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Critical role of the endogenous renin-angiotensin system in maintaining self-renewal and regeneration potential of epidermal stem cells

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ABSTRACT

A tightly controlled activity of renin-angiotensin system (RAS) including renin, angiotensin-converting enzymes (ACEs), and angiotensin II (Ang II) receptors is critical not only for maintaining systemic hemodynamics and blood volume but also for controlling cell proliferation, differentiation, and tissue remodeling in target organs. ACE inhibitors or Ang II receptor type 1 (AT1R) blockers are widely used as first line drugs for the treatment of cardiovascular diseases that are caused by chronical activation of RAS. However, about 15% of patients using ACE inhibitors develop side effects in the skin and the underlying mechanisms have been poorly understood or even neglected. Herein we show an endogenous RAS in maintaining self-renewal and regeneration potential of epidermal stem cells (ESCs) thereby contributing to wound healing. Firstly, we found that ESCs may express ACE, and its members in wound edges were positively associated with wound healing in Captopril-treated rats. Secondly, we demonstrated that human ESCs had a functional RAS including ACE1, ACE2, Ang II, AT1R, and AT2R. ACE-Ang II axis maintains human ESC function via activation of both AT1R and AT2R, which are negatively regulated by each other. Ang II-induced activation of extracellular signal-regulated kinase (ERK) and signal transducers and activators of transcription (STAT)1 and STAT3 was mediated by the negative cross-talk between AT1R and AT2R in human ESCs. These results suggest that Ang II is a critical regulator of ESC function and ESC-mediated epidermal regeneration. Inappropriate interruption of Ang II-operated signaling may prejudice ESC function leading to impaired skin wound healing or even disease.

1. Introduction

The renin-angiotensin system (RAS) consists of the angiotensin II (Ang II) precursor angiotensinogen, renin, angiotensin-converting enzyme (ACE), Ang II, and Ang II type 1 and type 2 receptors (AT_1R and AT_2R). Ang II is the main effector molecule of the RAS, deriving from angiotensinogen by successive enzymatic actions of renin and ACE. Mounting evidence demonstrated that tightly controlled RAS activity is critical not only for maintaining systemic hemodynamics and blood volume but also for controlling cell proliferation, differentiation, and tissue remodeling in target organs [1].

Chronic RAS activation in the cardiovascular system increases Ang II and plays a major role in the pathogenesis of cardiovascular diseases such as hypertension and heart failure [2]. Therapies that decrease Ang II clearly slow progression of these diseases, so ACE inhibitors are widely used as first-line treatments for hypertension and heart failure [3]. However, the overall incidence of ACE inhibitor therapy adverse effects is up to 28%. Approximately half of these side effects occur in the skin, including psoriasis and hair loss [4], and their underlying mechanisms are poorly understood.

Abbreviations: ACE, Angiotensin-converting enzyme; AT1R, Ang II receptor type 1; RAS, renin-angiotensin system; BrdU, 5-bromo-2′-deoxyuridine; K-SFM, keratinocyte-SFM; CFE, colony forming efficiency; ELISA, enzyme-linked immunosorbent assay; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinase; STAT1, signal transducers and activators of transcription

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A local RAS, including ACE and Ang II receptors, and local de novo Ang II production in human skin tissue has long been documented [5]. Growing evidence also revealed that ACE is involved in wound healing, inflammation, pathological scar formation, and carcinogenesis [6–8]. Moreover, ACE is a critical regulator of skin cell survival and tissue regeneration [9]. Notably, we demonstrated that ACE is epidermally expressed from early to late gestation [10]. and clear co-localization of ACE with putative epidermal stem cell (ESC) markers β1-integrins, K19, and P63 is documented [10]. Because ESCs may play critical roles in maintaining skin epidermis homeostasis [11,12], it is conceivable that ACE critically regulates ESC function and epidermal regeneration. Furthermore, ACE activity suppression may cause ESC dysfunction, leading to delayed skin wound healing. In the present study, we investigated the role of ACE in the regulation of ESC function and epidermal regeneration during wound healing.

2. Materials and methods

2.1. Animals

Male Wistar rats were purchased from Southern Medical University, Guangzhou, P.R. China and housed individually in a pathogen free animal facility under a12-hours light/dark cycle and an average temperature of 25 °C, with ad libitum access to standard laboratory food and water. All animal procedures were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Jinan University.

2.2. Measurement of BrdU-incorporated cells in skin

Rats were injected intraperitoneally (IP) with 5-bromo-2′-deoxyuridine (BrdU; Sigma Chemical Co., St Louis, MO, USA) at a dose of 50 mg/kg body weight (50 mg/kg) twice a day (at 8:00 AM and 6:00 PM) for 4 consecutive days. Two months later, they were prepared for the experiments [13].

2.3. Skin wound healing model in rats

Rats were anesthetized by a single IP injection of pentobarbital sodium (4 mg/kg) and ketamine (70 mg/kg). Two round full-thickness dermal wounds of 1.77 cm^2 (1.5 cm diameter) were generated on both sides of the dorsal trunk using skin punch equipment (Beijing Surgical Instrument, Beijing, P.R. China) after shaving the hair and disinfecting the skin with Softasept N (B Braun Melsungen, Melsungen, Germany). Each wound was well separated by at least 1.5 cm of unwounded skin. We standardized the wound biopsy by coring the skin until the biopsy punch reached the subcutaneous muscles (4–5 mm deep). At time intervals ranging from 3 days through to 11 days after wounding, the rats were euthanized after anesthetizing with 10% chloral hydrate (350 mg/kg), and wounds were excised with a 2-mm rim of surrounding tissue. Samples were fixed in 10% buffered formalin.

Wound healing was assessed by analysis of wound closure rate and migration of keratinocytes in the wounded site as described elsewhere [14]. Briefly, at the indicated time points after skin injury, skin wounds were photographed using a digital camera. Digital imaging software (SigmaScan; SPSS Science, Chicago, IL, USA) was used to measure the wound size. Wound closure rate was determined using the following formula: wound closure rate (%) = (1 - wound size/the original wound size) × %. The extent of migration of the keratinocytes was measured in sections stained with hematoxylin and eosin (H&E) and expressed as the length of epithelial tongue as described previously [14]. The length of the epithelial tongue was determined by the distance from the margin of the wound to the tip of the migrating keratinocytes. A total of six random fields from the wound bed were counted in each section. The average index of three sections was taken as the value for each wound. Five to seven rats were analyzed at each time point.

2.4. Administration of captopril

Animals were divided into two groups (n = 6–8 per group). One group was treated with an IP injection of captopril (Sigma Chemical Co.) at a dose of 10 mg/kg daily from 1 day before skin wounding. The other group was treated with an equal volume of 0.9% NaCl under the same conditions.

2.5. Immunohistochemistry

Five-μm sections were subjected to H&E staining or immunohistochemical staining as described previously [15]. The primary antibodies used in this study were mouse monoclonal anti-ACE antibody (ab-77,990, 1:100; Abcam Co, Cambridge, UK) and mouse monoclonal anti BrdU (sc-32,323, 1:100; Santa Cruz Biotechnology, Dallas, TX, USA).

2.6. Human skin biopsies

Foreskin tissue samples were obtained from plastic surgery operations carried out for the circumcision of normal and healthy children. Informed consent was obtained from each individual and their parents, and the study was approved by the Medical and Ethical Committees of the First Affiliated Hospital of Jinan University.

2.7. Isolation and culture of human ESCs

Human ESCs were isolated from foreskins as described previously [16,17]. Briefly, the epidermal layer was obtained from foreskin tissue which had been disinfected after an overnight incubation in 100 mg/L dispase (Roche, Mannheim, Germany) at 4 °C. The epidermis was placed in a sterile tube containing 0.25% trypsin and EDTA for 4 min and stirred rapidly with a pipette until dissociated into a single cell suspension. The trypsin was inactivated by addition of 10% fetal bovine serum (Invitrogen, Life Technologies, Carlsbad, CA, USA) in DMEM and the cells were centrifuged at 1000 rpm and resuspended in ESC culture medium; Keratinocyte-SFM (K-SFM; 17005-024, Gibco, Life Technologies) supplemented with 0.25 ng/ml human recombinant epidermal growth factor and 25 μg/ml bovine pituitary extract. The cells obtained were allowed to adhere for 20 min at 37 °C to culture dishes coated with the collagen IV (Sigma Chemical Co) substrate. Cells that did not adhere were gently rinsed off, adherent cells were subcultured by incubation in a solution of TGG (0.1% glucose, 0.25% trypsin, 0.5 μg/ml gentamicin and 0.02% EDTA) for 5 min at 37 °C, centrifuged, resuspended in ESC medium, and then cultured at 1×10^5 cells/ml, with a medium change every 3 days.

2.8. Immunofluorescence staining

Cultured ESCs at second passages were fixed in 4% paraformaldehyde for immunofluorescent staining as previously described [18]. Slides were incubated with primary antibodies (goat polyclonal anti-ACE antibody (sc-12,184), mouse monoclonal anti-K19 antibody (sc-51,584), and mouse monoclonal anti-β_1-integrin (sc-71,388) (Santa Cruz Biotechnology)) at 4 °C overnight, washed in PBS, followed by the addition of secondary antibodies fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulins (Jackson Immunoresearch, West Grove, PA, USA) for detecting K19, and Texas red-conjugated rabbit anti-mouse or anti-goat immunoglobulin for detecting β_1-integrin or ACE (Jackson Immunoresearch).

2.9. Flow cytometric analysis of ESCs

Cultured ESCs were fixed with 4% paraformaldehyde for 30 min,

permeabilized with 0.3% Triton™ X-100 for 10 min, blocked with blocking buffer (10% donkey serum in PBS) for 30 min. The cells were processed for two-color staining of β1-integrin and K19 for fluorescence-activated cell sorting (FACS) by incubating with goat anti-human β1-integrin receptor monoclonal antibody (1:100, sc-9936, Santa Cruz Biotechnology) and rabbit anti-human K19 receptor monoclonal antibody (1:100, ab52625, Abcam) overnight at 4 °C, after blocking in 3 ml blocking buffer (10% donkey serum in PBS) for 30 min as described previously [19]. Cells were washed twice with 0.01 M PBS and incubated with isotype-specific secondary antibodies (donkey anti-goat antibody, 1:50 donkey anti-rabbit antibody 1:50, Invitrogen) for 1 h at room temperature. Then, cells were fixed and resuspended at 1×10^6 cells per ml for flow cytometry. Cells were processed for double (β1-integrin-PE/K19-FITC) staining along with the appropriate negative controls to establish compensation setting on flow cytometer.

2.10. Flow cytometric analysis of the expression of ACE and K10

Cultured cells at second passages were processed for single (ACE or K10) staining along with the appropriate negative controls and single color positive controls to establish compensation settings as described previously [20].

2.11. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cultured cells at the second passage with TRIzol Reagent (Gibco-BRL). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described previously [15]. The following forward (5′-CAGTGCCCAGGACTCAAGGT-3′) and reverse (5′-GCAGCAGCCTCTTCTCATTCTC-3′) primers were used for PCR amplification of ACE1 (genbank: BC036375.2) to yield a 773 base pair (bp) product. Following The forward (5′-CCTGTTCCGATCATCTGTTG-3′) and reverse (5′- GACCAAATACACACTTTCCC-3′) primers were used for PCR amplification of ACE2 (genbank: BC048094.1) to yield a 767 bp product. The following forward (5′-CCTGGATACCGCAGCTAGGA-3′) and reverse (5′-GCGGCGCAATACGAATGCCCC-3′) primers were used for PCR amplification of β-actin to yield a 122 bp product. PCR amplifications were carried out with 35 cycles for ACE1 and ACE2 of 1 min of denaturation at 94 °C, 1 min of annealing at 55 °C, and 1 min of extension at 72 °C, followed by 5 min of final extension step at 72 °C.

2.12. Cell proliferation assay

ESCs (8000 cells/well) at second passage were seeded into 96-well flat-bottomed microtiter plates containing 200 μL of growth medium in the presence or absence of captopril, Ang II with or without the AT_1 receptor blocker valsartan (Sigma) or AT_2 receptor blocker antagonist PD123319 (Sigma) at 37 °C in 5% CO_2 after verifying cell viability by trypan blue dye exclusion test using Cellometer automatic cell counter (Nexcelom Inc., Lawrence, MO, USA). After an incubation period as indicated, 100 μL of XTT (Roche Applied Science, Mannheim, Germany) was added to each well, and plates were incubated at 37 °C for another four hours. The absorbance was measured at 450 nm using a microplate reader (DTX 880 Multimode Reader, Beckman Coulter, Indianapolis, IN, USA).

2.13. Colony forming efficiency (CFE)

Cells were seeded on 24-well culture plates with 100 cells per well and were treated with or without captopril at a concentration of 10^{-6} mol/L. After 10-days culture, clones were fixed with 4% formaldehyde (Sigma Chemical Co.) and stained with 1% rhodamine B (Sigma Chemical Co.), and the colony numbers were counted under a microscope. CFE values are expressed as the ratio of the number of colonies to the number of inoculated cells. Only the colonies that contained >50 cells were scored [21].

2.14. Apoptosis analysis

ESCs at second passage were seeded into 6-well plates at 5×10^6 cells/ml. Then cells were treated with or without captopril at a concentration of 10^{-6} mol/L and cultured for 5 days. Apoptosis assays were performed using an Annexin V-FITC apoptosis kit according to the manufacturer's instructions [22].

2.15. Cell migration assay in vitro

The effect of the ACE inhibitor captopril on ESC migration was measured by the scratch assay as described previously [23]. Cells were seeded in 6-well plates at a density of 5×10^6 cells/ml. A scratch was made through each confluent well using a sterile pipette tip at 24 h after seeding. The cells were then treated with captopril or Ang II with or without valsartan or PD123319. Pictures were taken at each time-point using a NikonDS-L2 camera. Wound closure areas were measured under the microscope (magnification 100×) immediately after scratch (0 h) and other time points as indicated using image analyzing software (NIH image). Experiments were carried out in triplicate and repeated at least five times.

2.16. Adhesion analysis

Collagen IV plates were prepared by coating six well plates with 2 ml of human collagen type IV (20 μg/ml; Collaborative Biomedical Products, Bedford, MA, USA) overnight at 4 °C. ESCs at second passage were cultured in six well plates with or without captopril in K-SFM medium until they reached confluence. The cells were then removed by 5 min trypsinization leaving the collagen IV. The reaction was quenched with bovine serum albumin (BSA) and cells washed with PBS. The cells were centrifuged at 1000 rpm and resuspended in K-SFM supplemented with 0.25 ng/ml human recombinant epidermal growth factor and 25 μg/ml bovine pituitary extract. After counting, 1×10^4 cells were seeded into each well. The cells obtained were allowed to adhere for 20 min at 37 °C to culture dishes coated with collagen IV. Cells were harvested by trypsinization after removing the nonadherent cells with gentle washing [20,24].

2.17. Enzyme-linked immunosorbent assay for Ang II

5×10^5 ESCs were plated in K-SFM with or without captopril at a concentration of 10^{-6} mol/L and cultured in a CO_2 incubator at 37 °C for 5 days. The culture media was collected and Ang II concentrations determined with a human Ang II enzyme-linked immunosorbent assay (ELISA) kit (XinRan Biological, P.R. China) [25].

2.18. Western blot analysis

Total proteins were prepared from the cultured cells, and western blot performed as previously described [15]. Immunoblotting was done using anti-extracellular signal-regulated kinase (ERK) (CST#9102), anti-phospho-ERK (ab4819), anti-signal transducer and activator of transcription (STAT)1 (CST#9172), anti-phospho-STAT1 (CST#9171), anti-STAT3 (CST#4904), anti-phospho-STAT3 (CST#9131), anti-Akt (CST#9272), anti-phospho-AKT (CST#9271) (Cell Signaling Technology, Danvers, MA, USA), and anti-beta-actin (ab8227) (Abcam) primary antibodies.

2.19. Statistical analysis

Values are expressed as the mean ± SEM in the text and figures. The data were analyzed by two-way ANOVA. If a statistically significant effect was found, *post-hoc* analysis was performed to detect the differences between the groups. A *P*-value of $P < 0.05$ was considered to indicate statistically significant differences.

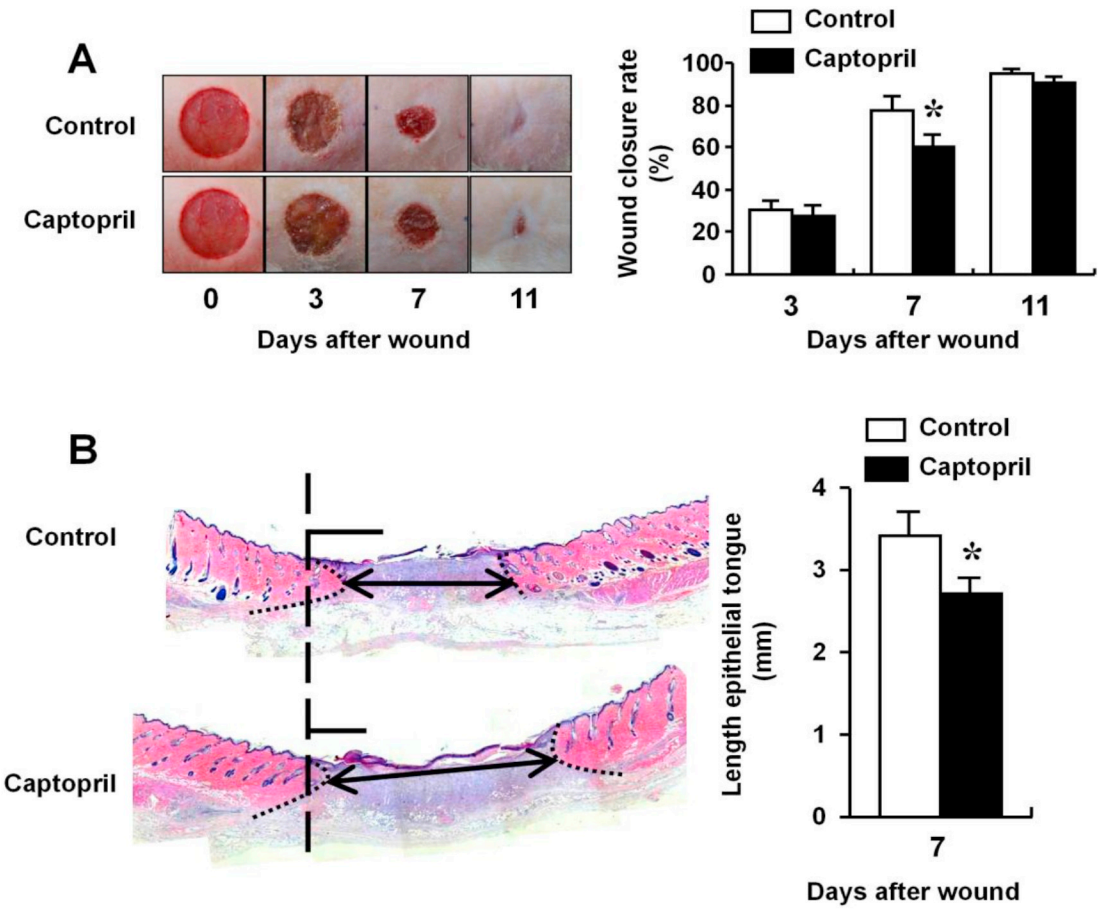


Fig. 1. Effect of captopril on cutaneous wound healing in rats.
Excision wounds were performed in Wistar rats, and the healing process was monitored at various time points.
(a) Left panels: Macroscopic observation of excisional control- and captopril-treated wounds at 0, 3, 7 and 11 days after wounding. Right panels: The wound closure in control- and captopril-treated groups were compared at 3, 7 and 11 days after wounding. The wound closure rate was calculated as described in the Materials and methods. Values are expressed as the mean ± SEM (n = 5–7). *$P < 0.05$ vs. control.
(b) Representative hematoxylin and eosin-stained sections showing a shorter migrating tongue in captopril-treated wounds. Scale bars indicate 50 μm. Values are expressed as the mean ± SEM (n = 6). *$P < 0.05$ vs control.

3. Results

3.1. A critical role for ACE in maintaining ESC wound healing function in rats

To study the potential mechanism by which ACE regulates skin wound healing, we established ACE inhibition-induced retardation of rat skin wound healing using captopril. We observed that 10 mg/kg/day IP administration of captopril inhibited skin healing (Fig. 1) without affecting blood pressure (data not shown). Captopril also inhibited increases in the epithelial tongue length in wounded skin (Fig. 1b). This was shown to indicate epithelial regeneration at the skin wound site [16], suggesting that captopril-induced ACE inhibition hinders skin wound healing by suppressing the regeneration of wound epithelia.

To further explore the underlying cellular mechanism, we determined whether captopril regulates ESC function to inhibit skin wound healing. Previous studies established that ESCs reside in the interfollicular epidermis and bulge region of the hair follicle and that they incorporate BrdU [26]. Therefore, we performed BrdU-retention analyses in the skin wounds of rats treated with or without captopril. As shown in Fig. 2a, the percentage of BrdU-incorporated cells in basal layers of normal rat skin was 1%–5% (mean, 2.25% ± 1.34%), while BrdU-retaining cells increased at the wound edge and peaked on day 7. Captopril administration markedly decreased the number of BrdU-incorporated cells 7 days after skin injury compared with controls (Fig. 2a). These results suggest that captopril delays skin wound healing partly via inhibiting ESC proliferation. To find a direct link between ACE and ESC function in skin wound healing, we examined ACE expression in wounded rat skin. In normal skin, ACE-positive cells were mainly seen in the basal layer; however, the number of ACE-positive cells was increased in wounded skin (Fig. 2b). Given that both BrdU-incorporated ESCs and ACE-positive cells reside and exhibit similar changes in cell number in the skin basal layer, it is likely that captopril suppresses ESC growth via inhibiting endogenous ESC ACE activity.

Collectively, these results suggest that the endogenous ESC ACE-Ang II axis is critical for maintaining function, and that Ang II signaling suppression may impair this and impede skin wound healing in rats. Thus, the observed ACE inhibitor- or AT1R blocker-induced retardation of skin wound healing is linked to the suppression of ECS function in rodent skin [27,28].

3.2. Characterization of ACE, AT_1 and AT_2 receptors in cultured human ESCs

Since we previously demonstrated that ACE-positive cells are also located in the basal layer of adult human skin [10], these findings in rats may be extrapolated to human skin wound healing. To explore this, we first determined RAS functionality in human ECS. After adaption of human ESCs as previously reported (Fig. S1), we determined ACE, AT_1R

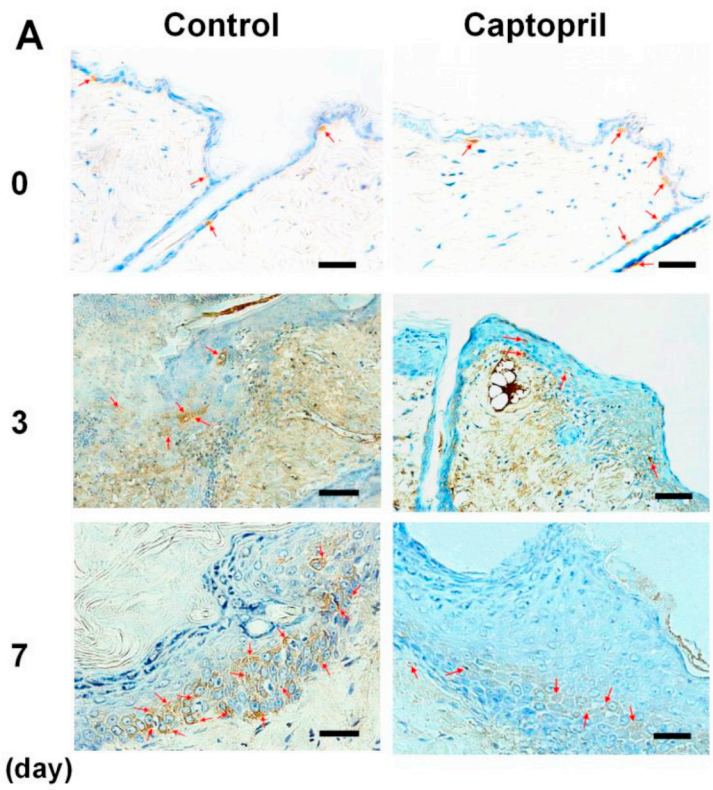
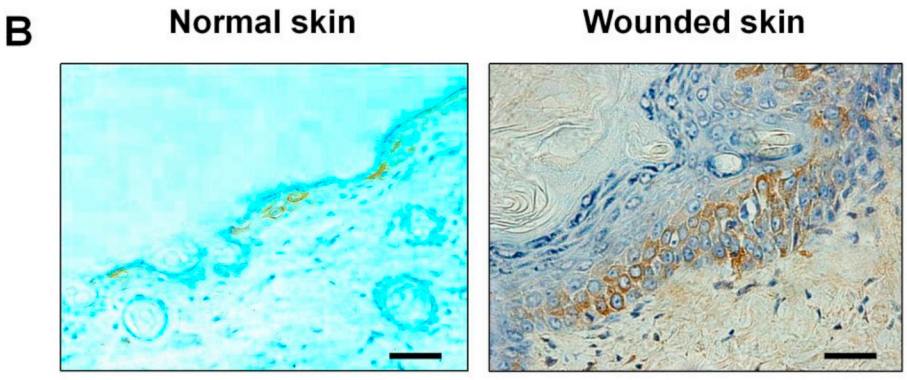


Fig. 2. Effect of captopril on the number of BrdU-incorporated cells at the wound edges of regenerative epithelia.
Proliferation analysis was performed as described in Materials and methods.
(a) Left panels: Representative immunostaining of paraffin-embedded sections, using mouse monoclonal antibodies against BrdU (red triangles). Right panels: The percentage of BrdU-incorporated cells was counted per high power view. Values are expressed as the mean ± SEM (n = 7). *$P < 0.05$ vs control. Scale bars indicate 50 μm.
(b) Immunostaining of ACE in the wounded skin of rats. Representative immunostaining of paraffin-embedded sections, using polyclonal antibodies directed against rat ACE. Positive ACE staining in normal skin was located only in the basal cell layer. Immunosignals for ACE were found in the spinous and granular layers of the regenerated epithelia in wounded sites at 7 days after wounding. Scale bars indicate 50 μm.



and AT_2R expression in these cells. PCR analysis demonstrated ACE1, ACE2, AT_1R and AT_2R mRNA levels (Fig. 3a). Immunocytochemical staining revealed that ACE was located mainly in the plasma membrane and cytoplasm, and flow cytometry demonstrated that up to 74% of cultured human ESCs were ACE-positive (Fig. 3b). Importantly, Ang II was secreted by cultured human ESCs, and was suppressed by the addition of captopril (Fig. 3c). These results show the existence of a functional RAS in human ESCs.

3.3. Critical role of the ACE-Ang II-Ang II R axis in maintaining human ESC function

Thereafter, we determined the functional significance of the ACE-Ang II signaling axis in the regulation of human ESC function in vitro. Suppression of Ang II production by captopril in human ESCs led to inhibition of their proliferation, colony formation, adhesion, and migration while minimally regulating their viability and differentiation (Fig. 4). In addition, valsartan (Kemikalieimport, Lyngby, Denmark), an AT_1R blocker, inhibited proliferation, colony formation, adhesion, and migration in human ESCs, while in contrast PD123319 (Kemikalieimport), an AT_2R blocker, exerted an opposite effect (Supplementary Fig. 2). These results demonstrate that Ang II-mediated actions are regulated by a negative cross-talk between AT_1R and AT_2R in human ESCs.

3.4. Ang II-induced signaling for maintaining human ESC function

Several signaling cascades, such as extracellular signal-regulated kinase (ERK), signal transducers and activators of transcription (STAT) 1 and STAT3, and Akt, were demonstrated to be critical for Ang II-mediated hemodynamic actions as well as cell proliferation, differentiation, and migration [29,30]. Therefore, we determined the effect of exogenous Ang II on the activation of mitogen-activated protein kinases (MAPK), STATs, and Akt in human ESCs. Exogenous Ang II mimicked endogenous Ang II-mediated ESC proliferation and migration (Fig. S2). Captopril inhibited ERK, STAT1, and STAT3 while minimally regulating Akt (Fig. 5), and Ang II activated ERK, STAT1, and STAT3, without affecting Akt activity (Fig. 5). Ang II-induced signaling was

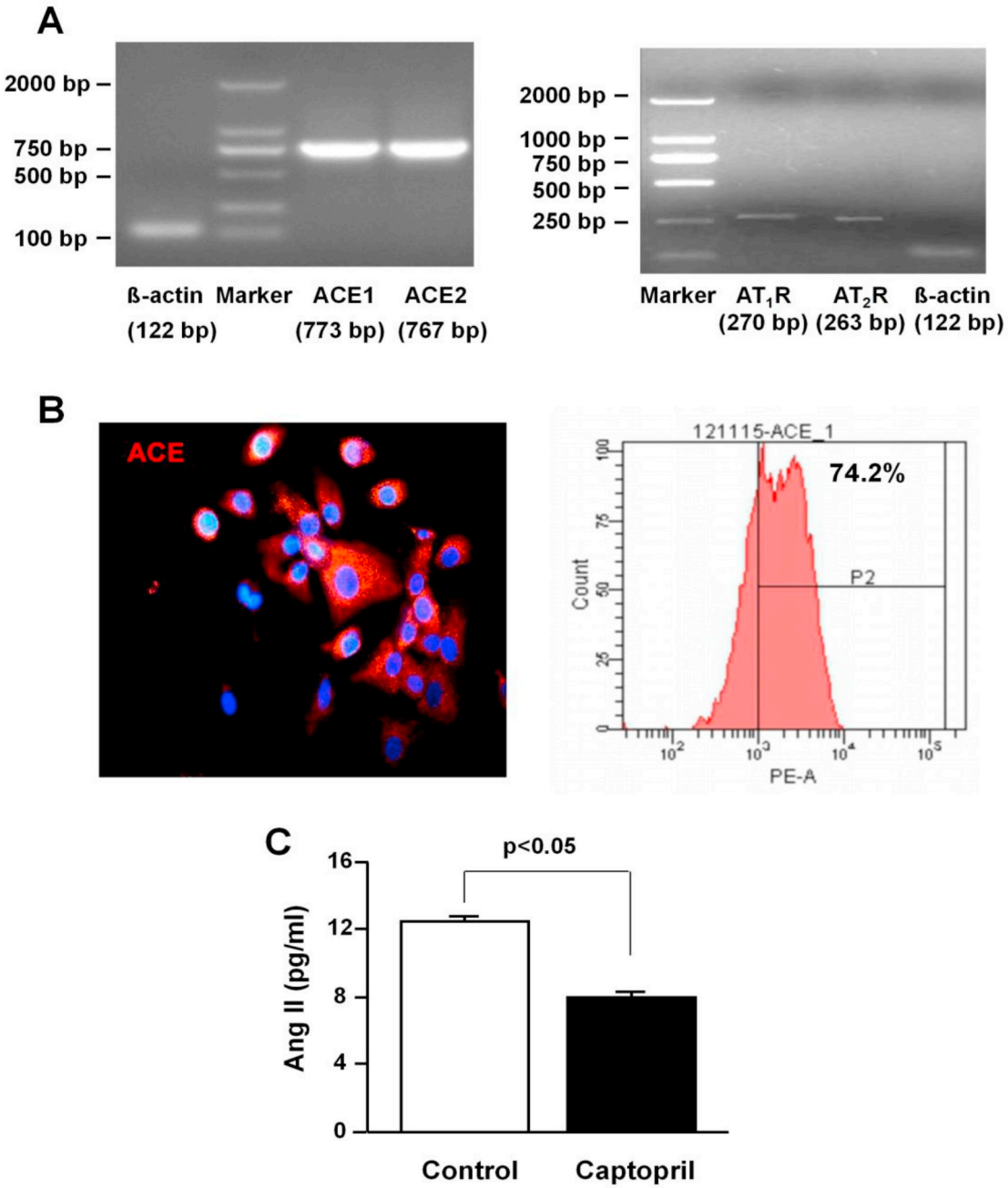


Fig. 3. Expression of ACE, AT1 and AT2 receptors in cultured human ESCs.
(a) RT-PCR showing the mRNA levels of ACE1 and ACE2 as well as AT1 and AT2 receptors.
(b) Representative immunofluorescence staining of paraffin-embedded sections, using polyclonal antibody directed against human ACE. Red indicates positive staining for ACE. Blue indicates staining of cell nuclei. The cultured ESCs were sorted using fluorescence-activated cell sorting, and 74.20% of the total cells were positive cells for ACE.
(c) Effect of captopril on the production of Ang II in cultured human ESCs. The production of Ang II in the media of cultured ESCs was measured by ELISA. Values are the mean ± SEM of three experiments. *$P < 0.05$ vs control.

ameliorated by valsartan and augmented by PD123319 (Fig. 5). These results indicate that Ang II maintains functional human ESC integrity at least partially by activation of ERK, STAT1 and STAT3 signaling via negative cross-talk between AT_1R and AT_2R.

4. Discussion

Herein, we provide the first evidence for an ACE-Ang II-Ang II R axis in maintaining ESC self-renewal and regeneration potential for skin wound healing. More specifically, captopril-induced retardation of epidermal regeneration was associated with a decreased number of BrdU-incorporated cells in the wound edge during rat wound healing. BrdU-positive and ACE-expressing cells resided in similar locations and exhibited similar cell number changes in wounded rat skin. Furthermore, an endogenous RAS in human ESCs played a critical role in maintaining their self-renewal and regeneration potential. Ang II induced ERK and STAT1 and STAT3 signaling via negative AT_1R and AT_2R cross-talk in human ESCs. These results highlight the functional significance of endogenous RAS in skin homeostasis and wound healing.

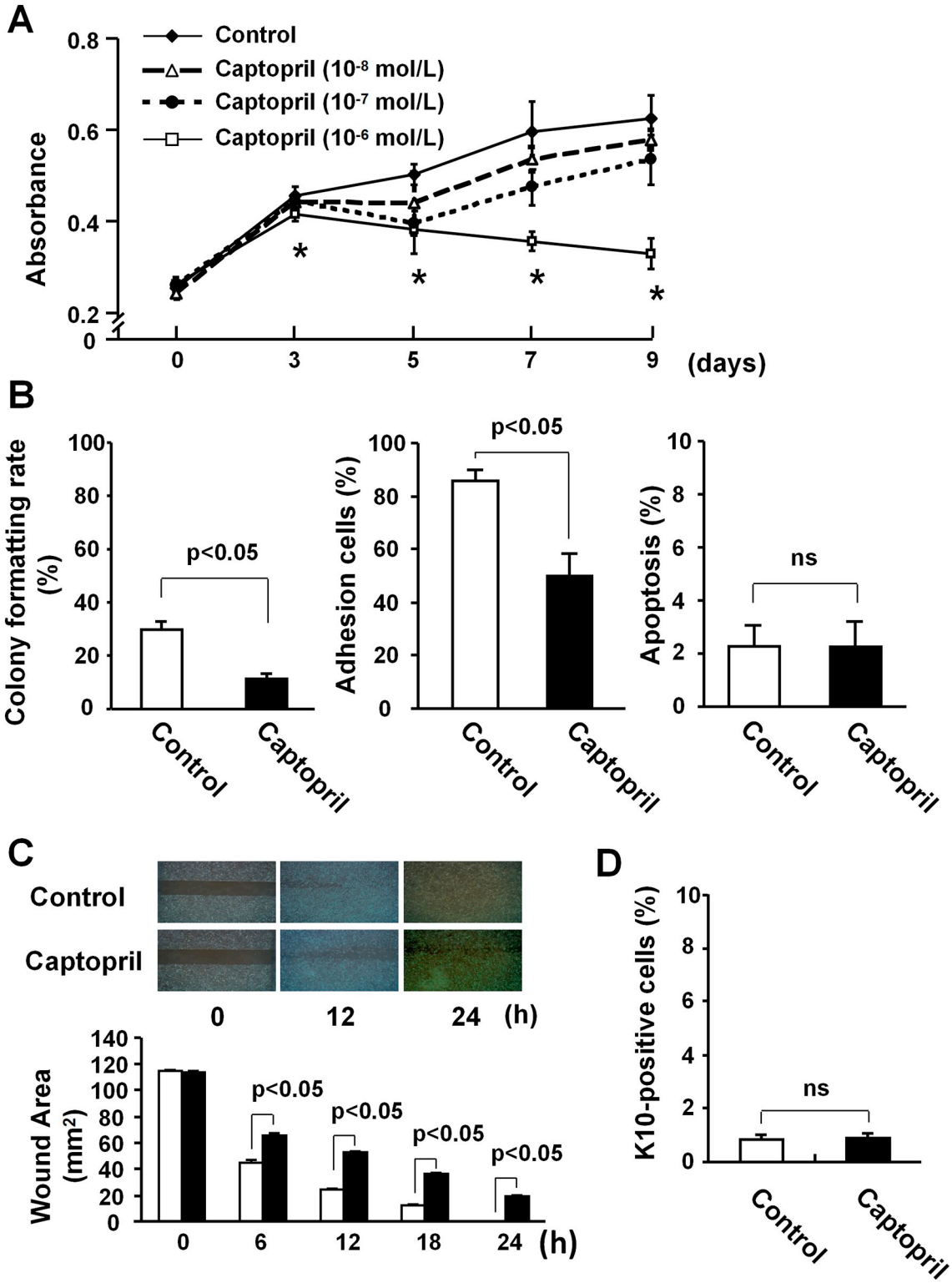


Fig. 4. Effect of captopril on the proliferation, colony-forming efficiency, adhesion, apoptosis, migration and differentiation of the cultured human ESCs.
(a) Effect of captopril on ESC proliferation. Values are means ± SEM of three independent experiments. $^{*}P < 0.05$ vs control.
(b) Effect of captopril on colony-forming efficiency, adhesion and apoptosis of cultured ESCs. Values are means ± SEM of three independent experiments. $^{*}P < 0.05$ vs control. $^{ns}P > 0.05$ vs control.
(c) Effect of captopril on ESC migration. Upper: Representative images 0, 12, and 24 h after wounding. Lower: Histogram of ESC migration after 0, 6, 12, 18 and 24 h. Values are means ± SEM of five samples. $^{*}P < 0.05$ vs control.
(d) Effect of captopril on ESC differentiation. Values are means ± SEM % of results in three replicate experiments. $^{ns}P > 0.05$ vs control.

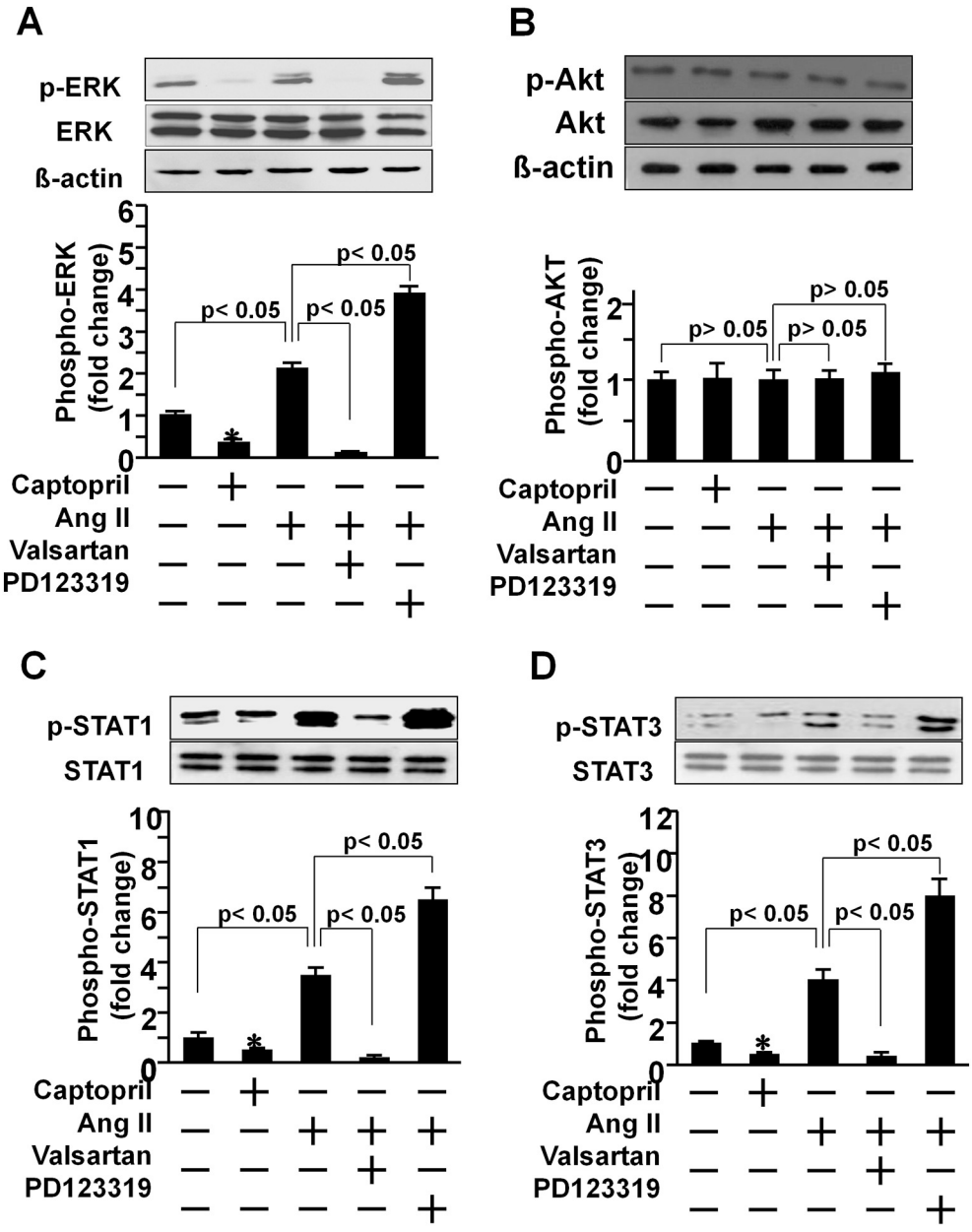


Fig. 5. Effect of captopril or Ang II on the phosphorylation of ERK, STAT and Akt in cultured human ESCs.
Cultured ESCs were treated with captopril (10^{-6} mol/L), Ang II (10^{-6} mol/L), valsartan (10^{-6} mol/L) and PD123319 (10^{-6} mol/L) for 5 min. ERK, STAT, and Akt phosphorylation was measured by western blotting. All values are means ± SEM. *$P < 0.05$ vs control. p, phosphorylation.
(a) Upper: Representative results from three separate experiments. Lower: Densitometric measurement of ERK.
(b) Upper: Representative results from three separate experiments. Lower: Densitometric measurement of Akt.
(c) Upper: Representative results from three separate experiments. Lower: Densitometric measurement of STAT1.
(d) Upper: Representative results from three separate experiments. Lower: Densitometric measurement of STAT3.

Many epidermis-resident stem cells are involved in skin wound healing [31]. ESCs are recruited to the epidermis and migrate to the wound center, thereby contributing to newly formed epidermis [32]. But the underlying mechanisms were unclear. Consist with our previous finding of ACE expression in proliferating human ESCs during epidermis development [10], we herein demonstrated that ACE activity is required for maintaining human ESC self-renewal and regeneration potential, suggesting that ACE promotes epidermal regeneration during wound healing. Additionally, Ang II maintained human ESC function by activating AT_1R and AT_2R which negatively regulate each other. This negative cross-talk was observed previously in Ang II-mediated proliferation and migration of human keratinocytes and dermal myofibroblasts [27,33] and in Ang II-mediated regulation of collagen production in neonatal mouse skin fibroblasts [34]. While AT_1R signaling inhibition may ameliorate Ang II-mediated beneficial effects in the skin, the consequences of AT_2R activation during AT_1R inhibition are uncertain. The topical application of 1% AT_1R inhibition valsartan gel, compared with other tested formulations or placebo, facilitated and significantly accelerated closure time and increased tensile strength in mice, and was validated in the porcine model [35]. The RAS is known to be dysregulated in diabetes, with increased AT_1R and decreased AT_2R expression in diabetic wound healing, which may play a role in the skin vulnerability associated with diabetes [36–38]. Diabetic patients exposed to ACE inhibitors are more likely to develop foot ulcers than those exposed to ARB, indicating a potential role of AT_2R-mediated protection in a diabetic setting [39]. at the same time, topical application of captopril has also already been shown to delay healing of wounds in diabetic mice [35]. Because diabetes could induce the dysfunction of several stem cells including ECSs [40–42], it is intriguing whether AT_2R activation in ESCs enhances its function, thereby facilitating healing of diabetic refractory skin wounds.

Skin and its specific receptors play an important role in tissue self-renewal and wound repair through paracrine and autocrine actions [43]. The RAS also functions in an autocrine and paracrine fashion in regulating local hemodynamics, cell growth and tissue remodeling, and neurotransmitter release [44]. ESCs regulate skin turnover, repair, and remodeling [45]. and serve as target cells for neuro-humoral endocrine effects. They also accept neurological signal transduction, and exert

endocrine effects through specific signaling regulation. Studies suggested that Ang II functions as a cell growth regulator, and that its signal-modulating pathway is mainly accomplished through Ang II receptors. Selective AT_1R blockers significantly inhibited the re-epithelialization and angiogenesis of keratinocytes during the healing of rat skin wounds, but inducing AT_1R signaling accelerated keratinocyte re-epithelialization, while AT_2R induction had the opposite effect. Thus, skin wound healing is regulated by the balance between AT_1R and AT_2R antagonism [46]. the specificity of PD123319 for AT2 has been questioned [47], further study need to be done to explore the possible effect of PD123319 on MAS and MrgD receptors during wound healing. We previously reported that AT_1 and AT_2 receptors play opposite roles in activating PI3K/Akt cascades of skin fibroblasts derived from patients with hypertrophic scarring [15]. Here, we propose that Ang II promotes ERK, STAT1, and STAT3 phosphorylation through AT_1R, whereas Ang II inhibits ERK, STAT1, and STAT3 phosphorylation through AT_2R. Ang II regulates the ESC function through AT1 and AT2 receptors, thereby affecting self-renewal and regeneration of skin epidermis after injury. Our study reveals a role for ACE in the repair of skin lesions, and identifies the potential mechanism of skin side effects produced by oral ACE inhibitors.

Transparency document

The Transparency document associated this article can be found, in online version.

Declaration of Competing Interest

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.07.006.

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