Platelet Rich Plasma-Derived Exosomal USP15 Promotes Cutaneous Wound Healing via Deubiquitinating EIF4A1

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Research

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Abstract

Background: Epithelial regeneration is an essential wound healing process, and recent work suggests that different types of exosomes (Exos) can be employed to improve wound repair outcomes by promoting such epithelial regeneration. Platelet-rich plasma (PRP) is known to facilitate enhanced wound healing, yet the mechanisms underlying its activity are poorly understood.

Results: We found that PRP-derived exosomes (PRP-Exos) effectively promoted the *in vitro* proliferation, migration, and wound healing activity of human immortalized keratinocytes (HaCaT cells). USP15 was further identified as a key mediator through which these PRP-Exos were able to promote tissue repair both *in vitro* and *in vivo.* At a mechanistic level, USP15 enhanced the functional properties of HaCaT cells by promoting EIF4A1 deubiquitination.

Conclusions: PRP-Exos and USP15 represent promising tools that can promote wound healing via enhancing epithelial regeneration.

Introduction

Chronic wounds are wounds that do not heal appropriately and that persist for 3 months or longer. [1,2] Such chronic wounds most commonly arise in the context of conditions such as diabetes, arterial ischemia, poor venous return, infection, pressure ulcers, and malignant tumors, resulting in pain for the affected patient while also compromising essential barriers to bacterial entry into the human body.[3] As such, chronic wounds can reduce patient quality of life while also imposing a significant economic burden on their families and on society as a whole.[4] Standard treatments for chronic wounds are still lacking, although keratinocytes are known to be essential mediators of normal wound healing processes.[5,6] Targeted efforts to accelerate re-epithelialization in the context of wound repair thus represent a key area of ongoing scholarly research.

Platelet-rich plasma (PRP) contains a diverse array of physiologically important growth factors at high concentrations, including transforming growth factor-β (TGF-β), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF).[7,8] The therapeutic application of PRP has been linked to accelerated angiogenesis and re-epithelialization, thereby promoting wound healing at a faster rate.[9] The mechanisms whereby PRP can promote such wound healing, however, are unclear. In addition to the abovementioned growth factors, platelets can produce a variety of different extracellular vesicles, including exosomes.[10] Exosomes, which are small vesicles that are approximately 100 nm in diameter (range: 40–160 nm), have been a focus of intensive research interest in recent years owing to their essential role as mediators of cell-cell communication, shuttling nucleic acids, proteins, metabolites, and other macromolecules between cells such that they hold promise as tools for treating a range of diseases. [11–14] Owing to the unique biological properties of PRP and of exosomes, further research is warranted to establish the ability of PRP-derived exosomes to shape wound healing processes *in vitro* and *in vivo.*

The present study was therefore designed to evaluate the impact of PRP-derived exosomes (PRP-Exos) on epithelial cell function to explore the mechanistic basis for such activity *in vitro* and *in vivo* in the context of wound healing.

Results

PRP-Exo characterization

Transmission electron microscopy (TEM, dynamic light scattering (DLS) analyses, and western blotting were initially used to characterize isolated PRP-Exo samples. The obtained PRP-Exos ranged from 40–100 nm in size (Fig. 1a, b), with the majority of these particles exhibiting an expected cup-shaped or spheroid morphology. Western blotting analyses indicated the presence of the exosomal markers CD9 and TSG101 in these samples (Fig. 1c), thus confirming the successful enrichment of exosomes from PRP samples.

PRP-Exo treatment enhances in vitro **keratinocyte responses**

The impact of PRP-Exo treatment on immortalized human keratinocytes (HaCaT cells) was next assessed. To assess the uptake of these exosomes by HaCaT cells, these particles were initially labeled using the lipophilic PKH26 dye, following by incubation in cell culture media for 8 h at which time the uptake of these fluorescent particles was clearly evident (Fig. 2a). HaCaT cells were then treated with PBS, PRP, or PRP-Exos, and their proliferation was assessed through a series of CCK-8 and EdU uptake assays, confirming that PRP-Exo exposure was associated with the enhanced proliferation of these keratinocytes (Fig. 2b, c). PRP-Exo treatment was associated with an increase in the frequency of HaCaT cells entering the S stage of the cell cycle (Fig. 2d). Following PRP-Exo treatment, higher levels of cell cycle-associated proteins were found to be expressed in these cells (Fig. 2e). In Transwell and wound healing assays, PRP-Exo treatment was also associated with significant improvements in HaCaT cell migration (Fig. 2f-h). Together, these data thus indicate that PRP-Exo treatment can significantly augment HaCaT cell proliferation and migration.

PRP-Exo treatment enhances in vivo **wound healing in mice**

An *in vivo* cutaneous wound model was next generated using C57BL/6 mice, with equal volumes of PBS, PRP, or PRP-Exo preparations being injected around the wound site in each animal to assess the impact of such treatment on wound healing. Relative to control animals, those treated with PRP and PRP-Exos exhibited faster wound repair, with such healing being even faster for PRP-Exo-treated mice relative to mice treated with PRP alone (Fig. 3A, B). The scars of mice in the PRP-Exo group were smaller than those of mice in any other group (Fig. 3C), and these mice exhibited the highest levels of collagen formation (Fig. 3D). Re-epithelialization plays an essential role in the wound healing process.[15] Deubiquitinases (DUBs) can alter protein stability by removing a ubiquitin chain from a given protein, thereby stabilizing it,[16] potentially accelerating the process of re-epithelialization in the context of wound healing. The

ubiquitin-specific protease (USP) family is the best-studied DUB family of proteins, of which USP15 is an important member.[17] As such, we evaluated the expression of USP15 in keratinocytes in wounded skin tissues, revealing it to be expressed at higher levels in the PRP-Exo treatment group (Fig. 3E).

USP15 is enriched in PRP-Exo preparations

To explore the functional importance of USP15 as a driver of PRP-Exo-mediated wound healing, we assessed the levels of this protein in PRP and PRP-Exo samples via Western blotting, revealing it to be present at significantly higher levels in PRP-Exos (Fig. 4a). Subsequent qPCR analyses additionally exhibited increased USP15 expression in PRP-Exos at the mRNA level (Fig. 4b). Similarly, higher USP15 protein levels were evident in epidermal keratinocytes in the PRP-Exo group *in vivo* (Fig. 4c, Fig. 3E), suggesting that USP15 may be linked to the ability of PRP-Exos to promote wound healing.

PRP-Exos promotes HaCaT cell migration and proliferation in a USP15-dependent manner

To understand the functional effects of USP15 on HaCaT cells, they were next treated with PBS, PRP, PRP-Exos. Western blotting indicated that USP15 levels were highest for HaCaT cells treated with PRP-Exos (Fig. 5a), As show in Fig. 1, HaCats treated with PRP-Exos can significantly augment cell proliferation and migration. In contrast, siUSP15 treatment had the opposite effect on HaCaT cell proliferation and migration in these same assay systems (Fig. 5b-e). As such, decreasing USP15 expression in HaCaT cells can reduce the beneficial impact of PRP-Exo treatment. Together, these findings suggest that USP15 is the primary mediator whereby PRP-Exos promote HaCaT cell migration and proliferation.

USP15 enhances HaCaT cell functionality by promoting EIF4A1 deubiquitination

We next explored the mechanisms whereby USP15 promotes HaCaT cell functionality. EIF4A1 is a key eukaryotic initiation factor complex component that has been linked to the proliferation of certain cell lines,[18,19] although it has not been studied in detail in HaCaT cells. As such, we hypothesized that USP15 may be able to promote HaCaT cell proliferation in part via altering EIF4A1 expression. To examine the effects of USP15 on EIF4A1 stability in HaCaT cells, we conducted Western blotting assays which revealed a significant decrease in both USP15 and EIF4A1 expression following siRNA-USP15 treatment (Fig. 6a, b), whereas a qPCR assay indicated that the EIF4A1 mRNA levels in these cells were unchanged (Fig. 6c).

We then assessed the functional importance of EIF4A1 in HaCaT cells by knocking down this gene with a specific siRNA construct. Subsequent CCK8 and EdU assays suggested that EIF4A1 knockdown was sufficient to suppress HaCaT cell proliferation (Fig. 6d, e), with Western blotting being used to confirm that EIF4A1 protein levels were reduced following siRNA transfection (Fig. 6f). In Transwell and wound healing assays, siRNA EIF4A1 treatment significantly reduced the migratory activity of these

keratinocytes (Fig. 6g, h). Together, these data suggested that USP15 can enhance the migration and proliferation of HaCaT cells by promoting EIF4A1 deubiquitination.

USP15 promotes wound healing in vivo

To examine the impact of USP15 on wound healing processes, equivalent amounts of PBS, siRNA-NC, siRNA-USP15, siRNA-USP15, or PRP-Exos were injected surrounding wound sites in C57/BL6 mice, as above. USP15 knockdown was found to significantly slow the wound healing process in these animals (Fig. 7a, b), and H&E staining confirmed that wounds in the siRNA-USP15 group were larger than in other groups (Fig. 7c). Masson's trichrome staining also revealed that collagen levels were lowest in the siRNA-USP15 group (Fig. 7d), and the number of USP15-positive cells in murine wound sites was lower in siRNA-USP15 treated mice relative to other groups (Fig. 7e, f). Together, these results thus demonstrated that USP15 knockdown was sufficient to largely ablate the beneficial effects of PRP-Exo treatment in the context of *in vivo* cutaneous wound healing.

Discussion

A number of clinical strategies have been employed in recent years in an effort to accelerate wound healing.[20] PRP-based therapy has been employed as one such approach to promoting wound healing, having been studied in the fields of orthopedics, dermatology, dentistry, and diabetic wound management. However, there have been few studies to date of PRP-Exos. Herein, we determined that PRP-Exo treatment was sufficient to promote wound healing in a manner more efficient than direct PRP treatment. The process of epidermal regeneration is a multi-step process that is regulated by a range of cytokine and cell types, ultimately leading to the reconstruction of the damaged skin barrier.[21] Exosomes derived from mesenchymal stem cells have been repeatedly shown to accelerate re-epithelialization and to thereby enhance wound healing.[22,23] Our present results indicated that PRP-Exo treatment similarly promoted enhanced epithelialization, highlighting the promise of these particles for use in the treatment of chronic wounds.

Exosomes are small, lipid bilayer-enclosed extracellular vesicles that are produced by most known cell types and that can transmit macromolecules and other compounds between cells.[24] When taken up by recipient cells, these exosomes can thus alter cellular functionality via the delivery of specific proteins, nucleic acids, and signaling molecules in a manner that makes them ideal for use as drug carriers in a range of disease types.[23] Herein, we found that HaCaT cells were able to efficiently internalize PRP-Exos.

Protein ubiquitination has been shown to play diverse regulatory roles in the context of wound healing.[25,26] USP family proteins, including USP15, are key mediators of protein deubiquitination. Others have reported that PRP can promote wound healing by driving accelerated epithelialization,[27] and we herein found that PRP-Exo treatment was superior to PRP treatment as a means of enhancing wound healing in a manner associated with increased USP15 protein levels in wound tissues relative to those detected upon PBS or PRP administration. Through a series of *in vitro* and *in vivo* experiments, we further

confirmed that USP15 was able to promote wound healing, suggesting that PRP-Exo-derived USP15 is a key mechanism driving this regenerative process. Tao et al. have reported that PRP-Exos can suppress apoptosis in a rat model of femoral head osteonecrosis via the Akt/Bad/Bcl-2 signal pathway,[28] while Shama et al. revealed the ability of PRP-Exo treatment to promote functional recovery following muscle injury.[29] Owing to their unique properties, exosomes have been shown to be of value for the treatment of many diseases, and thus warrant further clinical study.

In prior studies, USP15 was shown to interact with EIF4A1 and to thereby accelerate wound healing.[30] Consistent with such a model, we found that USP15 knockdown was sufficient to reduce EIF4A1 expression, while EIF4A1 knockdown directly impaired HaCaT cell migration and proliferation. Together, these results suggest that the USP15-EIF4A1 axis is a key mediator of the re-epithelialization process.

Conclusion

In conclusion, the results of this study indicate that PRP-Exo-derived USP15 is a key mediator of HaCaT cell survival and migratory activity, with EIF4A1 playing an important role in the process of USP15-induced epithelializationin (Fig. 8). Together, these findings provide a robust foundation for future studies of the therapeutic potential of PRP-Exo treatment as a means of promoting improved wound healing.

Material And Methods

Cell culture

HaCaT cells were purchased from the China Center for Type Culture Collection, Wuhan, China, and were cultured in DMEM containing 10%FBS in a 5% CO_2 incubator at 37°C. Lipofectamine was used to transfect cells with siRNA constructs (50 umol/L; GenePharma, Shanghai, China).

PRP preparation

Mice (n = 12) were anesthetized via intraperitoneal injection with 1% sodium pentobarbital, after which blood was collected from the abdominal vena cava into a 1 mL syringe containing 0.1 mL of anticoagulant. The collected blood was transferred into a fresh 1.5 mL tube and stored at 4°C until the collection was completed, at which time samples were spun for 10 min at 100 xg at 4°C. The supernatant and the layer containing the white blood cells were then collected from each tube and transferred to a new tube, leaving the red blood cell pellet undisturbed. In this case, normal plasma is obtained.These samples were then spun again for 10 min at 600 xg at 4°C, after which ¾ of the supernatant was discarded with the remaining sample being gently mixed to yield PRP.

PRP-Exo isolation and characterization

Samples of PRP (1.5 ml) were spun for 30 min at 2,000 xg, after which supernatants were collected, spun for 45 min at 12,000 xg at 4°C, and supernatants were then passed through a 0.45 μm filter membrane,

after which they were spun for an additional 80 min at 13,000 xg. Supernatants were then discarded, while pellets were resuspended in PBS, spun for 70 min at 110,000 xg, and the remaining particles were suspended in chilled PBS for subsequent analysis. For transmission electron microscopy analyses, these exosomes were combined for 30 min with osmium tetroxide (4%) at 4°C in a 50 μL volume, after which they were transferred onto a copper grid. Next, 1% phosphotungstic acid was utilized to stain these particles, and a transmission electron microscope (Hitachi, HT-7700) was used for their characterization. A NanoFCM ™ instrument (N30E) was used for DLS analyses. Exosome marker protein expression was assessed via Western blotting. Exosomes were pooled for use in subsequent experiments. Normal plasma exosomes were isolated in the same manner.

CCK-8 assay

HaCaT cells were cultured in 96-well plates (5×10^3) for 24, 48, or 72 h, after which they were treated with the CCK-8 reagent for 2 h (G4103, Servicebio). Absorbance was then measured at 450 nm to assess cell proliferation.

EDU assay

HaCaT cells were plated in 24-well plates (1×10^5 /well) and treated as appropriate for 24 h, after which EdU staining was performed based on provided directions (G1601, Servicebio).

Wound healing assay

Following appropriate treatments, a HaCaT cell monolayer cultured in 6-well plates was scratched with a sterile 10 μl pipette tip to generate a wound. Cells were then incubated for 12 or 24 h, after which they were imaged via inverted microscope.

Transwell migration assay

Cells were added to the upper chamber of a Transwell filter with an 8 μm pore size (3×10^4/well) in 200 μl of serum-free media, after which the lower chamber was filled with 600 μL of media containing Exos or other appropriate reagents. Following incubation for 24 h, the number of migratory cells was assessed via light microscopy.

Cell cycle analysis

Cell cycle progression was assessed via flow cytometry using a cell cycle and apoptosis analysis kit (G1700. Use G1700-50T, Servicebio) based on provided directions.

Western blotting

After extracting total protein, 40 μg of protein per sample was separated via 10% SDS-PAGE and transferred to PVDF membranes that were stained overnight at 4°C with primary antibodies, after which they were probed for 1 h with HRP-conjugated secondary antibodies at 37°C. The following antibodies

were used: anti-CD9 (1:1000, Abcam, ab223052), anti-TSG101 (1:1000, Abcam, ab125011), anti-Calnexin, (1:1000, Abcam, ab22595), anti-USP15 (1:500, Abcam, ab71713), anti-EIF4A1 (1:1000, Abcam, ab31217)

Quantitative real-time PCR (qPCR)

TRIzol (Invitrogen) was used to isolate total RNA from prepared samples, after which 1 μg of this RNA was used to prepare cDNA. A StepOne™ Real-Time PCR system (Life Technologies, CA, USA) was used to conduct all qPCR reactions, with the $2^{-\Delta\Delta Ct}$ method being used to assess relative gene expression and GAPDH serving as a normalization control. Primer sequences were as follows: USP15, Forward: 5'-AAAACCTCGCTCCGGAAAGG-3', Reverse: 5'-CCACCTTTCGTGCTATTGG-3', EIF4A1, Forward: 5'-TGTCTGCGAGCCAGGATTCCC – 3', Reverse: 5'- AGATGCCACGGAGAAGGGACTC – 3'

Murine cutaneous wounding model establishment

Male C57BL/6 male mice (6–8 weeks old) from the Center of Experimental Animals, Tongji Medical College, Huazhong University of Science and Technology were anesthetized using pentobarbital sodium (50 mg/kg), after which a 10 mm-diameter full-thickness excisional skin wound was generated on the dorsum of each animal. Mice were then randomly assigned to five treatment groups that were treated with PBS (100 μl), PRP-Exos (100 μg PRP-Exos in 100 μl PBS), siRNA-NC (in PBS), siRNA-USP15 (in PBS), or siRNA-USP15 + PRP-Exos (in PBS). For siRNA-NC and siRNA-USP15 treatments, animals were administered 100 μl of a 20 μmol/L preparation of the corresponding siRNA, while for the siRNA-USP15 + PRP-Exos treatment group, mice were administered 100 μl of a 10 μmol/L siRNA preparation and 10μmol/L of PRP-Exos in PBS. All prepared solutions were injected subcutaneously in appropriate mice at four sites adjacent to the wounded area (25 μl/site). Images of the wounds were captured on days 0, 3, 7, 10, and 14 post-wounding. Animals were euthanized on day 14, at which time skin samples were collected for downstream analyses. Wound area was measured with the ImageJ software, and wound healing was calculated as follows: $\text{Wound healing} = \frac{Wound\ area\ on\ Day\ n}{Wound\ area\ on\ Day\ 0} \times 100$

The Animal Care and Use Committee of the Tongji Medical College, Huazhong University of Science and Technology approved all animal studies detailed herein.

Hematoxylin and eosin staining, Masson's trichrome staining, and immunohistochemical staining USP15

Paraffin-embedded tissue sections (7 μm-thick) were subjected to hematoxylin and eosin (H&E) and Masson's trichrome staining. An immunofluorescent approach was used to detect USP15 in prepared tissue sections. Briefly, prepared sections were blocked for 30 min with 1% BSA, probed overnight with anti-USP15 (1:500, Abcam, ab71713), stained for 1 h with an appropriate secondary antibody, and then USP15-positive cells area in three random fields of view were analysis. All stained tissue sections were independently assessed by three observers blinded to experimental treatment protocols.

Statistical analysis

Data are means ± SD, and were compared via Student's t-tests or ANOVAs with Tukey's post hoc test as appropriate. All analyses were performed with GraphPad Prism 8.0 (GraphPad Software, CA, USA), with $P < 0.05$ as the threshold of significance.

Declarations

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Author' contributions

DD, CXdeveloped designofthis study.YX, ZL, LHwere responsible for conducting experiments and data collection.YX, LO, ZL comtributed to the draft and pictures.DD, CX revised the manuscript and figures.All authors agree to submit this manuscript.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Notapplicable

Consent for publication

Allauthorsdeclare that they agree to be published

Competing interests

All authors report no conflicts of interest in this work.

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Figures

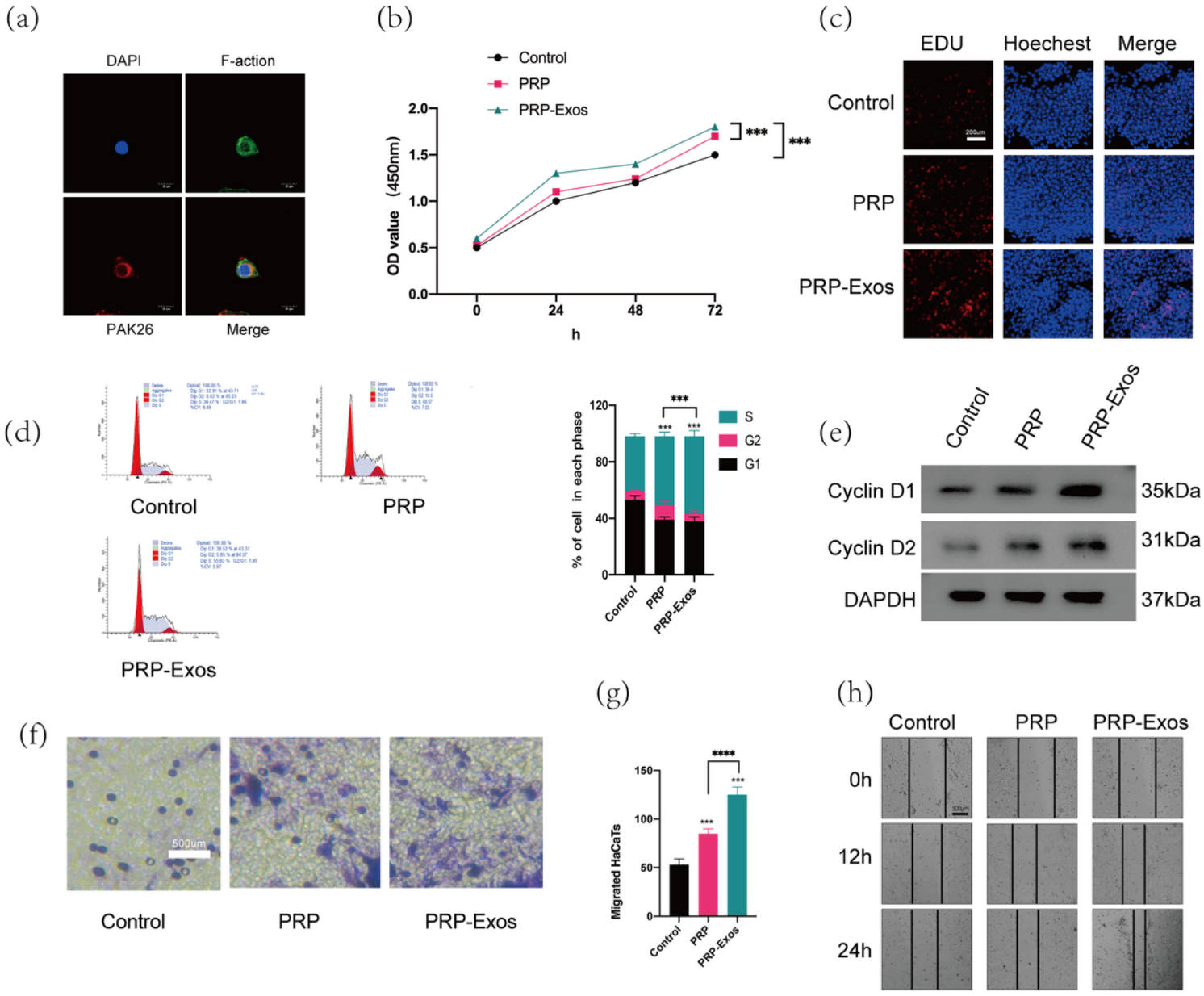

Figure 1

PRP-Exo characterization. (a) TEM images of PRP-Exos. (b) PRP-Exo size distributions, as measured via DLS. (c) Western blotting analyses of proteins within PRP-Exos.

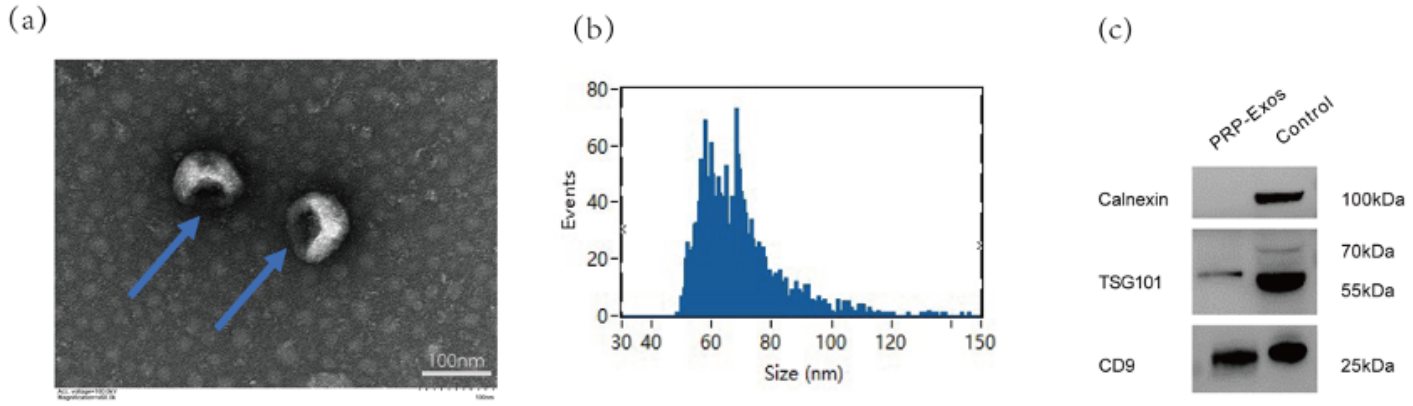

Figure 2

PRP-Exos enhance HaCaT cell proliferation and migration. (a) HaCaT cells were able to take up PKH26-labelled PRP-Exos. (b, c) CCK8 andEdU uptake assays were used to assess the impact of PRP-Exo treatment on HaCaT cells. (d) Flow cytometry was used to assess how PRP-Exos affected cell cycle progression. (e) Western blotting was used to assess levels of cell-cycle associated proteins (Cyclin D1 and cyclin D3) following PRP-Exo treatment. (f) Wound healing and (G, H) Transwell assays were used to gauge the impact of PRP-Exos on HaCaT cell migration. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

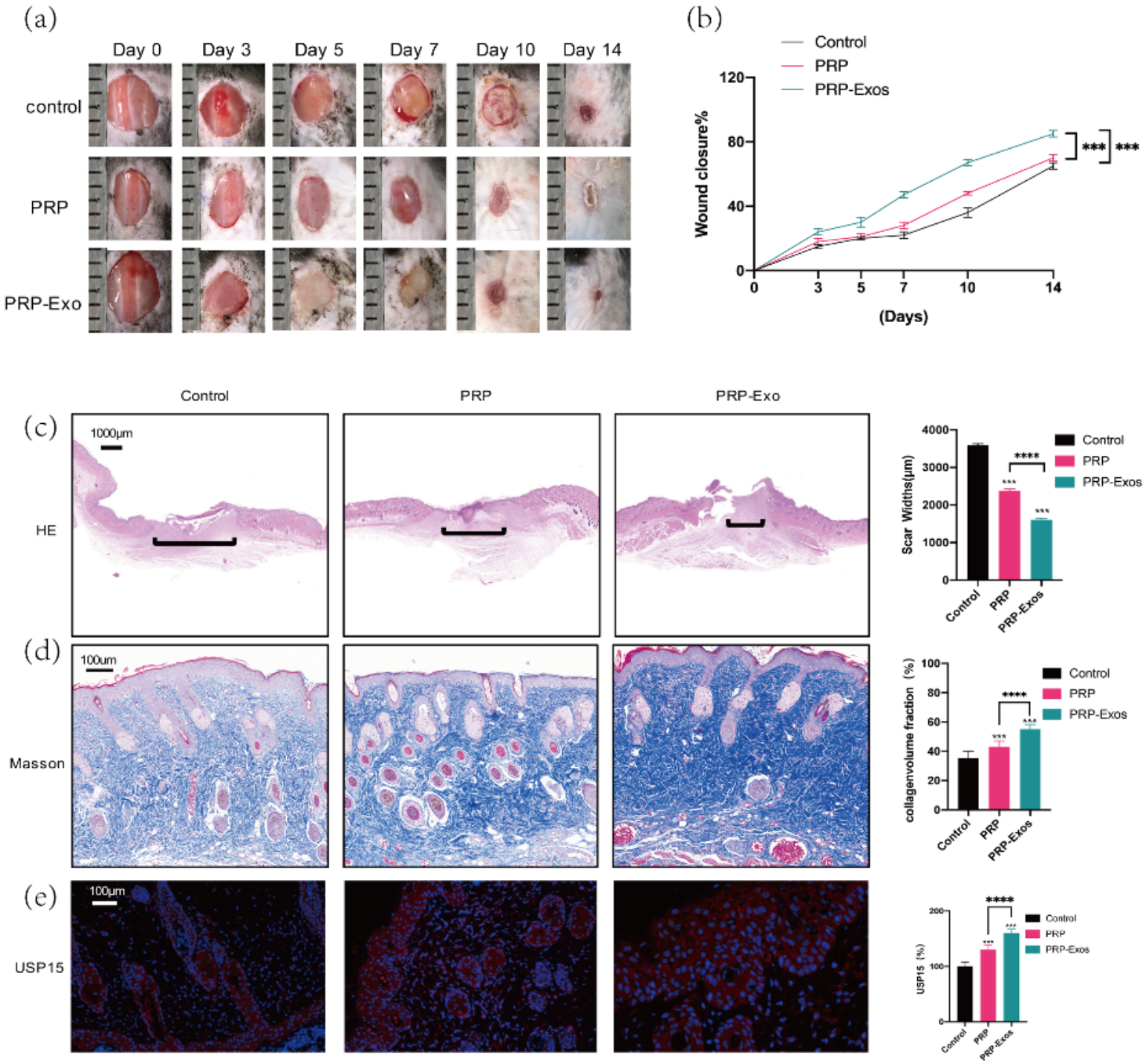

Figure 3

PRP-Exos promote wound healing in C57BL/6 mice. (a) Images of wounds in representative mice. (b) Wound healing rates in the control, PRP, and PRP-Exo treatment groups. (c, d) H&E staining and Masson's staining results from the three treatment groups with corresponding quantification results.(e) USP15 immunohistochemistry results for wound sections in the three treatment groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.

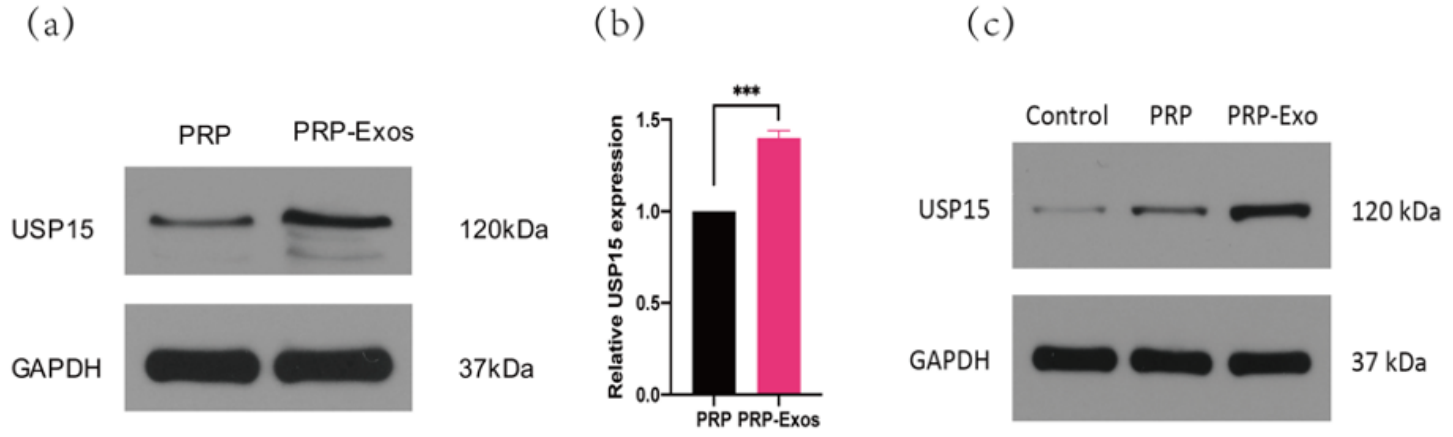

Figure 4

PRP-Exos contain high levels of USP15, which promotes enhanced HaCaT cell functionality. (a) Western blot results of PRP and PRP-Exos USP15 levels.(b) USP15 mRNA levels were assessed via qPCR. (c) USP15 levels in murine wound tissues were assessed via Western blotting.

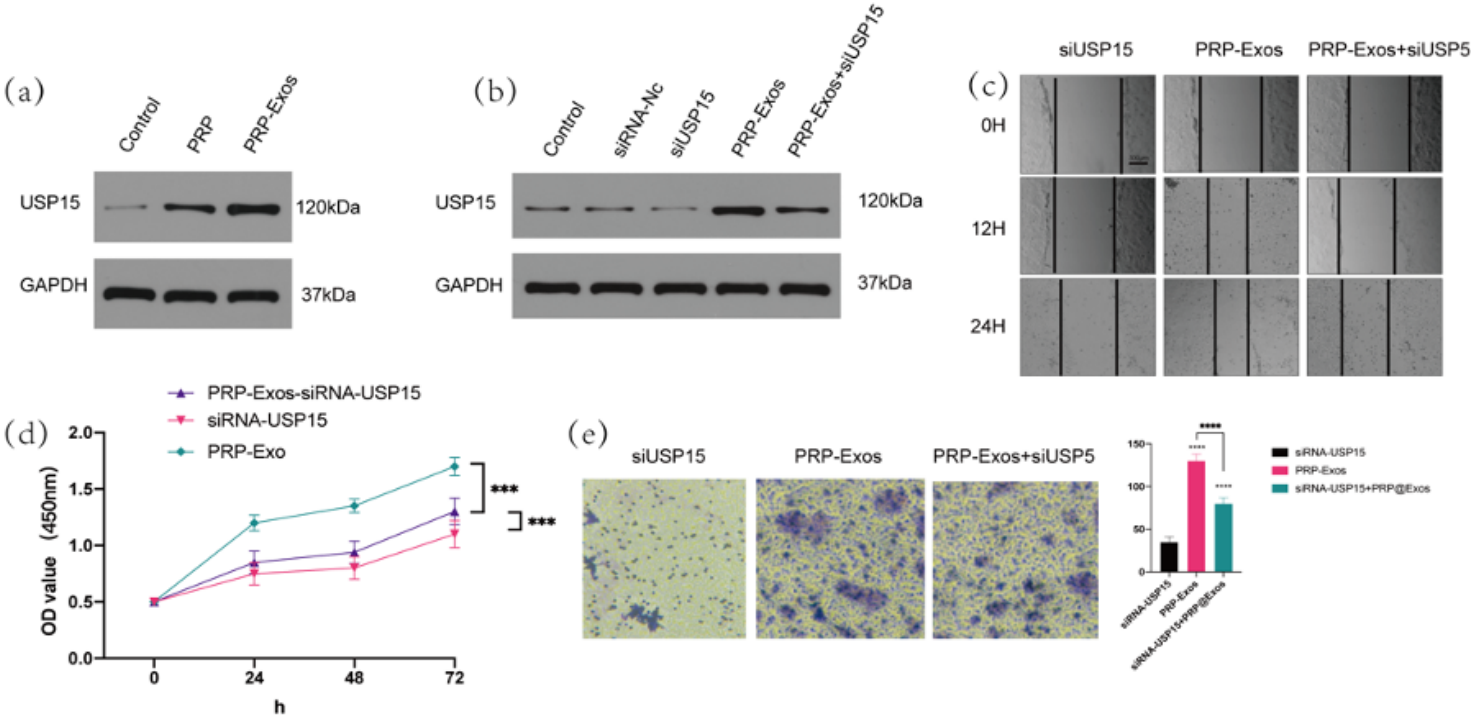

Figure 5

USP15 promotes in vitrowound healing. (a) Western blotting data demonstrating USP15 levels in Control, PRP, PRP-Exos groups. (c, e) Wound healing and Transwell assays were used to assess the migratory

activity of HaCaT cells. (d) The proliferation of HaCaT cells in the indicated treatment groups was assessed via CCK-8 assay.

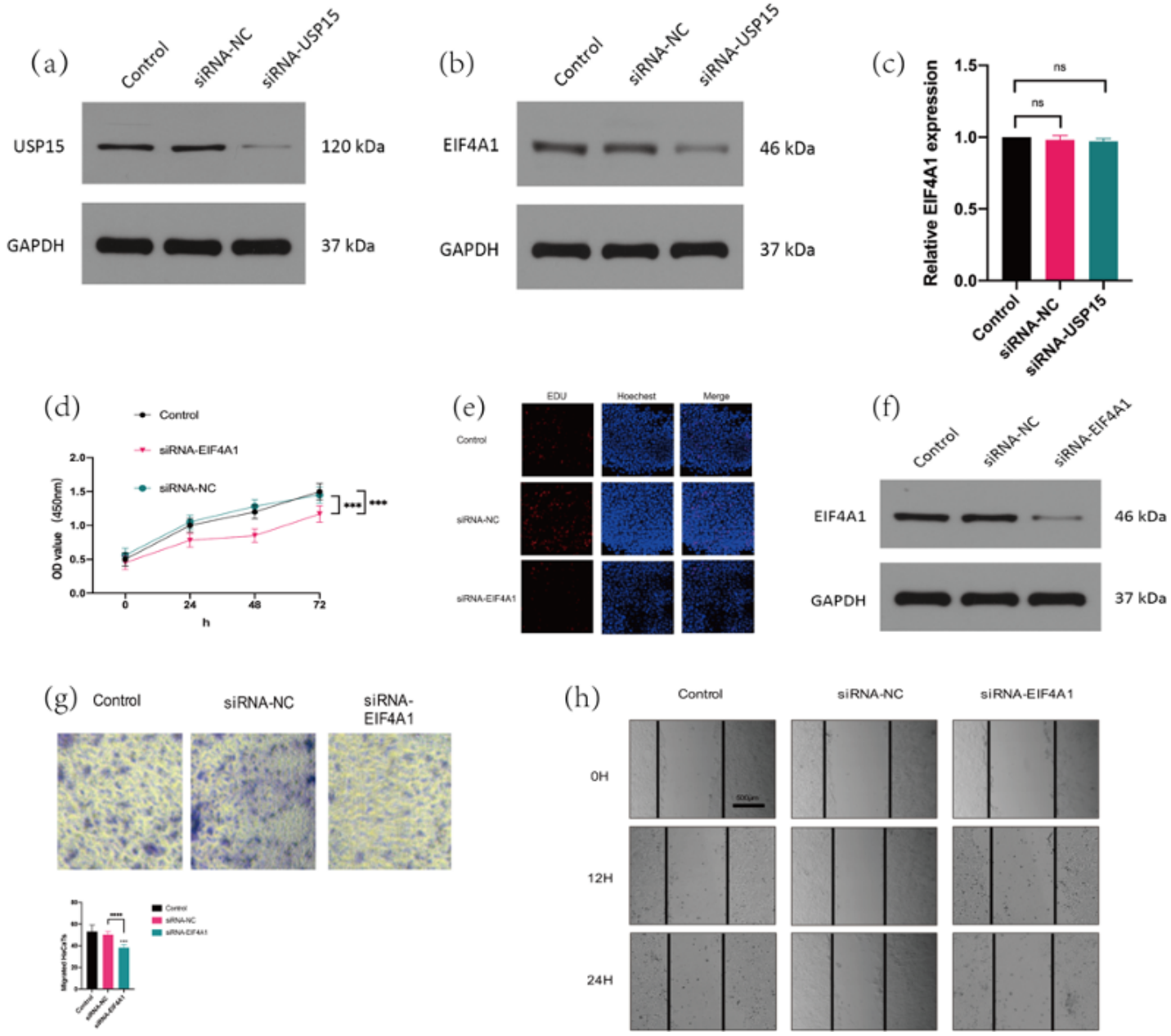

Figure 6

USP15 promotes EIF4A1 deubiquitination to enhance HaCaT cell functionality. (a, b) Western blotting results demonstrating USP15 and EIF4A1 levels in HaCaT cells following siRNA-USP15 treatment. (c) EIF4A1 levels HaCaT cells following siRNA-USP15treatment, as assessed via qPCR. (d, e) HaCaT cell proliferation was assessed via CCK-8 and EdU assays following siRNA-EIF4A1 treatment. (f) Western blotting analyses were used to assess EIF4A1 expression HaCaT cells following siRNA-EIF4A1 treatment. (g, h) Wound healing and Transwell assays were used to assess the migratory activity of HaCaT cells following siRNA-EIF4A1 treatment.

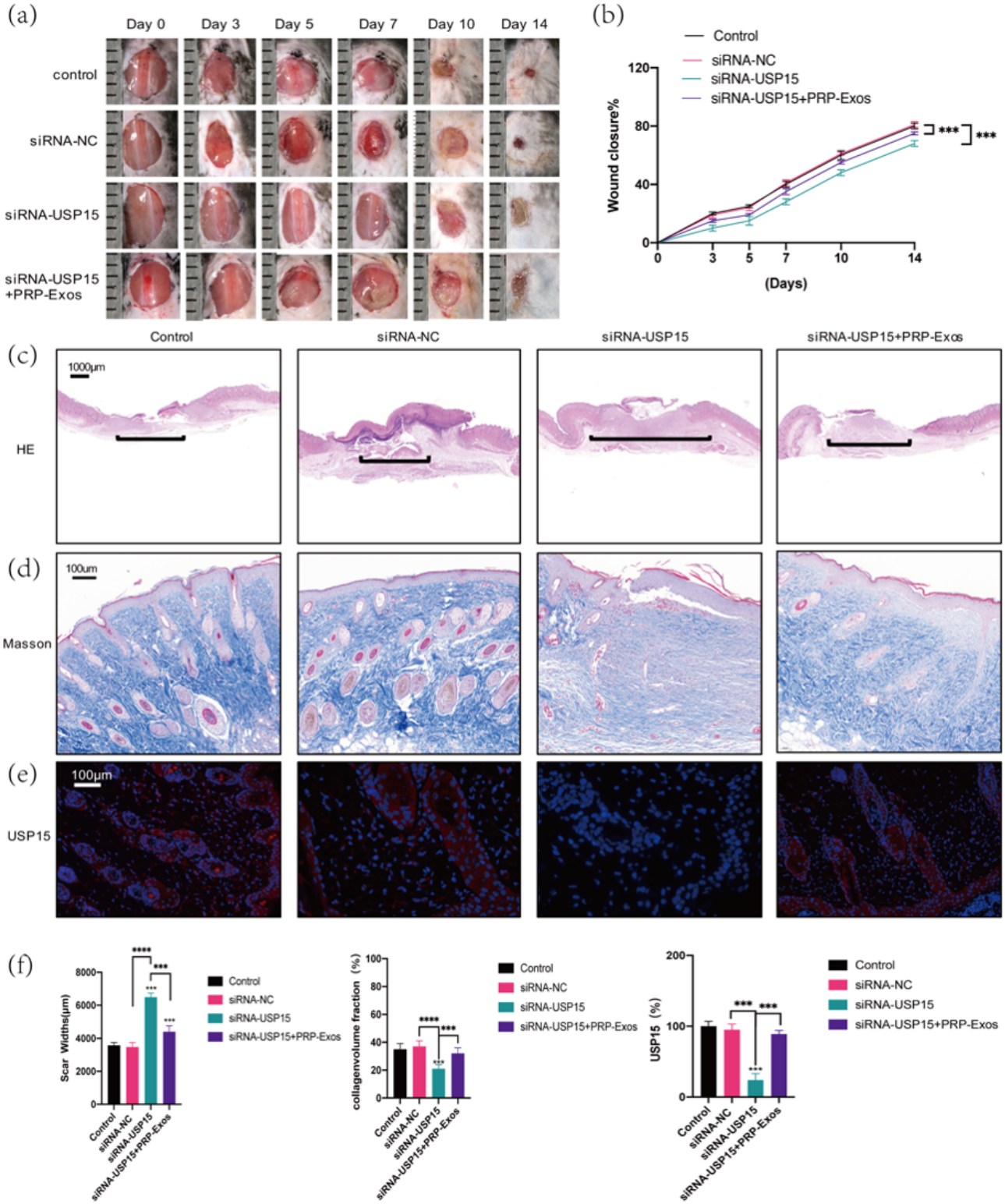

Figure 7

USP15 promotes in vivowound healing. (a) Images of mice in the three treatment groups. (b) Wound closure rates for mice in the three treatment groups. (c) H&E staining results from the three treatment groups. (d) Masson's trichrome staining results for wounds in the three treatment groups. (e) USP15 immunohistochemical staining results for the three groups. (f) Quantification results for data in the three treatment groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. n=6.

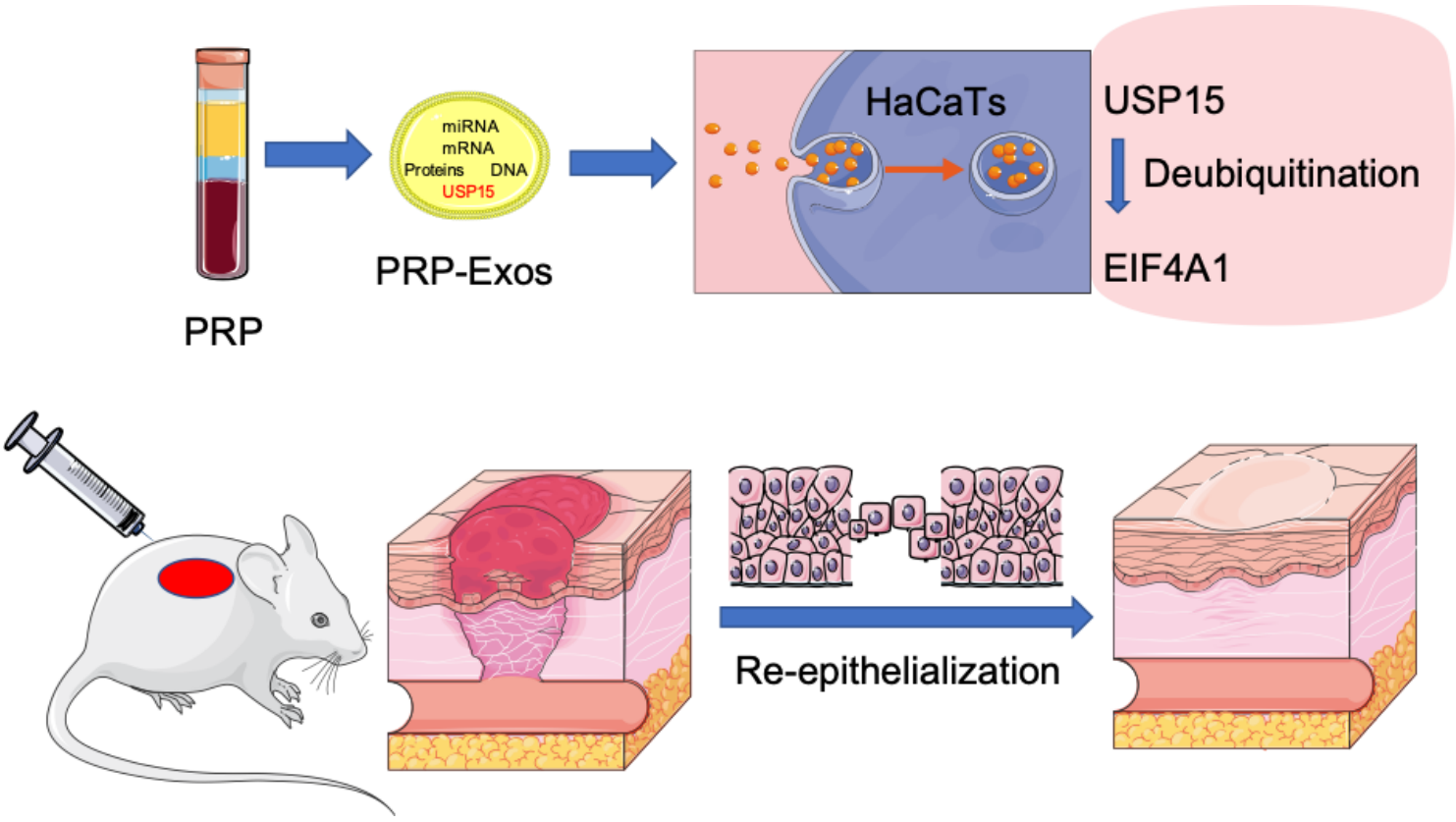

Figure 8

USP15 is one of main mRNA inPRP-Exos,which could be take into HaCaTs, subsequently deubiquitinating EIF4A1, then, accelerate re-epithelialization and promote wound healing.

Supplementary Files

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- GraphicalAbstract.docx