescence regulators: p16 and p21 are well known proteins in regulating cell cycle and senescence (31, 32); as far as our assay systems are concerned, p16 is, as opposed to a recognition supported by certain experimentation, not playing a role in establishing the transient senescence that readies wounds to undergo healing. From all criteria, p16 is actually an offsetting factor for therapeutic values of ceAF. Our data (Figure 4C; second last panel) support a notion that p21 might as well play compensatory role(s) by itself or in conjunction with other factor(s), e.g., a non-redundant function afforded by the third component of the INK4-ARF locus, INK4B.

Further Discussion and Perspectives

Intrinsic to and secreted by developing chick embryos 6-8 days post fertilization, ceAF exhibits powerful wound healing capacities apparent in mammalian cutaneous wound healing models, in which ceAF with no preservatives/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 signalling molecules at biologically homeostatic levels to support embryogenesis. This view is especially important given a manifestation of the attenuating activities at high doses (Figure 1B). Of note, given that the control for establishing the expression profiling with culture cells (Figure 4C/D) 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/B and Figure 3B. Recently, occurrence of senescence and roles of SASP factors during embryogenesis (33) and in limiting fibrosis upon tissue injury (34, 35) 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.

Conceivably, at least in our assay systems and as in many signalling cascades, signals from ceAF, however chick-embryo-derived, obviously can be received and amplified by cells in the wound/surrounding areas of a murine model to facilitate the wound healing. From a biochemical, mechanistic and therapeutic perspective, chromatographic isolation of active fractions as well as reconstitution to formulate an efficient cocktail starting from ceAF and, probably much more importantly, efforts to rid of attenuating activities (Figure 1B) also by biochemical isolation, are a daunting challenge yet obviously a worthy goal.

Conclusion

Our studies link an INK4/ARF locus-mediated signalling to cutaneous wound healing, implicate the therapeutic potentials of ceAF exerting functions likely by driving transient senescence and expediting cell proliferation, and conceptualize a homeostatic and/or balanced dosage strategy in medical intervention.

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 are listed in Table 2S(B). HeLa 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.

Chick early amniotic fluid (ceAF) preparation

Fertilized chick eggs were incubated at $38 \pm 1^\circ\text{C}$ and a 50% humidity, and ceAF was collected from eggs between days 6-8. After centrifugation of the samples at 2500g for 20 min, supernatants were filtered over a 0.22 μm sterilization device (Millipore China) 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), they were kept in the Animal Facility, Zhejiang University (Permit #: ZJU20170013) with a 12-hr day/light cycle and 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. Anaesthesia (Phentolamine; 8mg/kg) was administered intraperitoneally (36). Step-wisely, procedures were conducted aseptically as: a full-thickness wound (10x10mm) was excised from the dorsum of mice after hair removal; excessive panniculus carnosus layer's contraction was controlled by silicon disks cut/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 a 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 1ng/ 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. Briefly, embedded in paraffin, samples were sectioned in 3 μm thickness, mounted on glass slides and, after deparaffinizing, stained with haematoxylin-Eosin and Masson's trichrome. The density of inflammatory cells and blood vessels density in dermis were analysed using M-42 system (37). 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

HeLa and HaCaT cells, a gift from YU Faxing (Fudan University), were experimentally confirmed free of mycoplasma and assured to have normal/expected 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) antibodies and Senescence Activated β -Galactosidase Staining Kit (#9860) [Cell Signalling Technology, Danvers, MA]. 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. RNase free double distilled water, 4x gDNA wiper mix and 1 μ g total RNA were sequentially pipetted into a tube and incubated at 42°C for 2 minutes. Then 5X 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 2S(B).

Cell scratch assay

HaCaT cells were cultured in 6-well plates to attain a ~80% confluence, and starved for 24 hours. 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. In the p16 minus samples was added the vector plasmid. After 24 hours, scratches were drawn with a 10 μ l pipette-tip. The readings/cell migration rates at the “wounded” sites were recorded at 0, 24 and 48 hours.

Western blotting

Aspirated of media, cells were rinsed with PBS twice and lysed with HEPES lysis buffer (115 mM NaCl, 1.2 mM CaCl_2 , 1.2 mM MgCl_2 , 2.4 mM K_2HPO_4 , 20 mM Hepes-KOH, pH7.0, 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 hours 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 CO_2 until colour manifestation.

Trans-well migration assay

Cells were collected/suspended at a density of 3×10^4 cells in 200 μ l serum-free medium after transfection with or without p16-expressing plasmid for 24 hours, and seeded in the upper chamber. Media with indicated doses of ceAF (600 μ l in total) were suspended in the lower chamber. After 24 hours, 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 μ m diameter) were rinsed with PBS thrice, fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet. Number of cells that transwell-migrated was observed/counted/recorded by a microscope (Olympus BX51).

Cell cycle analysis

Cells were suspended at $1-2 \times 10^6$ cells/ml, transferred into 5 ml polystyrene tubes and washed with ice-cold PBS. After centrifugation at 1000 rpm for 5 minutes 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 hours. Cells were washed twice with PBS, centrifuged at 3000 rpm for 5 minutes at 4°C, and re-suspended in 200 μ l of propidium iodide (Sigma-Aldrich) supplemented with 30 μ 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 was 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.

Abbreviations

ceAF (chick early amniotic fluid), SASP (senescence associated secretory phenotypes), RB (retinoblastoma), CDK4/6 (cyclin dependant 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).

Declarations

Concent to publish

*Not applicable

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Ethics approval and consent to participate

All necessary ethical approvals were obtained from animal facility center, ZJU (Permit #: ZJU20170013) before conducting experiment.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declaration of interest statement

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.

Author contributions:

AM, JQ, YS and YL jointly conceived the project; guided by YL, AM and YS designed and carried out the experiments; XZ and KZA provided technical help and were involved in statistical analyses and the graphical abstract design; AM and YL co-wrote the paper, in which YS helped in editing and figures.

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Figures

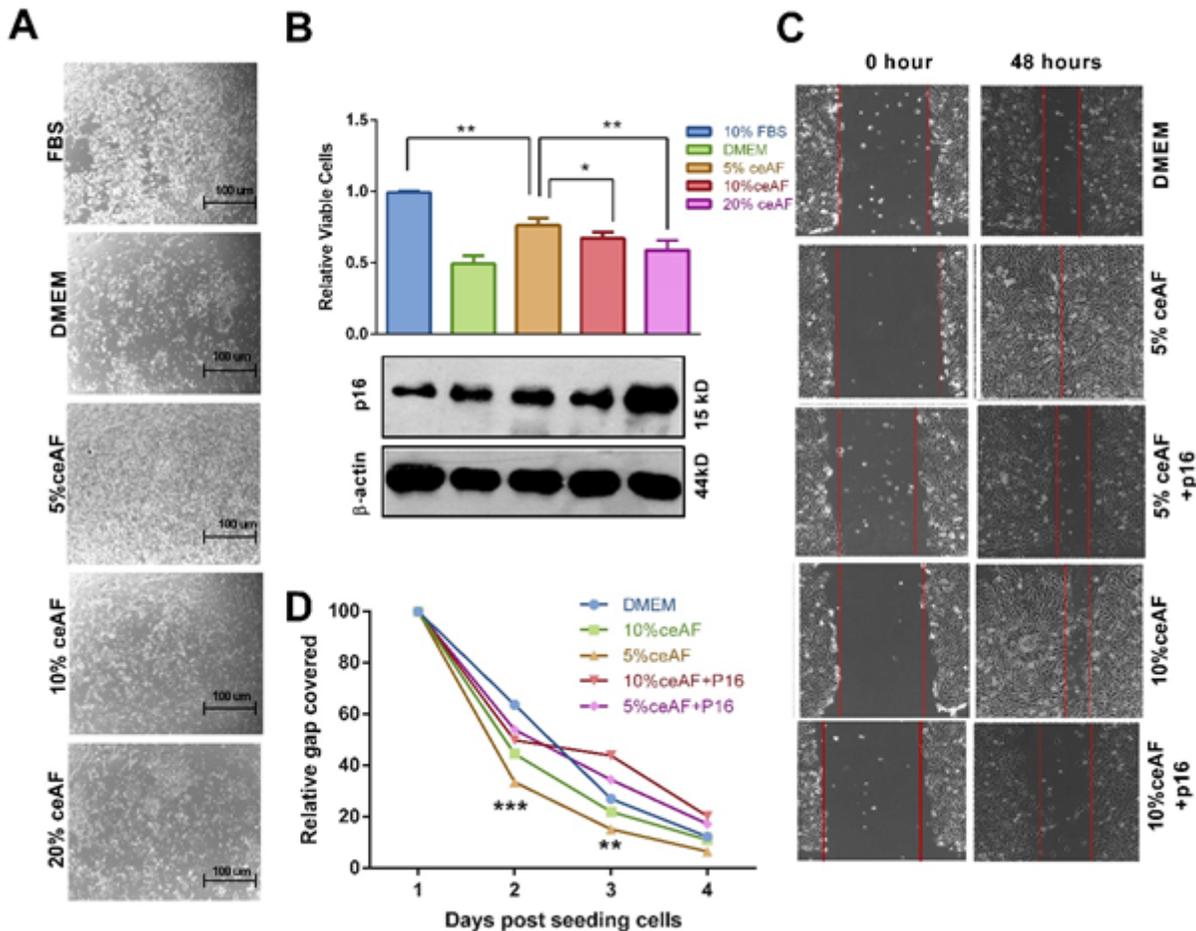


Figure 1

ceAF supports cell growth and promotes wound healing in vitro. A) Snapshots of a typical cell proliferation assay with increasing ceAF (0, 5%, 10% and 20%) comparing efficacies among doses and with 10% FBS to support the growth of HeLa cells (scale bar at 100 μ m). Images taken 48 hours post treatment. B) Quantification of relative number of viable cells post treatment with ceAF vs. FBS (upper panel); expression of endogenous p16 induced by higher doses of ceAF (bottom panel). C) Wound healing scratch assay on confluent/24-hr 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 hours post scratch formation as indicated on top. D) Healing quantification as assessed by measuring the reduced gap distance within the specified time frames. Experiment was repeated at least three times.

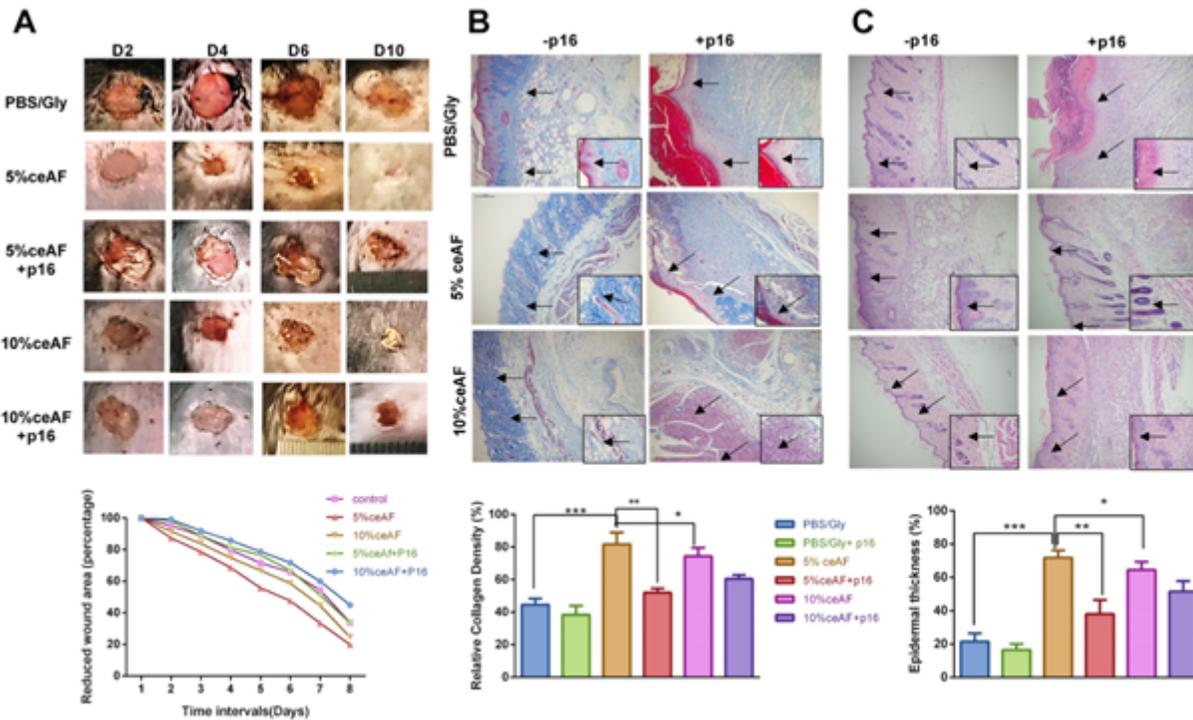
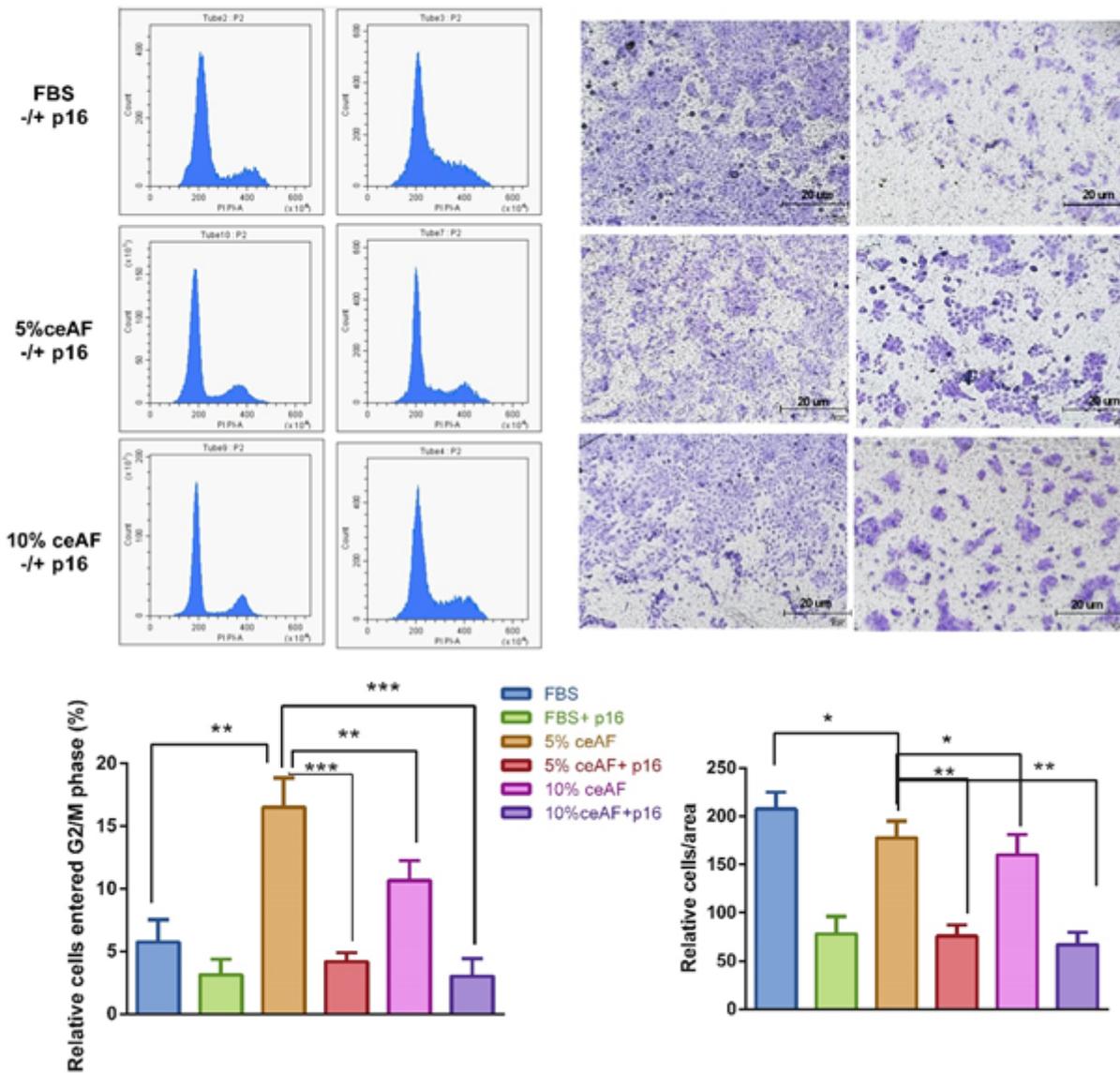


Figure 2

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 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 (C). 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.

A**B****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 a 24-hr incubation. The bottom panel shows percentiles of cells at G2/M. (n=3). B) Trans-well migration assay for cell groups (scale bar 50 μ m) as in (A). The bottom panel shows the quantification of cells per field. The error bars represent standard deviation of three different experiments.

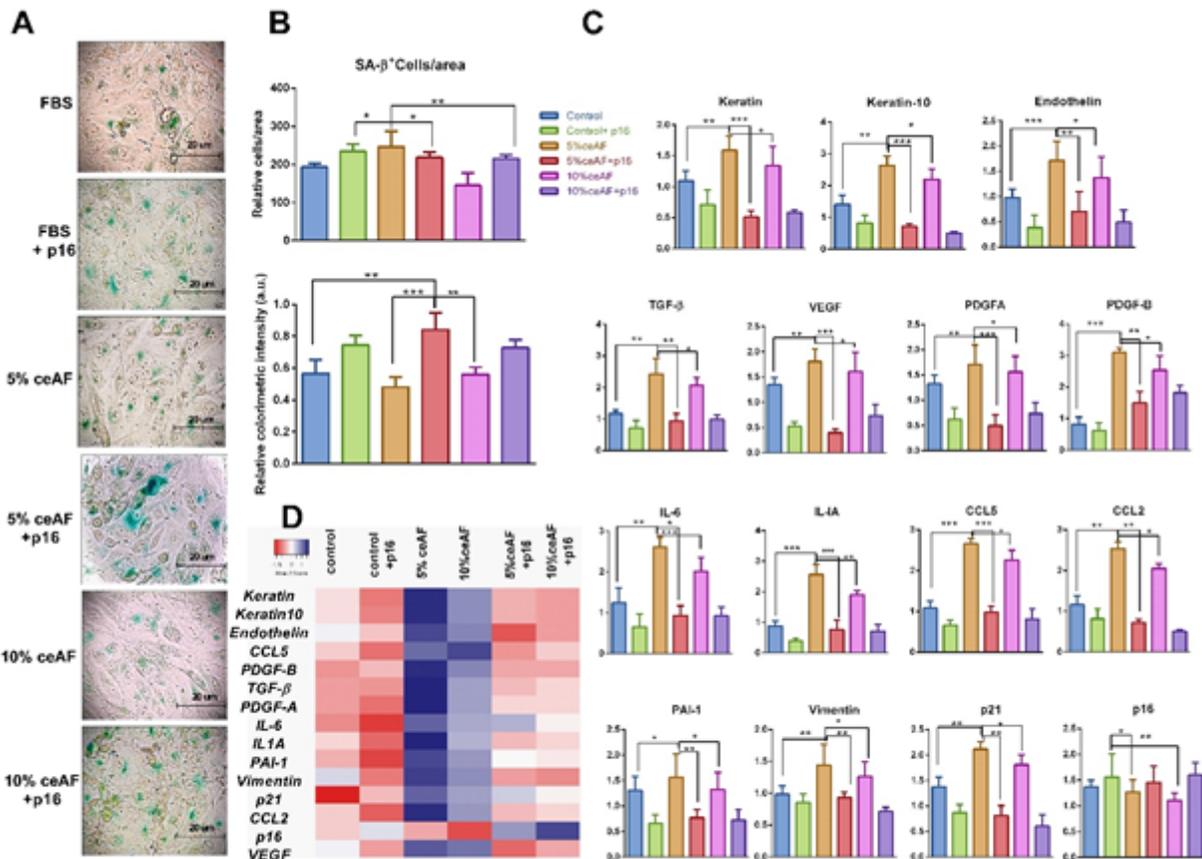


Figure 4

SASP factors mediate and contribute to facilitating the cutaneous wound healing and transient senescence. A) SA-β-Gal activity in HaCaT cells treated as indicated for 48 hours and maintained in fresh media for 2 days. Micrographs were obtained with phase contrast microscopy. B) Quantification of SA-β-Gal⁺ cells. Upper panel: number of cells; bottom panel: intensities of positive signals. C) 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-A, TGF-β, IL-1A and IL-6. The experiments were conducted in triplicates. * p<0.05, **p<0.01 and ***p<0.001. D) Heatmap analyses for qRT-PCR of factors as listed in (C).

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