Journal of Dental Sciences 20 (2025) 487–501

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

Heat stress promotes osteogenic and odontogenic differentiation of stem cells from apical papilla via glucose-regulated protein 78-mediated autophagy

Xiaolan Zhang [a], Zhou Wei [b], Yunlong Xu [c*]

[a] *Department of Stomatology, Wuxi No.2 People's Hospital, Wuxi, China*
[b] *Department of Stomatology, Wuxi City Rehabilitation Hospital, Wuxi, China*
[c] *Endodontic Department, Changzhou Stomatological Hospital, Changzhou, China*

Received 17 March 2024; Final revision received 8 May 2024
Available online 15 May 2024

KEYWORDS
Heat stress;
Osteogenic differentia-tion;
GRP78;
Autophagy

Abstract *Background/purpose:* Heat stress is essential for improving the efficacy of mesenchymal stem cell (MSC)-based regeneration medicine. However, it is still unclear whether and how heat stress influences the differentiation of stem cells from apical papilla (SCAPs). This research aimed to explore the potential mechanism of glucose-regulated protein 78 (GRP78) in regulating differentiation under heat stress in SCAPs.
Materials and methods: The proliferation ability was assessed using the 5-Ethynyl-2'- deoxyuridine (EdU) assay, cell counting kit assay (CCK-8), and flow cytometry (FCM). The osteogenic and odontogenic differentiation capacities were investigated through Western blot, quantitative reverse transcription polymerase chain reaction (qRT-PCR), alkaline phosphatase (ALP) staining and activity assay, alizarin red S (ARS) staining, as well as immunofluorescence staining. Western blot and transmission electron microscopy (TEM) were used to detect autophagy.
Results: Heat stress enhanced the osteogenic and odontogenic differentiation of SCAPs, but it did not significantly affect proliferation. Besides, GRP78 has been confirmed to modulate the differentiation induced by heat stress. Autophagy triggered by GRP78 enhanced osteogenic and odontogenic differentiation of SCAPs, while the knockdown of GRP78 or the inhibitor of autophagy suppressed the differentiation.
Conclusion: Heat stress induces osteogenic and odontogenic differentiation of SCAPs through GRP78-mediated autophagy.

* Corresponding author. Endodontic Department, Changzhou Stomatological Hospital, 61 Beizhi Street, Changzhou 213000, China.
E-mail address: 15950555383@163.com (Y. Xu).

https://doi.org/10.1016/j.jds.2024.05.007

Introduction

With the exacerbation of the aging population, the prevalence of bone diseases is increasing rapidly and has become a growing public health problem worldwide, leading to serious health, economic, and social burdens.[1–3] Among them, the most critical pathologic conditions are large bone defects caused by osteoporosis, bone fractures, and tumor resection.[4] Fortunately, mesenchymal stem cells (MSCs)-based regenerative medicine has shown its notable capacity for bone remodeling and regeneration over the past years.[5,6] MSCs are primitive cells with the ability to self-renew and differentiate into multiple cell types. They can be obtained from various sources, including bone marrow, adipose tissue, peripheral blood, and teeth.[7] Research has shown that stem cells from apical papilla (SCAPs), derived from the apex of immature permanent teeth, are highly promising candidates for regenerative medicine.[8,9] SCAPs have the capacity to differentiate into odontoblasts, osteoblasts, and chondrocytes, contributing not only to dentin formation but also to the regeneration of bone tissue.[10] Furthermore, compared to other MSCs, SCAPs are characterized by a great proliferative potential and little inherent immunogenicity. As a result, SCAPs are widely used for regenerative endodontic treatment (RET) and bone regeneration.[11,12]

However, despite the remarkable efficacy of MSCs in clinical studies, there are still certain challenges to address, such as limited viability and loss of stemness.[13] To achieve the desired regenerative potential, hyperthermia therapy has been adopted, which is a protective response caused by low doses of heat stress.[14] Recently, hyperthermia therapy has attracted mounting attention because of its precise spatiotemporal control, which depends on the rapid development of photothermal technology.[15,16] Additionally, several findings have indicated that mild heat stress can dramatically optimize the potency and differentiative capacity, promoting the angiogenesis and osteogenesis.[17–19] Therefore, gaining a better understanding of the differentiation mechanism of MSCs under heat stress might offer new perspectives on MSCs-based regeneration.

Glucose-regulated protein 78 (GRP78), also referred to as BiP or HSPA5, is an endoplasmic reticulum (ER) chaperone and a member of the heat shock protein (HSP) 70 family.[20] It has been reported that GRP78 can be strengthened by heat stress, which has piqued our interest.[21] GRP78 is a multifunctional protein that actively participates in various cellular processes.[22,23] It facilitates the folding of newly synthesized proteins and targets misfolded proteins for degradation, enabling cells to adapt to adverse stress conditions. It is also one of the calcium-binding proteins, contributing to calcium homeostasis in the ER.[24] Moreover, GRP78 participates in cellular signaling, migration, proliferation, apoptosis, and immunity.[25,26] Notably, it has been suggested that there is a feasible link between GRP78 and the differentiation of MSCs.[27]

Autophagy, a cellular degradation mechanism conserved across eukaryotes, serves as an adaptive response to cellular stress. It involves the delivery of non-essential macromolecules and detrimental cytoplasmic entities to the lysosome or vacuole for degradation, to hereby ensuring cellular homeostasis.[28,29] Autophagy plays a crucial role in various physiological processes, whereas impaired autophagy has been implicated in numerous diseases such as cancer, neurodegenerative disorders, and autoimmune conditions.[30,31] Coincidentally, accumulating evidence has revealed a close connection between both heat stress and GRP78 with autophagy.[32–34] Additionally, autophagy plays a significant role in maintaining the self-renewal potential of stem cells and is crucial in bone regeneration.[35–37] Our previous research has demonstrated the positive effect of autophagy on the osteogenic and odontogenic differentiation in SCAPs.[38] Therefore, the objective of this present study is to investigate the impact of GRP78 on the osteogenic and odontogenic differentiation of SCAPs during heat stress, as well as the regulatory role of autophagy in the committed differentiation under this condition.

Materials and methods

Cell culture

Premolars and third molars were obtained from patients aged 13–20 who provided informed consent. The study received approval from the Ethics Committee of Jiangsu Provincial Stomatological Hospital. Initially, the teeth were washed with PBS (Gibco, Grand Island, NE, USA), followed by meticulous isolation of the apical papilla from immature roots. The tissue was subjected to cutting and digestion in a medium containing 3 mg/mL collagenase type I (Sigma–Aldrich, St. Louis, MO, USA) and 4 mg/mL trypsin (Gibco) at a temperature of 37 °C for a duration of 20 min. The cells were centrifuged and subsequently seeded in a culture dish with CM. Finally, the cells were transferred to a humidified incubator with a temperature of 37 °C and a carbon dioxide concentration of 5%. The SCAPs used in this study were obtained from passages 3–5. GRP78 small interfering RNAs (siGRP78) and overexpression plasmid were designed (Ribobio, Guangzhou, China). Once the cell density reached to approximately 70%, SCAPs were transfected with of the corresponding vector.

Flow cytometry

SCAPs were subjected to incubation with conjugated primary antibodies, including CD29-APC, CD34-PE, CD45-PE, CD73-PE, CD90-PE, and CD105-PerCP-Cy5.5, all of which were obtained from BD Biosciences. This incubation process occurred in a dark environment for a duration of 30 min. Following this, the stained cells were resuspended in PBS for the purpose of final flow cytometric analysis. For the examination of the cell cycle phase, the cells were fixed in 70% ethanol at a temperature of 4 °C for a duration of 30 min. Following this fixation process, the cells were

stained with propidium iodide (PI) in order to determine the various cell cycle phases, including G0/G1, S, and G2/M.

Heat exposure

The presence of heat stress was confirmed based on previous studies.[39,40] Subsequently, cells were transferred to an incubator with preset temperatures of 39 °C, 41 °C, and 43 °C for a duration of 1 h every 3 days. To prevent evaporation, the culture media was promptly refreshed following heat exposure, after which the cells were returned to the 37 °C incubator. In order to maintain consistent experimental conditions, the control group (37 °C) also underwent a change in medium. Following several tests, the 41 °C group was designated as the heat stress (HS) group, while the 37 °C group remained as the non-heat stress (NC) group.

Alkaline phosphatase (ALP) staining and activity assay

After a period of 7 days of osteogenic induction, ALP staining and ALP activity were conducted. ALP staining was performed in accordance with the user manual of the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Shanghai, China). SCAPs were fixed using 4% PFA and subsequently stained with the ALP detection solution for a duration of 5–30 min under conditions of darkness. The stained cells were then observed using an inverted microscope. The quantification of ALP activity was assessed using the Alkaline phosphatase assay kit (Jiancheng, Nanjing, China). The optical density (OD) value was measured following the manufacturer's instructions at 520 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). ALP activity was normalized to the total protein content.

Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was extracted from SACPs using the Trizol reagent and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the total RNA was reverse-transcribed into cDNA using a HiScript II Q RT SuperMix for qPCR (Vazyme, Nanjing, China). The cDNA was then analyzed using the ChamQ Universal SYBR qPCR Master Mix (Vazyme) and a QuantStudio™ 7 Flex following the provided protocol. GAPDH was utilized as a reference gene, and relative mRNA expression levels were determined using the $2^{-\Delta\Delta CT}$ method. All primer sequences could be found in Table 1.

Western blot analysis

The protein samples were collected from SCAPs in different groups using RIPA (Beyotime) and their concentrations were determined with BCA protein assay kit (Beyotime). The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF (Millipore, Billerica, MA, USA). After being blocked with 5% skim milk for 2 h at room temperature, the membranes were incubated with specific primary antibodies overnight at 4 °C. Subsequently, membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 60 min before exposure and imaging.

5-Ethynyl-2′-deoxyuridine (EdU) assay

Cell proliferation was assessed using the Cell-Light™ EdU Apollo In Vitro Kit (Ribobio). SCAPs were incubated with a 50 μM EdU solution for a duration of 2 h and subsequently fixed with 4% PFA for 30 min. To neutralize any excess aldehyde, a 2 mg/mL glycine solution was employed. Permeabilization was achieved by treating the cells with 0.5% TritonX-100 (Beyotime). Following this, the cells were stained with 1 × Apollo and 1 × Hoechst 33342 for a duration of 30 min each. Images were captured using a fluorescence microscope, and data analysis was performed using Image J.

Cell counting kit-8 assay

In accordance with the manufacturer's instructions for CCK-8, SCAPs were seeded in 96-well plates at a density of 2000 cells per well. Subsequently, on days 0, 1, 3, 5, 7, and 9, SCAPs were subjected to incubation with the CCK-8

Table 1 Primer sequences for qRT-PCR analysis of gene expression. DSPP, dentin sialophosphoprotein; GRP78, glucose-regulated protein 78; ALP, alkaline phosphatase; RUNX2, RUNX family transcription factor 2; OSX, osterix.

Target gene	Primers	Sequences (5′–3′)
DSPP	Forward	ATATTGAGGGCTGGAATGGGGA
	Reverse	TTTGTGGCTCCAGCATTGTCA
GRP78	Forward	GCTCGACTCGAATTCCAAAG
	Reverse	TTTGTCAGGGGTCTTTCACC
ALP	Forward	GACCTCCTCGGAAGACACTC
	Reverse	TGAAGGGCTTCTTGTCTGTG
RUNX2	Forward	TCTTAGAACAAATTCTGCCCTTT
	Reverse	TGCTTTGGTCTTGAAATCACA
OSX	Forward	CCTCCTCAGCTCACCTTCTC
	Reverse	GTTGGGAGCCCAAATAGAAA
GADPH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAGATGGTGATGGGATTTC

reagent at a temperature of 37 °C for a duration of 2 h. Following this incubation period, the optical density (OD) value was measured at a wavelength of 450 nm. Furthermore, on days 0, 3, and 7, SCAPs were subjected to exposure at either 37 °C or 41 °C for a duration of 1 h subsequent to the measurement of absorbance.

Immunofluorescence staining

SCAPs were grown on the glass coverslips placed in a 12-well plate. After cell attachment, SCAPs were subjected to heat stress or transfection. 3 days later, cells were fixed with 4% PFA and permeabilized with Triton X-100 solution (Beyotime). Subsequently, cells were sealed with goat serum at 37 °C for 1.5 h and incubated with ALP, RUNX2, and GRP78 primary antibodies at 4 °C overnight, followed by the incubation with a mixture of secondary antibody with fluorochrome and phalloidine. The nucleus was stained with DAPI and then the images for ALP, RUNX2, and GRP78 were taken under the fluorescence microscope.

Statistical analysis

All experiments were repeated at least in triplicate. Data were analyzed by SPSS (version 20.0) and presented as mean ± standard deviation (SD). Student's t-test was used to compare the difference between the two groups, while one-way analysis of variance (ANOVA) was used for multiple groups. $P < 0.05$ was deemed as statistically significant.

Results

Cells culture and identification

Primary SCAPs crawled out of the tissue after 3 days of the initial seeding (Fig. 1A). SCAPs at passage 3 exhibited a spindle-like morphology (Fig. 1B). Flow cytometry analysis revealed that SCAPs were highly positive for MSC surface markers including CD29, CD73, CD90, and CD105 while negative for the hematopoietic cell marker CD34 and leukocyte marker CD45 (Fig. 1C). Results of Alizarin Red S staining, Oil Red O staining, and Alcian Blue staining confirmed that SCAPs could differentiate into osteoblasts, adipocytes, and chondrocytes, respectively (Fig. 1D and F). All these characteristics were similar to the findings of SCAPs described previously.

To determine the optimal temperature, ALP staining and ALP activity assay were performed on the 7th day of osteogenic induction. Results indicated that an increase in temperature within the range of 37 °C—41 °C resulted in enhanced ALP staining and ALP activity. However, temperatures exceeding 41 °C did not yield any further enhancement (Fig. 2A and B). Consistent findings were observed through Western blot and qRT-PCR analyses (Fig. 2C and E). Collectively, these experiments demonstrated that the 41 °C group exhibited the highest ALP activity, leading to the selection of 41 °C as the optimal temperature for subsequent investigations. The 41 °C group was then named as the HS group.

Heat stress had no effect on the proliferation of SCAPs

In order to assess the influence of heat stress on the proliferation of SCAPs, the researchers conducted an EdU assay, CCK-8 assay, and flow cytometry analysis. The results from the EdU assay and CCK-8 assay demonstrated no statistically significant difference in cell proliferation between the NC group and HS group (Fig. 2F—H). Additionally, the flow cytometry analysis revealed no variation in the proliferation index (PI = G2M + S) (Fig. 2I—J). Consequently, these findings suggest that heat stress does not have a detrimental effect on SCAPs.

Heat stress enhanced the osteogenic and odontogenic differentiation of SCAPs

The results depicted in Fig. 2A—E demonstrate a significant increase in the expression of ALP under heat stress conditions. Consequently, further investigations were carried out to ascertain the potential of heat stress in augmenting osteogenic and odontogenic differentiation. Western blot analysis presented in Fig. 3A and B revealed that heat stress positively influenced the protein levels of DSPP, RUNX2, and OSX. Additionally, in line with the observed protein promotion, the transcript levels of DSPP, RUNX2, and OSX were significantly upregulated by heat stress, as depicted in Fig. 3 C. The results of Alizarin red S staining demonstrated a higher capacity for mineralization in the HS group, as evidenced by the presence of more calcium nodules compared to the NC group (Fig. 3D and E). Additionally, immunofluorescence staining revealed an increased abundance of ALP and RUNX2 proteins in the HS group relative to the NC group (Fig. 3F and G). Taken together, these findings strongly suggest that heat stress significantly promotes the differentiation of osteoblasts and odontoblasts.

GRP78 promoted osteogenic and odontogenic differentiation of SCAPs

The protein and mRNA expression levels of GRP78 were found to be enhanced by heat stress, as depicted in Fig. 4A—D. This finding is consistent with the expression patterns observed during osteogenic and odontogenic differentiation. Based on these observations, it can be inferred that GRP78 potentially plays a role in promoting osteogenic and odontogenic differentiation. To further assess the differentiation capacity of GRP78, gain- and loss-of-function experiments were conducted. The siGRP78 group exhibited downregulated expression of GRP78, while the GRP78-over group showed upregulated expression, as shown in Fig. 5A—C, 5H and Fig. 6A—C, 6H. In the siGRP78 group, the expression of DSPP, ALP, RUNX2, and OSX was significantly suppressed, as evidenced by Western blot analysis (Fig. 5A and B, Fig. 6A and B). These findings were further confirmed by qRT-PCR, which showed a distinct reduction in the transcript levels of DSPP, ALP, RUNX2, and OSX upon knockdown of GRP78 (Fig. 5C). Conversely, the overexpression of GRP78 resulted in a significant increase in

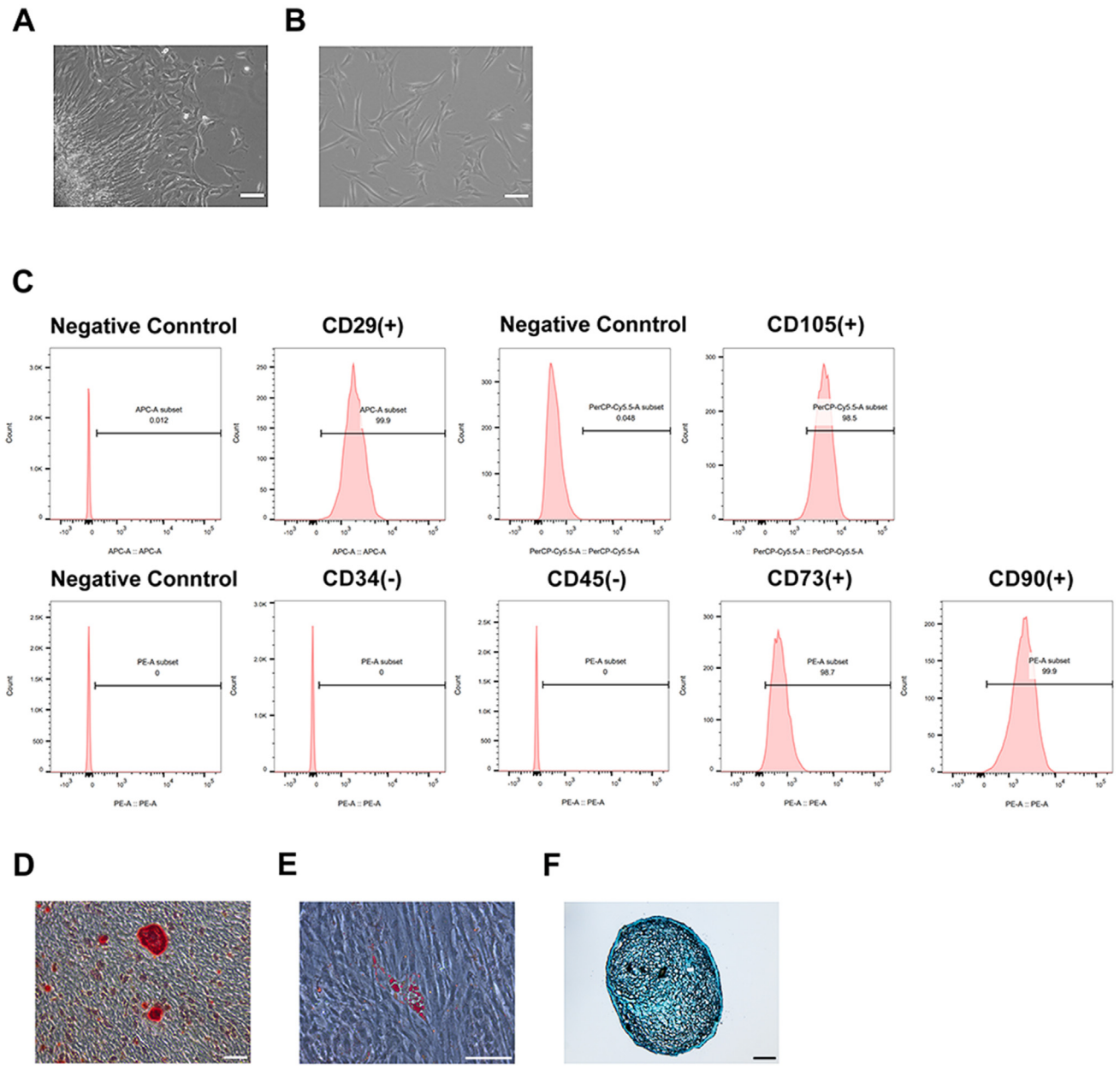

Figure 1 Identification of SCAPs. (A) Morphology of primary SCAPs. (B) Morphology of SCAPs at passage 3. (C) Flow cytometry analysis of CD29, CD34, CD45, CD73, CD90 and CD105 on SCAPs. (D) Alizarin red S staining of cells cultured in osteogenic medium. (E) Oil red O staining of cells cultured in adipogenic medium. (F) Alcian blue staining of cells cultured in chondrogenic medium. Scale bar = 100 μm.

the expression of these genes (Fig. 6C). Additionally, the downregulation of GRP78 led to a decrease in ALP expression, as observed through ALP staining and ALP activity assay, while the overexpression of GRP78 reversed this effect (Fig. 5D and E; Fig. 6D and E). In contrast to the siGRP78 group (Fig. 5F and G), enhanced mineralized nodule formation was observed in the GRP78-over group (Fig. 6F and G). Additionally, immunofluorescence staining demonstrated that GRP78 increased the immunoreactivity of ALP and RUNX2 (Fig. 5H; Fig. 6H). These findings collectively indicate that GRP78 plays a significant role in activating the osteogenic and odontogenic differentiation of SCAPs. However, the impact of heat stress-induced GRP78 on these processes remains uncertain.

GRP78 siRNA reversed the effects of heat stress on committed differentiation of SCAPs

To investigate the potential role of heat stress-induced GRP78 in the differentiation of SCAPs into osteogenic and odontogenic lineages, additional experiments were conducted. The Western blot analysis revealed a significant increase in the protein levels of DSPP, ALP, RUNX2, and OSX

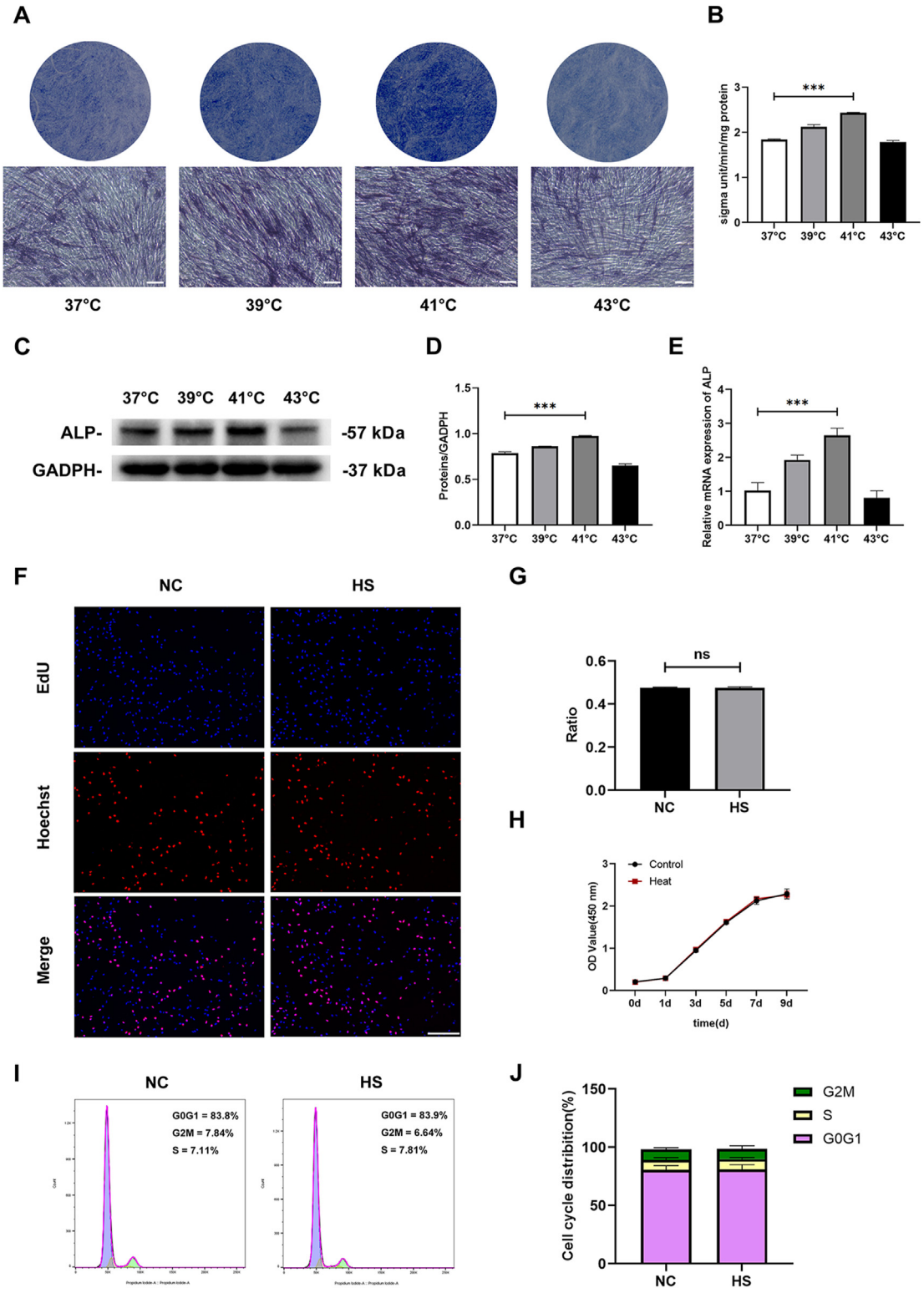

Figure 2 Screening for the optimal temperature and investigating the effect of heat stress on the proliferation of SCAPs. (A—B) ALP staining and ALP activity assay in SCAPs exposed to different temperatures. SCAPs were cultured in osteogenic medium (OM) for 7 days. Scale bar = 100 μm. (C) Western blot of ALP in SCAPs exposed to different temperatures. (D) Quantitative analysis of Western blot in (C). (E) qRT-PCR analysis of ALP in SCAPs exposed to different temperatures. GAPDH was used for normalization. (F—J) Cell proliferation of SCAPs was evaluated by EdU assay, CCK-8 assay, and flow cytometry. Scale bar = 200 μm ***$P < 0.001$.

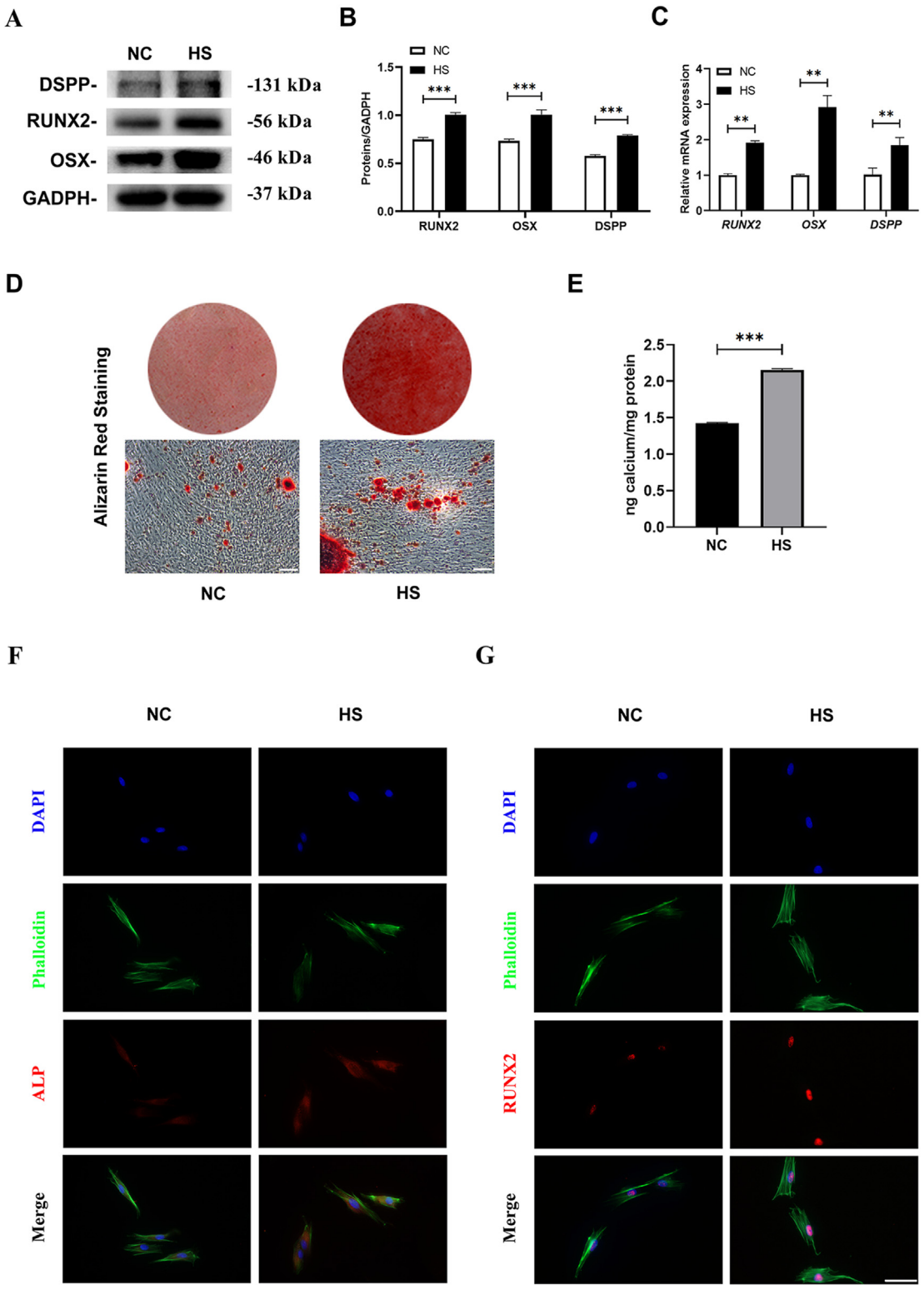

Figure 3 Heat stress enhanced osteogenic and odontogenic differentiation of SCAPs. (A) Western blot analysis of DSPP, RUNX2, and OSX in the NC group and HS group. (B) Quantitative analysis of Western blot in (A). (C) qRT-PCR analysis of DSPP, GRP78, RUNX2, and OSX in the NC group and HS group. (D) Image of Alizarin red S staining after being cultured in OM for 14 days. Scale bar = 100 μm. (E) Quantitative analysis of ARS staining in (D). (F–G) Immunofluorescence staining and quantification of ALP, RUNX2. Scale bar = 50 μm $^{**}P < 0.01$, $^{***}P < 0.001$.

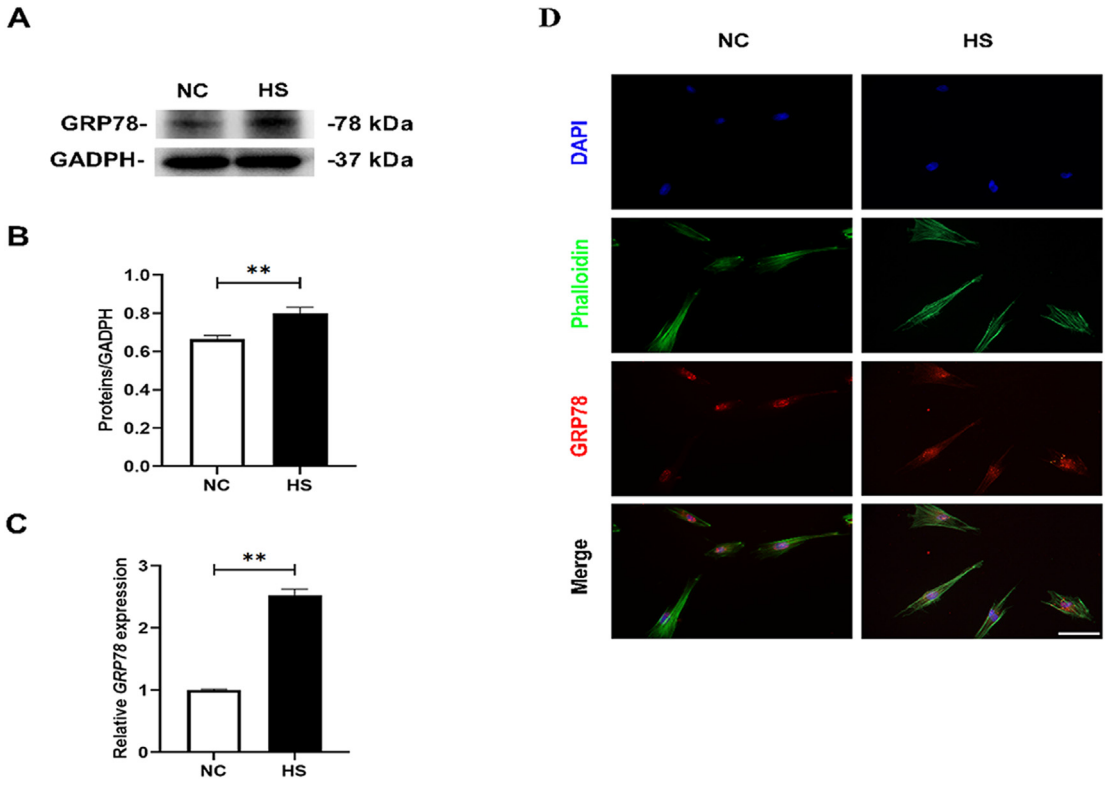

Figure 4 Heat stress upregulated the expression of GRP78. (A) Western blot analysis of GRP78 in the NC group and HS group. (B) Quantitative analysis of Western blot in (A). (C) qRT-PCR analysis of GRP78 in the NC group and HS group. (D) Immunofluorescence staining of GRP78 in the NC group and HS group. Scale bar = 50 μm $^{**}P < 0.01$.

in SCAPs subjected to heat stress, which were subsequently reduced upon silencing of GRP78 (Fig. 7A and B). Furthermore, heat stress prominently enhanced ALP staining and activity, while interference with GRP78 effectively counteracted this enhancement (Fig. 7C and E). This phenomenon was further confirmed by Alizarin red S staining. The knockdown of GRP78 attenuated the promotion of mineralization induced by heat stress (Fig. 7D and F). These findings suggested that the GRP78 upregulation mediated by heat stress was strongly associated with osteogenic and odontogenic differentiation.

Heat stress-induced GRP78 increased the committed differentiation of SCAPs by activating autophagy

In order to examine the impact of heat stress and GRP78 on autophagy in SCAPs, an autophagy inhibitor called 3-methyladenine (3-MA) was utilized to inhibit autophagy. The protein level of Beclin-1 and the ratio of LC3 II/I exhibited a time-dependent increase (Fig. 8A and B). However, the increase induced by heat stress was nullified by the presence of 3-MA, indicating that heat stress is capable of inducing autophagy and this induction can be mitigated by 3-MA (Fig. 8C and D). To explore the correlation between GRP78 and autophagy under heat stress conditions, Western blot and TEM analyses were conducted. The results of the Western blot analysis demonstrated that the application of GRP78 siRNA effectively inhibited the heat stress-induced enhancement of DSPP, RUNX2, ALP, and OSX at the protein level (Fig. 8E and F). Furthermore, the transmission electron microscopy (TEM) images revealed a higher presence of autophagosomes in SCAPs subjected to heat stress, which was subsequently suppressed by the administration of GRP78 siRNA and 3-MA (Fig. 8G). These findings strongly indicate that GRP78, induced by heat stress, plays a crucial role in initiating autophagy.

To further investigate the potential involvement of autophagy in the differentiation of SCAPs into osteogenic and odontogenic lineages, subsequent experiments were conducted. Western blot analysis revealed a significant upregulation of DSPP, ALP, RUNX2, and OSX protein levels in response to heat stress, while the enhanced capacity was downregulated by 3-MA (Fig. 8H and I). Additionally, the results obtained from ALP staining, ALP activity assay, and Alizarin red S staining were consistent with the aforementioned findings (Fig. 8J–M). Collectively, these findings provide evidence that heat stress-induced GRP78 is associated with the osteogenic and odontogenic differentiation of SCAPs through activation of autophagy.

Discussion

In recent years, there has been a growing interest in the field of MSCs-based regeneration medicine as a means to address a range of diseases and disorders.[41,42] However, it is important to acknowledge that the effectiveness of stem cells may be hindered by factors such as the age of the

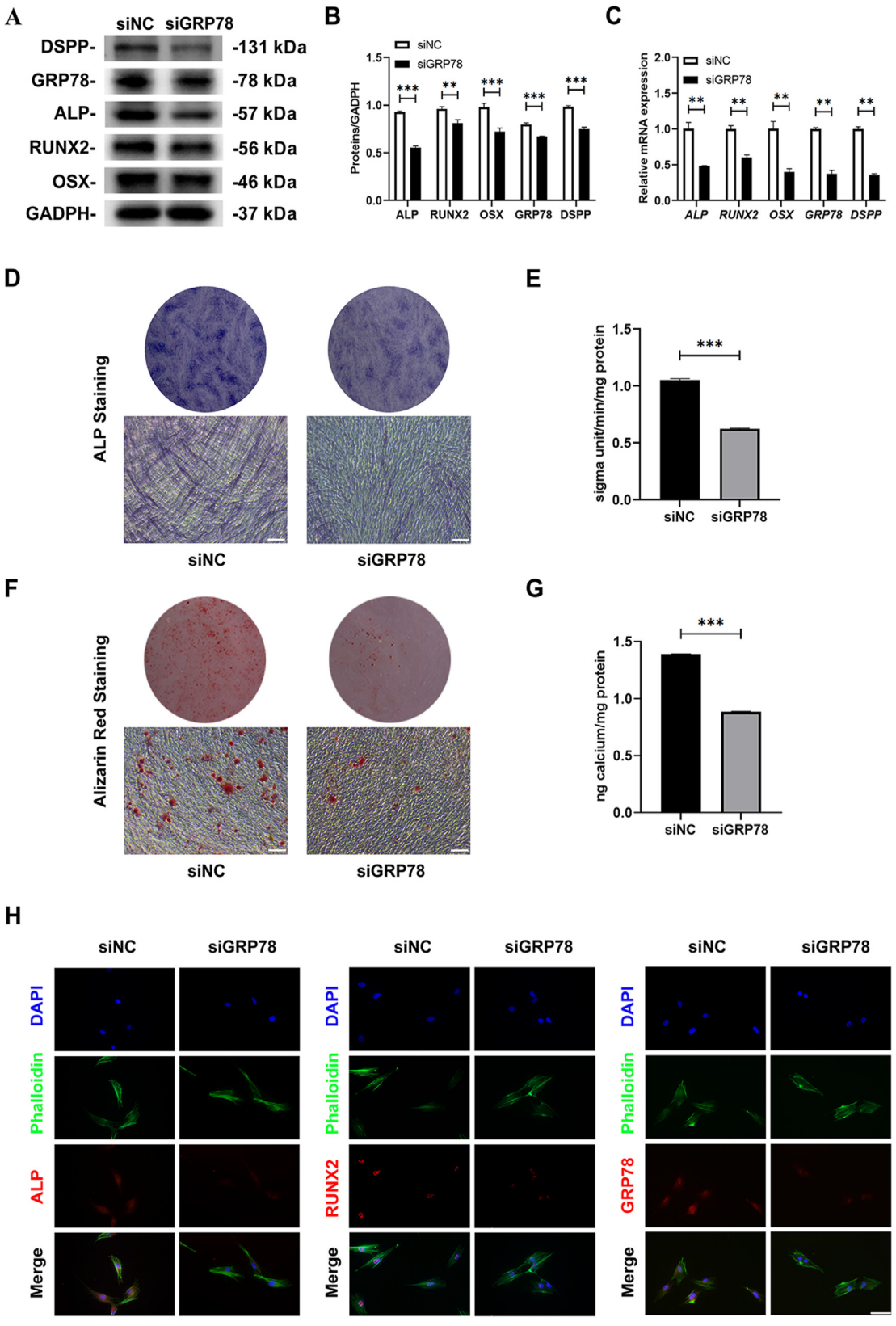

Figure 5 Knockdown of GRP78 suppressed osteogenic and odontogenic differentiation of SCAPs. (A) Western blot analysis of DSPP, GRP78, ALP, RUNX2, and OSX in SCAPs transfected with or without GRP78 siRNA. (B) Quantitative analysis of Western blot in (A). (C) qRT-PCR analysis of DSPP, GRP78, ALP, RUNX2, and OSX in SCAPs transfected with or without GRP78 siRNA. (D–E) ALP staining and ALP activity assay after 7 days of osteogenic induction. Scale bar = 100 μm. (F–G) Image and quantification of Alizarin red S staining after 14 days of osteogenic induction. Scale bar = 100 μm. (H) Immunofluorescence staining of ALP and RUNX2. Scale bar = 50 μm $^{**}P < 0.01$, $^{***}P < 0.001$.

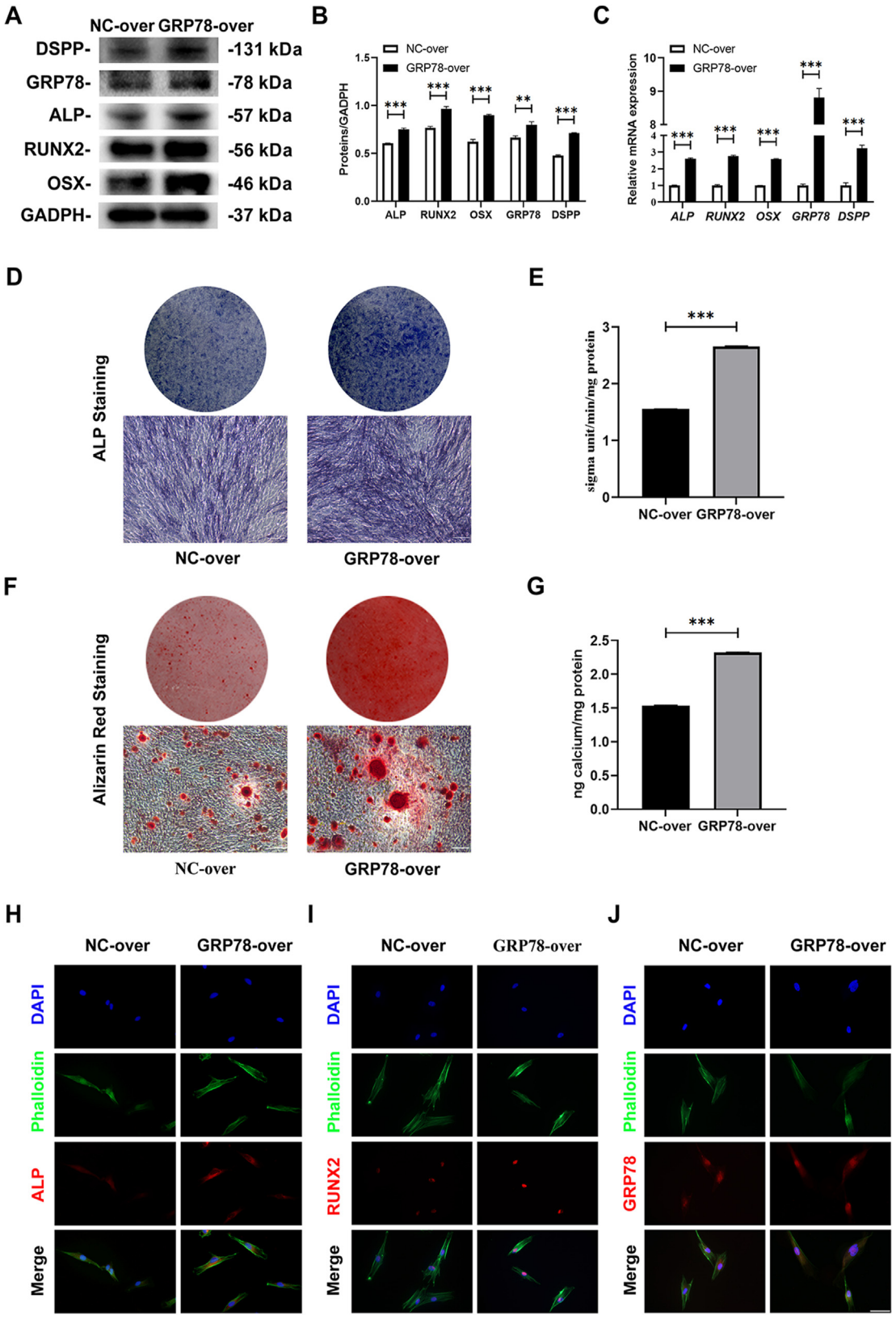

Figure 6 Overexpression of GRP78 promoted osteogenic and odontogenic differentiation of SCAPs. (A) Western blot analysis of DSPP, GRP78, ALP, RUNX2, and OSX in the NC-over group and GRP78-over group. (B) Quantitative analysis of Western blot in (A). (C) qRT-PCR analysis of DSPP, GRP78, ALP, RUNX2, and OSX in the NC-over group and GRP78-over group. (D—E) ALP staining and ALP activity assay on day 7 after osteogenic induction. Scale bar = 100 μm. (F—G) Image and quantification of Alizarin red S staining on day 14 after osteogenic induction. Scale bar = 100 μm. (H) Immunofluorescence staining of ALP, RUNX2, and GRP78. Scale bar = 50 μm $^{**}P < 0.01$, $^{***}P < 0.001$.

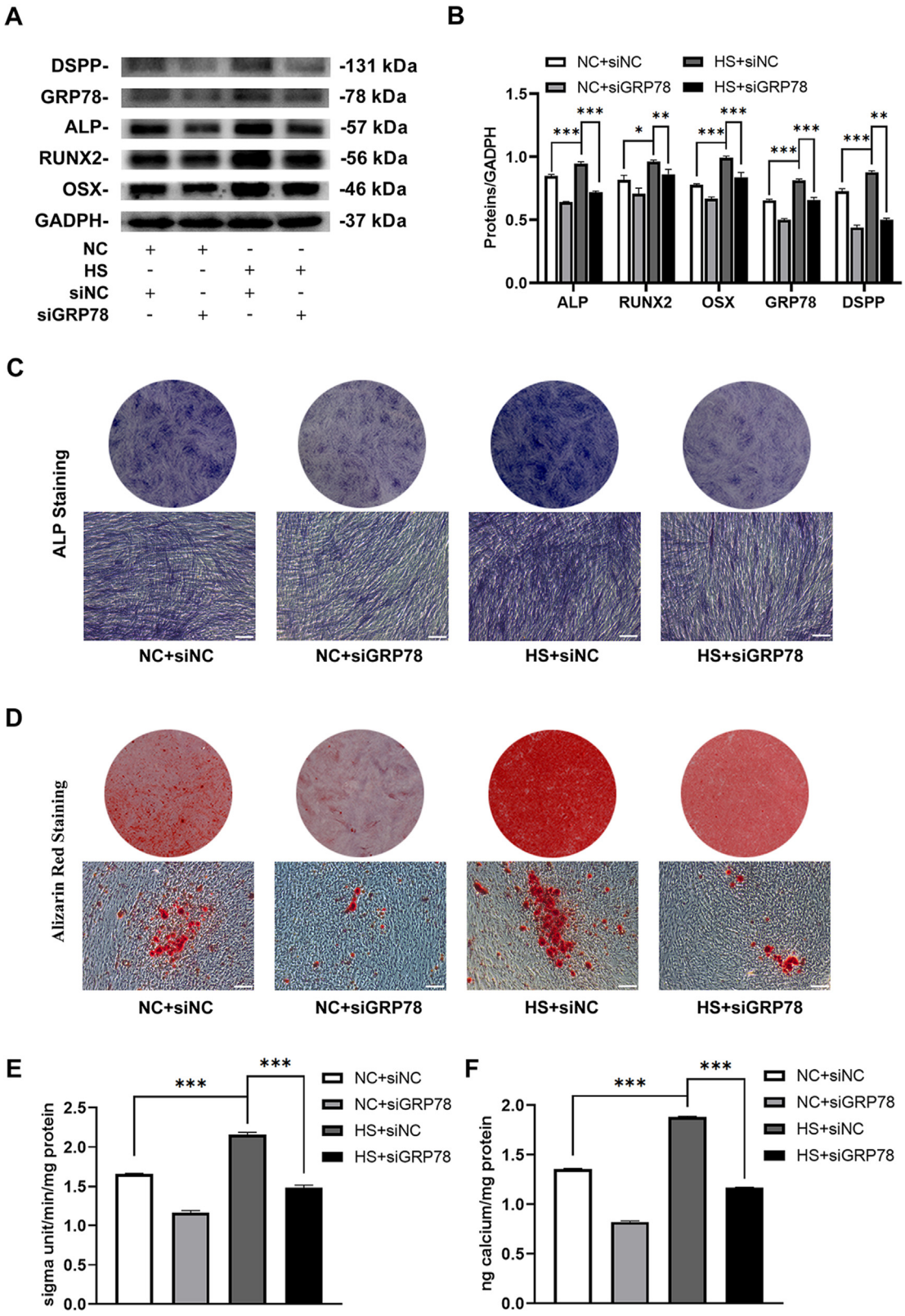

Figure 7 GRP78 siRNA reversed the role of heat stress on committed differentiation of SCAPs. (A) Western blot analysis of DSPP, GRP78, ALP, RUNX2, and OSX in SCAPs with or without heat stress and GRP78 siRNA. (B) Quantitative analysis of Western blot in (A). (C, E) ALP staining and ALP activity assay in SCAPs. Scale bar = 100 μm. (D, F) Image and quantification of Alizarin red S staining. Scale bar = 100 μm *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

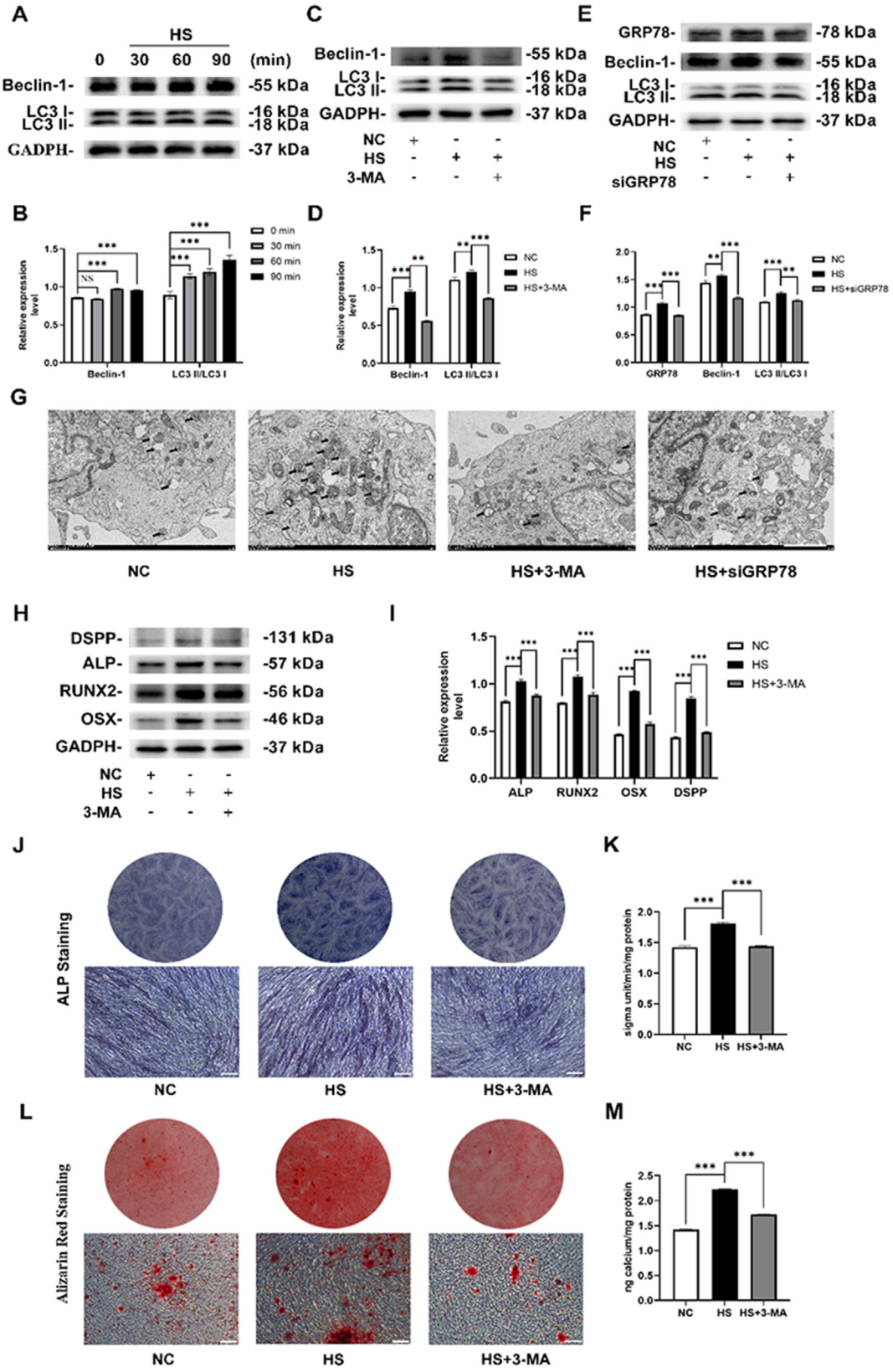

Figure 8 Heat stress-induced GRP78 increased the committed differentiation of SCAPs by activating autophagy. (A) Western blot analysis of Beclin-1 and LC3 II/I in SCAPs exposed to heat stress, followed by recovery at 37 °C for 0, 3, 6, or 12 h. (B) Quantitative analysis of Western blot in (A). (C) Western blot analysis of Beclin-1 and LC3 II/I in SCAPs exposed to heat stress with or without 3-MA pretreatment. (D) Quantitative analysis of Western blot in (C). (E) Western blot analysis of GRP78, Beclin-1, and LC3 II/I in SCAPs exposed to heat stress with or without GRP78 siRNA pretreatment. (F) Quantitative analysis of Western blot in (E). (G) TEM analysis

donor, the presence of disease, and the process of in vitro expansion.[14] In order to enhance the therapeutic potential of MSCs, various strategies have been implemented, including the use of a hypoxic microenvironment, the application of Janus electro-microenvironment (JEM), pharmacological mobilization, and mild period heat stress.[43,44] Among the various therapeutic approaches, heat stress emerges as a prevalent physical stressor, demonstrating notable spatial and temporal precision through the application of Near-infrared (NIR) light to attain predetermined temperatures.[45] Consequently, heat stress has garnered significant attention and has been extensively employed in diverse musculoskeletal disorders to enhance bone remodeling and regeneration.[17,39] Notably, the cellular response to heat stress exhibits variability across different cell types.[46,47] In this study, it was confirmed that the differentiative capacity of SCAPs was significantly enhanced following exposure to heat stress at a temperature of 41 °C for a duration of 1 h. Consequently, this condition was determined to be the optimal choice for inducing heat stress in subsequent experiments. Previous research has documented that both BMSCs and hMSCs exhibit significant proliferation after exposure to heat stress.[19,48] However, our findings indicate that heat stress had no noticeable impact on the proliferation of SCAPs. We hypothesize that the disparity in these results may be attributed to variations in cell types, as well as differences in thermal dosing and frequencies employed in the respective studies.

GRP78 primarily localizes in the endoplasmic reticulum (ER) lumen, functioning as a crucial molecular chaperone that maintains ER homeostasis. Additionally, it participates in various physiological and pathological processes. Recent research has indicated a negative correlation between GRP78 and the aging process as well as age-related diseases.[23,49] GRP78 serves as a representative marker of senescence, with both its gene and protein expression significantly decreasing as senescence progresses in TMSCs.[50] Furthermore, the absence of GRP78 leads to aging-related ailments, such as impaired stem cell potency in Grp78 KO AT2 cells and a severe fibrotic response in Grp78 KO mice.[51] Furthermore, GRP78 possesses a distinctive association with osteogenic differentiation, in addition to its anti-aging property. Specifically, when relocated to the cellular surface, GRP78 assumes a receptor-like role, exhibiting specific binding capabilities to DMP1 and playing a crucial role in the formation of mineralized matrix.[52] Throughout the formation of mineralized tissues, GRP-78 is detected in various regions such as the pulp, dentin matrix, alveolar bone matrix, and cartilage.[53] Moreover, owing to its capacity to bind calcium, GRP-78 facilitates the nucleation of calcium phosphate crystals in vitro.[54] In consistence with the above evidence, we confirmed that GRP78 was responsible for the osteogenic and odontogenic differentiation of SCAPs. Furthermore, we found that the knockdown of GRP78 could reverse the committed differentiation, suggesting GRP78 accounted for the differentiation induced by heat stress.

The understanding of the impact of heat stress on osteogenic and odontogenic differentiation has primarily focused on the expression of HSP70.[47] HSP70 plays a crucial role in maintaining the protein self-stabilization system, facilitating faster biochemical reactions, increased protein synthesis, and enhanced protein aggregations during stem cell differentiation.[55] However, limited research has been conducted on alternative mechanisms. In this study, we propose a novel molecular mechanism involving GRP78 and autophagy.

Furthermore, it has been established that GRP78 plays a crucial role in stress-induced autophagy and is directly involved in the formation of autophagosomes.[33] Various studies have demonstrated that heat stress can induce autophagy in different cell types, including C2C12 myoblasts, *C. elegans*, and retinal pigment epithelium cells.[32,56,57] In lung cancer cells, GRP78 has been found to activate autophagy flux by inhibiting mTOR signaling.[58] Suppression of GRP78 has been shown to decrease the protein level of Beclin-1 and inhibit the conversion of LC3 II/I.[59] Beclin-1 is a protein that plays a crucial role in initiating autophagy and promoting the formation of phagophore membranes. During heat stress, the conversion of cytosolic LC3-I to membrane-bound LC3-II occurs, which serves as an indicator of autophagic activity facilitating the transportation of autolysosomes to lysosomes for the degradation of captured cargos.[60,61] Given the potential enhancement of GRP78 by heat stress, it is hypothesized that the ER chaperon GRP78 may play a pivotal role in the activation of autophagy during heat stress. Subsequently, we confirmed that autophagy is indeed mediated by GRP78 under heat stress conditions. Autophagy is a critical process that operates in both physiological and stressful environments, serving as an inherent mechanism for repair.[62] Efficient autophagic flux facilitates the proper recycling of nutrients and the elimination of aggregated proteins and damaged organelles.[32] The presence of defective autophagy is associated with the development of various bone-related diseases, whereas increased autophagy significantly enhances osteoblast differentiation. The negative regulation of autophagy by RUBCN positions it as a restrictive regulator of mineralization and bone formation.[37] METTL14 impedes the progression of osteoporosis by targeting beclin-1 to activate the autophagy signaling pathway.[63] Autophagy has been widely proposed as a mechanism through which multiple MSCs can exert their differentiative capacity.[64–66] Consistent with previous studies, our findings demonstrate that GRP78-mediated autophagy plays a role in facilitating the osteogenic and odontogenic differentiation of SCAPs.

Taken together, we demonstrated that both heat stress and GRP78 could contribute to the committed

of autophagosomes in SCAPs exposed to heat stress after pretreatment with GRP78 siRNA or 3-MA. Autophagosomes were indicated by black arrows. Scale bar = 2 μm; (H) Western blot analysis of DSPP, ALP, RUNX2, and OSX in SCAPs exposed to heat stress with or without 3-MA pretreatment. (I) Quantitative analysis of Western blot in (H). (J–K) ALP staining and ALP activity assay on day 7 after osteogenic induction. Scale bar = 100 μm. (L–M) Image and quantification of Alizarin red S staining on day 14 after osteogenic induction. Scale bar = 100 μm. NS, no significance, $^{**}P < 0.01$, $^{***}P < 0.001$.

differentiation of SCAPs, and the enhancement was at least partially mediated by activation of autophagy. GRP78 may be a crucial participant for RET and bone regeneration in the future, and we expect this study can provide references for the regulatory impact of GRP78 on the biological behavior of SCAPs.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgements

This research was funded by Science and Technology Project of Changzhou Health Commission (QN202124).

References

1. Biermann JS, Chow W, Reed DR, et al. NCCN guidelines insights: bone cancer, version 2.2017. *J Natl Compr Cancer Netw* 2017;15:155—67.
2. Gopinath V. Osteoporosis. *Med Clin* 2023;107:213—25.
3. Zheng K, Bai J, Yang H, et al. Nanomaterial-assisted theranosis of bone diseases. *Bioact Mater* 2023;24:263—312.
4. Ye C, Leslie WD. Fracture risk and assessment in adults with cancer. *Osteoporos Int* 2023;34:449—66.
5. Oryan A, Kamali A, Moshiri A, Baghaban Eslaminejad M. Role of mesenchymal stem cells in bone regenerative medicine: what is the evidence? *Cells Tissues Organs* 2017;204:59—83.
6. Kim MS, Chung HJ, Kim KI. Optimal concentration of mesenchymal stem cells for fracture healing in a rat model with long bone fracture. *World J Stem Cell* 2022;14:839—50.
7. Zhou H, He Y, Xiong W, et al. MSC based gene delivery methods and strategies improve the therapeutic efficacy of neurological diseases. *Bioact Mater* 2023;23:409—37.
8. Zhang SY, Ren JY, Yang B. Priming strategies for controlling stem cell fate: applications and challenges in dental tissue regeneration. *World J Stem Cell* 2021;13:1625—46.
9. Sonoyama W, Liu Y, Yamaza T, et al. Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod* 2008;34:166—71.
10. Ebadi M, Miresmaeili A, Shojaei S, Farhadi S, Rajabi S. Isolation and characterization of apical papilla cells from root end of human third molar and their differentiation into cementoblast cells: an in vitro study. *Biol Proced Online* 2023;25:2.
11. Chen Y, Wang X, Wu Z, Jia S, Wan M. Epigenetic regulation of dental-derived stem cells and their application in pulp and periodontal regeneration. *PeerJ* 2023;11:e14550.
12. Mavinga M, Palmier M, Rémy M, et al. The journey of SCAPs (stem cells from apical papilla), from their native tissue to grafting: impact of oxygen concentration. *Cells* 2022;11:4098.
13. Li M, Jiang Y, Hou Q, Zhao Y, Zhong L, Fu X. Potential preactivation strategies for improving therapeutic efficacy of mesenchymal stem cells: current status and future prospects. *Stem Cell Res Ther* 2022;13:146.
14. Choudhery MS. Strategies to improve regenerative potential of mesenchymal stem cells. *World J Stem Cell* 2021;13:1845—62.
15. Tong L, Liao Q, Zhao Y, et al. Near-infrared light control of bone regeneration with biodegradable photothermal osteoimplant. *Biomaterials* 2019;193:1—11.
16. Xue Y, Zhang L, Liu F, et al. Surface bandgap engineering of nanostructured implants for rapid photothermal ion therapy of bone defects. *Adv Healthcare Mater* 2022;11:e2200998.
17. Li M, Fuchs S, Böse T, et al. Mild heat stress enhances angiogenesis in a co-culture system consisting of primary human osteoblasts and outgrowth endothelial cells. *Tissue Eng C Methods* 2014;20:328—39.
18. Shi W, Wang Z, Bian L, et al. Periodic heat stress licenses EMSC differentiation into osteoblasts via YAP signaling pathway activation. *Stem Cell Int* 2022;2022:3715471.
19. Sunderic K, Li C, Ahmed AHR, et al. Tuning thermal dosage to facilitate mesenchymal stem cell osteogenesis in pro-inflammatory environment. *J Biomech Eng* 2021;143:011006.
20. Xiao Y, Ren L, Wang Y, et al. Biochemical characterization and functional analysis of glucose regulated protein 78 from the silkworm bombyx mori. *Int J Mol Sci* 2023;24:3964.
21. Alemu TW, Pandey HO, Salilew Wondim D, et al. Oxidative and endoplasmic reticulum stress defense mechanisms of bovine granulosa cells exposed to heat stress. *Theriogenology* 2018;110:130—41.
22. Gonzalez-Gronow M, Pizzo SV. Physiological roles of the autoantibodies to the 78-kilodalton glucose-regulated protein (GRP78) in cancer and autoimmune diseases. *Biomedicines* 2022;10:1222.
23. Pfaffenbach KT, Lee AS. The critical role of GRP78 in physiologic and pathologic stress. *Curr Opin Cell Biol* 2011;23:150—6.
24. Dudek J, Benedix J, Cappel S, et al. Functions and pathologies of BiP and its interaction partners. *Cell Mol Life Sci* 2009;66:1556—69.
25. Gonzalez-Gronow M, Gopal U, Austin RC, Pizzo SV. Glucose-regulated protein (GRP78) is an important cell surface receptor for viral invasion, cancers, and neurological disorders. *IUBMB Life* 2021;73:843—54.
26. Ibrahim IM, Abdelmalek DH, Elfiky AA. GRP78: a cell's response to stress. *Life Sci* 2019;226:156—63.
27. Merkel A, Chen Y, Villani C, George A. GRP78 promotes the osteogenic and angiogenic response in periodontal ligament stem cells. *Eur Cell Mater* 2023;45:14—30.
28. Zhen Y, Stenmark H. Autophagosome biogenesis. *Cells* 2023;12:668.
29. Yamamoto H, Zhang S, Mizushima N. Autophagy genes in biology and disease. *Nat Rev Genet* 2023;24:382—400.
30. Dikic I, Elazar Z. Mechanism and medical implications of mammalian autophagy. *Nat Rev Mol Cell Biol* 2018;19:349—64.
31. Aman Y, Schmauck-Medina T, Hansen M, et al. Autophagy in healthy aging and disease. *Nat Aging* 2021;1:634—50.
32. Summers CM, Valentine RJ. Acute Heat exposure alters autophagy signaling in C2C12 myotubes. *Front Physiol* 2019;10:1521.
33. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. *Cell Death Differ* 2008;15:1460—71.
34. Zhang H, Sun X, Li J, Shan W, Yang J, Zuo Z. Endoplasmic reticulum stress-activated neuronal and microglial autophagy contributes to postoperative cognitive dysfunction in neonatal rats. *Neurochem Res* 2023;48:1835—47.
35. Ma Y, Qi M, An Y, et al. Autophagy controls mesenchymal stem cell properties and senescence during bone aging. *Aging Cell* 2018;17:e12709.
36. Rajabian N, Choudhury D, Ikhapoh I, et al. Reversine ameliorates hallmarks of cellular senescence in human skeletal myoblasts via reactivation of autophagy. *Aging Cell* 2023;22:e13764.
37. Yoshida G, Kawabata T, Takamatsu H, et al. Degradation of the NOTCH intracellular domain by elevated autophagy in osteoblasts promotes osteoblast differentiation and alleviates osteoporosis. *Autophagy* 2022;18:2323—32.
38. Pan Y, Li Z, Wang Y, et al. Sodium fluoride regulates the osteo/odontogenic differentiation of stem cells from apical

papilla by modulating autophagy. *J Cell Physiol* 2019;234: 16114—24.
39. Sayed S, Faruq O, Hossain M, Im SB, Kim YS, Lee BT. Thermal cycling effect on osteogenic differentiation of MC3T3-E1 cells loaded on 3D-porous biphasic calcium phosphate (BCP) scaffolds for early osteogenesis. *Mater Sci Eng C* 2019;105: 110027.
40. Shui C, Scutt A. Mild heat shock induces proliferation, alkaline phosphatase activity, and mineralization in human bone marrow stromal cells and Mg-63 cells in vitro. *J Bone Miner Res* 2001;16:731—41.
41. Hong CG, Chen ML, Duan R, et al. Transplantation of nasal olfactory mucosa mesenchymal stem cells benefits alzheimer's disease. *Mol Neurobiol* 2022;59:7323—36.
42. Shu Y, Ma M, Pan X, Shafiq M, Yu H, Chen H. Cobalt protoporphyrin-induced nano-self-assembly for CT imaging, magnetic-guidance, and antioxidative protection of stem cells in pulmonary fibrosis treatment. *Bioact Mater* 2023;21: 129—41.
43. Lai C, Cheng M, Ning C, et al. Janus electro-microenvironment membrane with surface-selective osteogenesis/gingival healing ability for guided bone regeneration. *Mater Today Bio* 2022; 17:100491.
44. Todorova VB, Baxan N, Delahaye M, Harding SE, Rankin SM. Drug-based mobilisation of mesenchymal stem/stromal cells improves cardiac function post myocardial infarction. *Dis Model Mech* 2023;16:dmm049630.
45. Wan Z, Zhang P, Lv L, Zhou Y. NIR light-assisted phototherapies for bone-related diseases and bone tissue regeneration: a systematic review. *Theranostics* 2020;10:11837—61.
46. Rühle A, Thomsen A, Saffrich R, et al. Multipotent mesenchymal stromal cells are sensitive to thermic stress - potential implications for therapeutic hyperthermia. *Int J Hyperther* 2020;37:430—41.
47. Li C, Sunderic K, Nicoll SB, Wang S. Downregulation of heat shock protein 70 impairs osteogenic and chondrogenic differentiation in human mesenchymal stem cells. *Sci Rep* 2018;8: 553.
48. Wang Q, Li X, Wang Q, Xie J, Xie C, Fu X. Heat shock pretreatment improves mesenchymal stem cell viability by heat shock proteins and autophagy to prevent cisplatin-induced granulosa cell apoptosis. *Stem Cell Res Ther* 2019;10:348.
49. Naidoo N, Zhu J, Galante RJ, et al. Reduction of the molecular chaperone binding immunoglobulin protein (BiP) accentuates the effect of aging on sleep-wake behavior. *Neurobiol Aging* 2018;69:10—25.
50. Choi DH, Lee KE, Oh SY, et al. Tonsil-derived mesenchymal stem cells incorporated in reactive oxygen species-releasing hydrogel promote bone formation by increasing the translocation of cell surface GRP78. *Biomaterials* 2021;278:121156.
51. Borok Z, Horie M, Flodby P, et al. Grp78 loss in epithelial progenitors reveals an age-linked role for endoplasmic reticulum stress in pulmonary fibrosis. *Am J Respir Crit Care Med* 2020;201:198—211.
52. Ravindran S, Narayanan K, Eapen AS, et al. Endoplasmic reticulum chaperone protein GRP-78 mediates endocytosis of dentin matrix protein 1. *J Biol Chem* 2008;283:29658—70.
53. Ravindran S, Gao Q, Ramachandran A, Sundivakkam P, Tiruppathi C, George A. Expression and distribution of grp-78/bip in mineralizing tissues and mesenchymal cells. *Histochem Cell Biol* 2012;138:113—25.
54. Ravindran S, Gao Q, Ramachandran A, Blond S, Predescu SA, George A. Stress chaperone GRP-78 functions in mineralized matrix formation. *J Biol Chem* 2011;286:8729—39.
55. Jawed A, Ho CT, Grousl T, et al. Balanced activities of Hsp 70 and the ubiquitin proteasome system underlie cellular protein homeostasis. *Front Mol Biosci* 2022;9:1106477.
56. Kumsta C, Chang JT, Schmalz J, Hansen M. Hormetic heat stress and HSF-1 induce autophagy to improve survival and proteostasis in C. elegans. *Nat Commun* 2017;8:14337.
57. Amirkavei M, Plastino F, Kvanta A, Kaarniranta K, André H, Koskelainen A. Hormetic heat shock enhances autophagy through HSF1 in retinal pigment epithelium cells. *Cells* 2022; 11:1778.
58. Wang L, Li D, Su X, et al. AGO4 suppresses tumor growth by modulating autophagy and apoptosis via enhancing TRIM21-mediated ubiquitination of GRP78 in a p53-independent manner. *Oncogene* 2023;42:62—77.
59. Zhang XY, Zhang TT, Song DD, et al. Endoplasmic reticulum chaperone GRP78 is involved in autophagy activation induced by ischemic preconditioning in neural cells. *Mol Brain* 2015;8: 20.
60. Ye J, Zheng M. Autophagosome trafficking. *Adv Exp Med Biol* 2021;1208:67—77.
61. Adel N, Abdulghaffar S, Elmahdy M, Nabil M, Ghareeb D, Maghraby H. Autophagy-related gene and protein expressions during blastocyst development. *J Assist Reprod Genet* 2023;40: 323—31.
62. Zhong Y, Tang K, Nattel S, et al. Myosin light-chain 4 gene-transfer attenuates atrial fibrosis while correcting autophagic flux dysregulation. *Redox Biol* 2023;60:102606.
63. He M, Lei H, He X, et al. METTL14 regulates osteogenesis of bone marrow mesenchymal stem cells via inducing autophagy through m6A/IGF2BPs/Beclin-1 signal axis. *Stem Cells Transl Med* 2022;11:987—1001.
64. Pei T, Su G, Yang J, et al. Fluid shear stress regulates osteogenic differentiation via AnnexinA6-mediated autophagy in MC3T3-E1 cells. *Int J Mol Sci* 2022;23:15702.
65. Ames K, Kaur I, Shi Y, et al. PI3-kinase deletion promotes myelodysplasia by dysregulating autophagy in hematopoietic stem cells. *Sci Adv* 2023;9:eade8222.
66. Zhang X, Huang S, Niu X, Li S, Wang J, Ran X. miR-103-3p regulates the differentiation and autophagy of myoblasts by targeting MAP4. *Int J Mol Sci* 2023;24:4130.