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RESEARCH ARTICLE

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Phosphorylated heat shock protein 27 improves the bone formation ability of osteoblasts and bone marrow stem cells from patients with adolescent idiopathic scoliosis

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Abstract

Background: Adolescent idiopathic scoliosis (AIS) is a scoliotic deformity of unknown etiology that occurs during adolescent development. Abnormal bone metabolism is closely related to AIS, but the cause is uncertain. Recent studies have shown that heat shock protein 27 (HSP27) and its phosphorylation (pHSP27) play important roles in bone metabolism. However, whether HSP27 and pHSP27 are involved in abnormal bone metabolism in AIS is unclear.

Methods: Osteoblasts (OBs) and bone marrow stem cells (BMSCs) were extracted from the facet joints and bone marrow of AIS patients and controls who underwent posterior spinal fusion surgery. The expression levels of HSP27 and pHSP27, as well as the expression levels of bone formation markers in OBs from AIS patients and controls, were examined by quantitative real-time PCR (qRT–PCR) and Western blotting. The mineralization ability of OBs from AIS patients and controls was analyzed by alizarin red staining after osteogenic differentiation. Heat shock and thiolutin were used to increase the levels of pHSP27 in OBs, and the levels of bone formation markers were also investigated. In addition, the levels of pHSP27 and the bone formation ability of BMSCs from AIS patients and controls were investigated after heat shock treatment.

Results: Lower pHSP27 levels and impaired osteogenic differentiation abilities were observed in the OBs of AIS patients than in those of controls. Thiolutin increased HSP27 phosphorylation and increased the mRNA levels of *SPP1* and *ALPL* in OBs from AIS patients. Heat shock treatment increased *SPP1* and *HSP27* mRNA expression, pHSP27 levels, OCN expression, and mineralization ability of both OBs and BMSCs from AIS patients.

Conclusion: Heat shock treatment and thiolutin can increase the levels of pHSP27 and further promote the bone formation of OBs and BMSCs from AIS patients. Therefore, decreased pHSP27 levels may be associated with abnormal bone metabolism in AIS patients.

KEYWORDS
adolescent idiopathic scoliosis, bone formation, bone marrow stem cells, heat shock protein 27, osteoblast, phosphorylation

1 | INTRODUCTION

Adolescent idiopathic scoliosis (AIS) is a scoliotic deformity of unknown etiology that occurs during adolescent development, with an incidence of approximately 1% to 3% in adolescents aged 10–16 years.[1,2] An increasing number of studies have confirmed that abnormal bone metabolism is closely related to AIS.[3–7] AIS with abnormal bone metabolism mtay lead to a more serious clinical manifestation, such as a larger curve.[8–10] Two long-term follow-up studies showed that approximately half of AIS patients treated with surgery will develop osteopenia or osteoporosis in adulthood, which makes some patients with AIS less able to exercise in adulthood.[11,12] Therefore, bone metabolism has a great influence on the pathogenesis, development, and long-term prognosis of AIS.

Bone metabolism includes bone formation and bone resorption, for which osteoblasts and osteoclasts are required.[13] Bone formation is currently considered to be an important factor in improving the prognosis of spinal fusion surgery.[14] Therefore, it is necessary to find more ways to stimulate bone formation. Recently, Sayed et al. found that in response to regular mild heat shock stimulation, osteoblast-like cells upregulate the mRNA of heat shock protein 27 (*HSP27*) and heat shock protein 70 (*HSP70*), exhibit more calcium deposition and mineralization and express more osteospecific markers.[15] Other studies also showed that heat shock protein exerts a good effect on bone formation.[16–19]

HSP27, a 27 kDa heat shock protein, is always found in the cytoplasm and nucleus.[20] HSP27 is found in the nucleus under heat stress or other stress conditions, its main function is facilitating protein folding, and it is involved in various cellular processes.[21,22] Mild heat shock, such as heat shock at temperatures of 40°C–43°C, is beneficial to some cellular activities,[23] including bone formation.[15] Omagari et al. found that higher *HSP27* mRNA expression could increase the mRNA expression of the osteogenic genes alkaline phosphatase (*ALP*) and bone salivary protein (*BSP*).[24] Kenji Kato et al. found that lower phosphorylated HSP27 levels could decrease the expression of osteocalcin (OCN).[25] These studies showed that HSP27 and its phosphorylation played an important role in bone metabolism. Moreover, Zhuang et al. found that HSP27 was decreased in bone marrow mesenchymal stem cells (BMSCs) from AIS patients compared to those from non-AIS patients with lower-leg fracture.[26] However, no studies have shown that HSP27 is related to the abnormal bone metabolism of AIS.

Our study aimed to determine the relationship between HSP27 and AIS, and provide ideas to improve bone formation in patients with AIS.

2 | MATERIALS AND METHODS

2.1 | Ethical approval

This study was conducted in accordance with the principles of the Declaration of Helsinki II and approved by the medical ethics committee of Xiangya Hospital, Central South University (ethical code, 201703359). Written informed consent was obtained from each patient (or their parents and legal guardians) to authorize treatments and access imaging findings and biological specimens.

2.2 | Subjects

The facet joints were harvested during posterior spinal fusion surgery at Xiangya Hospital (Changsha, Hunan, China) and the patients were divided into two groups (8 AIS patients and 4 controls). The bone marrow was collected during bone marrow aspiration and the patients were divided into two groups (8 AIS patients and 4 controls). All the patients were diagnosed by medical imaging, and diagnosis was confirmed by three experienced radiologists. The control patients were age-matched and suffered from herniated discs, spinal injuries, spinal spondylolisthesis, and spinal bone destruction. The bone mineral density of AIS patients was collected by the Z value which is a gold standard for determination of child osteopenia using a dual-energy x-ray bone densitometer, Z value ≤ -2 indicates osteoporosis, $-2 <$ Z value ≤ -1 indicates osteopenia, Z value > -1 indicates normal bone mineral density.[27] We collected the lumbar total Z value to determine the bone mineral density.

2.3 | Cell isolation and culture

Human primary osteoblasts were collected from the facet joints as described previously.[28] The osteoblasts were cultured on proper dishes in F12/DMEM (HyClone, Logan, USA) supplemented with 10% foetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% penicillin–streptomycin double antibiotics (P/S, Gibco, Carlsbad, CA, USA) in an incubator (37°C with 5% CO_2). The medium was changed every 3–4 days.

Primary BMSCs were collected from the bone marrow as described previously.[28] BMSCs were cultured in 15% fotal bovine serum in F12/DMEM with 1% P/S in an incubator (37°C with 5% CO_2). The medium was replaced every 3–4 days.

2.4 | Heat shock and phosphorylation treatment

When the osteoblasts and BMSCs at passage 2 reached 80% confluence, the medium was changed to Human Mesenchymal Stem Cell

Osteogenic Differentiation Complete Medium (OriCell, Guangzhou, China). The heat shock (HS) group was subjected to heat shock treatment in the incubator at 41 1°C for 1 h once a day. After heating, the medium was replaced, and the cells were returned to the 37°C incubator. The not heated (NH) group was kept in the 37°C incubator with osteogenic differentiation medium.

For phosphorylation treatment, a rapid phosphorylation-inducing reagent that is specific for HSP27 called thiolutin was used as described previously.[29,30] When the osteoblasts at passage 2 reached 80% confluence, the medium was changed to 1 μM thiolutin (MCE, Shanghai, China) in F12/DMEM supplemented with 10% fetal bovine serum and 1% P/S, the cells were incubated at 37°C for 1 h, and total mRNA and total protein were harvested for subsequent experiments.

2.5 | Cell viability analysis

The level of cell viability was analyzed using a Viability/Cytotoxicity Assay Kit for Animal Live & Dead Cells (Proteintech, China) which is also applicable to human cells. After a 1-h heat shock, osteoblasts and BMSCs were incubated with Calcein AM and EthD-I staining solution for 20 min. Live cells (494/517 nm) and dead cells (528/617 nm) were observed in 5 different fields of view under a fluorescence microscope (magnification 100×).

2.6 | Quantitative real-time PCR

According to the instructions, total mRNA was extracted from the osteoblasts and BMSCs described above using TRIzol reagent (CWBio, Beijing, China). Then, the total mRNA was reverse transcribed into cDNA via a HiFiScript cDNA Synthesis Kit (CWbio, Beijing, China). Quantitative real-time PCR was performed by SYBR qPCR SuperMix Plus (NovoStart, China) and an Applied Biosystems 7500 instrument. The sequences of the primers used in the experiment are shown in Table 1. The RNA levels were normalized by *GAPDH* and the data were analyzed by delta Ct method.

TABLE 1 Primers for quantitative real-time PCR (qPCR).

Gene	Primer sequences (5′–3′)	
GAPDH	F	TGACCCCTTCATTGACCTCA
	R	ATCGCCCCACTTGATTTTGG
HSP27	F	AGGATGGCGTGGTGGAGA
	R	GGGAGGAGGAAACTTGGGTG
ALPL	F	CCACAAGCCCGTGACAGA
	R	GGGCGGCAGACTTTGGTT
SPP1	F	GACAGCCAGGACTCCATT
	R	GATGTCAGGTCTGCGAAA

2.7 | Western blotting

Total proteins were extracted from the preceding osteoblasts and BMSCs using RIPA Lysis Buffer reagent (CWbio, Beijing, China) containing phosphatase inhibitor cocktail (CWbio, Beijing, China) and phenylmethanesulfonylfluoride (Servicebio, China). The concentrations of the isolated proteins were normalized to 1 μg/μL, and the proteins were separated by 10% SDS–PAGE gels. Then, the proteins were transferred to PVDF membranes, which were blocked using skim milk for 1 h at room temperature. The membranes were incubated on a rocker overnight at 4°C with polyclonal rabbit anti-β actin (1:2000, Cell Signaling Technology, USA), polyclonal rabbit anti-HSP27 (1:4000, Proteintech, China), polyclonal rabbit anti-phospho-HSP27 (Ser78) (1:1000, Proteintech, China), and polyclonal rabbit anti-osteocalcin (1:2000, Servicebio, China), which were diluted in primer antibody diluent. After secondary antibody incubation for 2 h at room temperature, the bands were visualized using BeyoECL Plus (Beyotime, China) with Image Lab 3.0 software.

2.8 | Alizarin red staining

The treated cells were fixed in 4% paraformaldehyde for 30 min at room temperature and incubated with Alizarin Red reagent (OriCell, Guangzhou, China) for 10 min. Mineralization was measured by calculating the percentage of the area that positively stained for calcium mineral deposits in 5 different views under bright-field microscopy (magnification 100×) with ImageJ software.

2.9 | Statistical analysis

All experiments were repeated three independent times. Statistical analyses were performed using IBM SPSS Statistics 25.0 software (SPSS Inc., Chicago, IL, USA). The Shapiro–Wilk test was used to determine if the data were normally distributed. Student's *t* test was used to compare two independent sample groups. A paired *t* test was used to compare paired sample groups. A nonparametric rank sum test was used to compare two independent sample groups that were not normally distributed. The chi-square test was used to analyze the gender difference. All the results were expressed as the mean ± standard error of the mean. Differences were considered significant if the *p*-value <0.05.

3 | RESULTS

3.1 | Lower mineralization ability and lower levels of phosphorylated HSP27 in osteoblasts and BMSCs from AIS patients

We collected human primary osteoblasts and BMSCs from patients who underwent posterior spinal fusion surgery at Xiangya Hospital

TABLE 2 Information on the AIS patients and controls.

Characteristics	OB		BMSC		
	Control	AIS	Control	AIS	*p*-Value
Age	18.00 ± 1.83	15.63 ± 2.33	15.75 ± 4.65	16.13 ± 3.52	0.1067 ^{P1} 0.8781 ^{P2}
Number (male/female)	4 (2/2)	8 (2/6)	4 (2/2)	8 (3/5)	0.5475 ^{P1} >0.9999 ^{P2}
Major Cobb	-	51.78 ± 8.54	-	41.01 ± 8.36	-
Bone mineral density (lumbar total Z value)	0.4 ± 0.78	−1.19 ± 0.83	0.28 ± 1.05	−1.15 ± 0.87	<0.01 ^{P1} <0.05 ^{P2}

Note: P_1 represents difference between the control group and AIS group in OB; P_2 represents difference between the control group and AIS group in BMSC.

Abbreviations: AIS, adolescent idiopathic scoliosis; BMSC, bone marrow mesenchymal stem cell; OB: osteoblast.

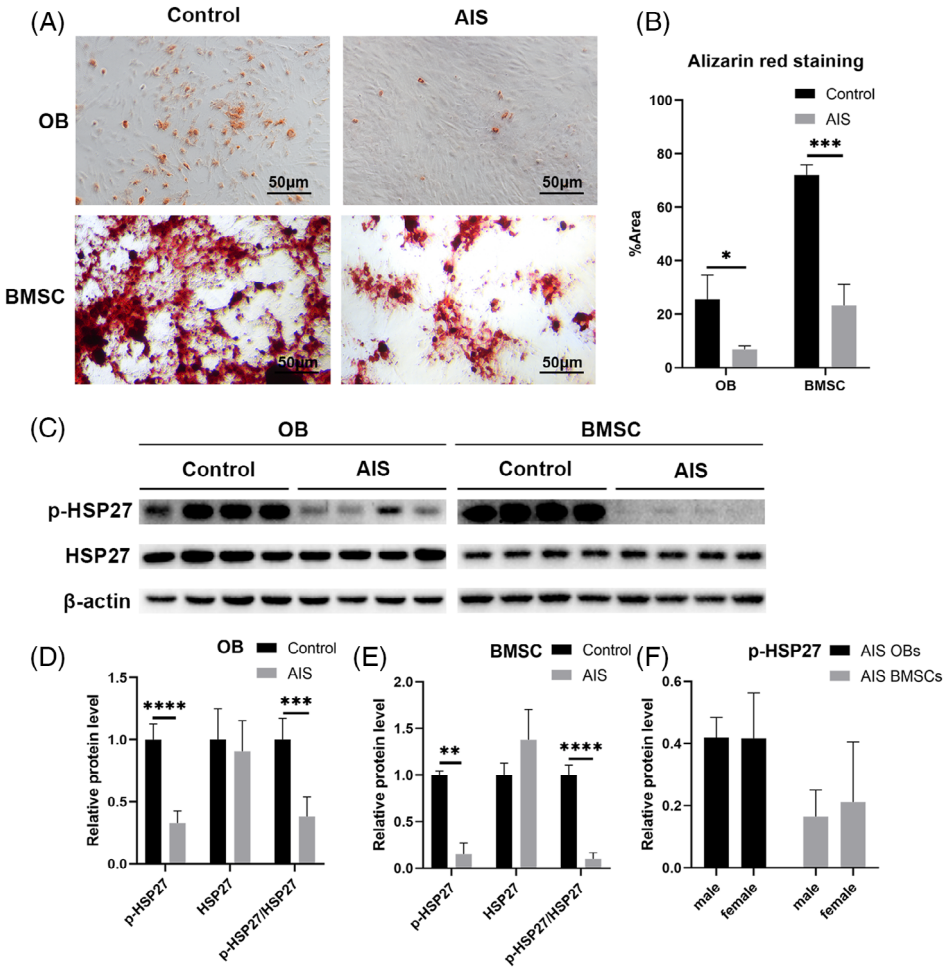

FIGURE 1 Lower mineralization ability and lower expression levels of phosphorylated HSP27 in OBs and BMSCs from AIS patients. (A) Alizarin red staining for mineralization ability of OBs and BMSCs from patients with AIS ($n = 3$) and controls ($n = 3$). (B) The percentage of Alizarin red staining area. (C–E). The protein levels of p-HSP27, HSP27, and p-HSP27/HSP27 ratio in OB and BMSC from patients with AIS ($n = 8$) and controls ($n = 4$). (F). the protein levels of p-HSP27 in OB (males, $n = 2$, females, $n = 6$) and BMSC (males, $n = 3$, females, $n = 5$) from different gender with AIS. AIS, adolescent idiopathic scoliosis; BMSC, bone marrow mesenchymal stem cell; HSP27, heat shock protein 27; OB, osteoblast; p-HSP27: phosphorylated HSP27 (Ser78). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

(Table 2). Among the patients who provided primary osteoblasts, age, and sex were not different between the control group (18.00 ± 1.83 years old, 2 males and 2 females) and the AIS group (15.63 ± 2.33 years old, 2 males and 6 females, $p = 0.11$ and $p = 0.55$). Among the patients who provided BMSCs, age, and sex were not different between the control group (15.75 ± 4.65 years old, 2 males and 2 females) and the AIS group (16.13 ± 3.52, 3 males and 5 females, $p = 0.88$ and $p > 0.99$). The major cobb angle of AIS patients who provided osteoblasts was 51.78° ± 8.54° and that of the patients who provided BMSCs was 41.01° ± 8.36°. The Z value showed AIS patients had lower bone mineral density than controls in both who provided osteoblasts (AIS: −1.19 ± 0.83 and controls: 0.4 ± 0.78, $p < 0.01$) and BMSCs (AIS: −1.15 ± 0.87 and controls: 0.28 ± 1.05, $p < 0.05$).

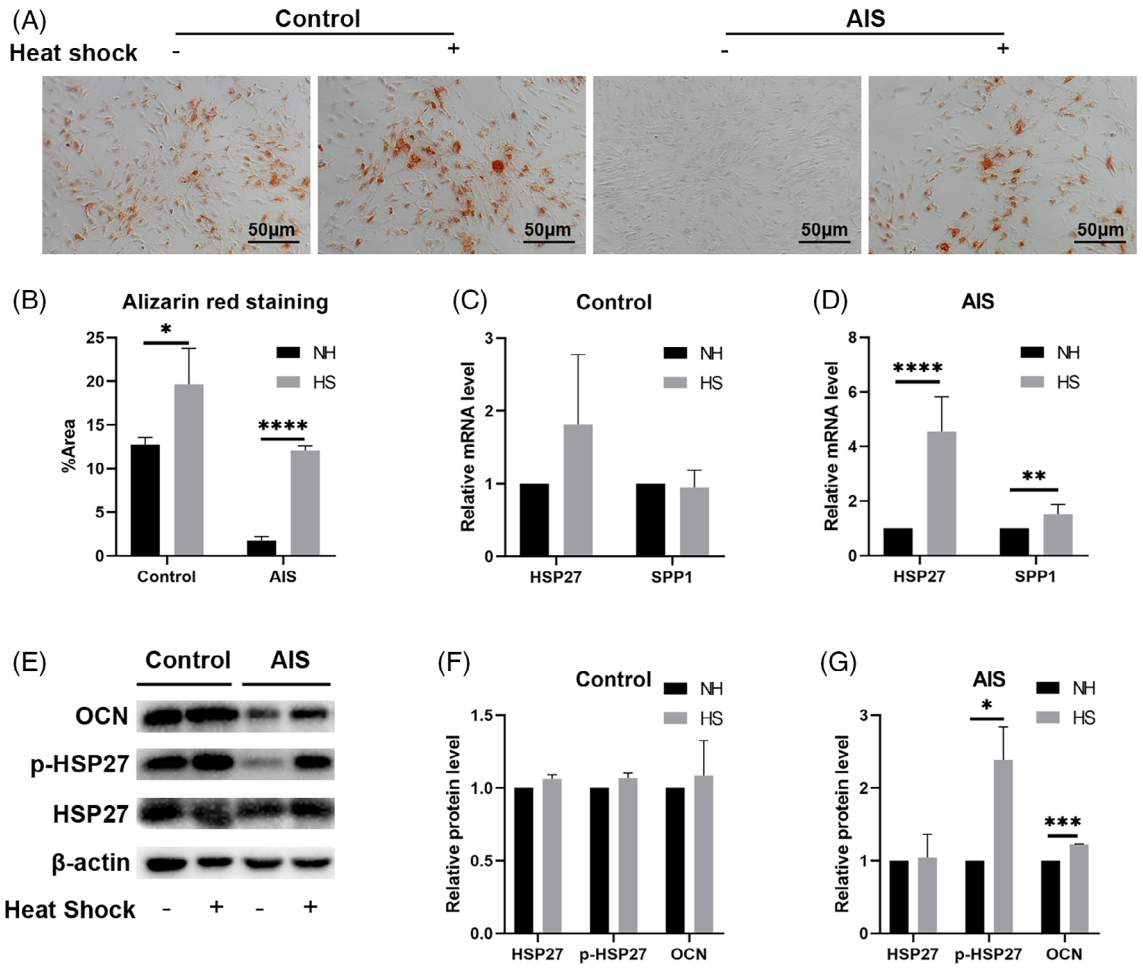

FIGURE 2 Heat shock treatment increases the phosphorylated heat shock protein 27 (HSP27) levels and promotes the bone formation in the osteoblasts from adolescent idiopathic scoliosis (AIS) patients. (A) Alizarin red staining for mineralization ability of osteoblasts (controls, $n = 3$, AIS, $n = 3$). (B) The percentage of Alizarin red staining area. (C) The mRNA level of *HSP27* and *SPP1* between the Control osteoblasts with heat shock (HS) and the Control osteoblasts not heated (NH) (controls, $n = 3$, AIS, $n = 3$). (D) The mRNA level of *HSP27* and *SPP1* between the AIS osteoblasts with HS and the AIS osteoblasts with NH (controls, $n = 3$, AIS, $n = 3$). (E–G) The protein levels of OCN, p-HSP27, HSP27 in Control osteoblasts with/without heat shock and AIS osteoblasts with/without heat shock (controls, $n = 3$, AIS, $n = 3$). p-HSP27, phosphorylated HSP27 (Ser78). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

The Alizarin red staining results showed fewer calcium mineral deposits in both osteoblasts and BMSCs from AIS patients (Figure 1A,B, $p < 0.05$ and $p < 0.001$).

Then, we extracted the total proteins. Western blotting was used to determine the expression levels of phosphorylated HSP27 and HSP27 (Figure 1C). We found that the levels of phosphorylated HSP27 were lower in both osteoblasts and BMSCs from AIS patients (Figure 1D,E, $p < 0.0001$ and $p < 0.01$), and the levels of HSP27 were not different in either osteoblasts or BMSCs between the AIS group and the control group (Figure 1D,E, $p = 0.56$ and $p = 0.05$). Moreover, the p-HSP27/HSP27 ratio was lower in both osteoblasts and BMSCs from AIS patients (Figure 1D,E, $p < 0.001$ and $p < 0.0001$), and p-HSP27 in osteoblasts from the concave and convex sides of the AIS group were not different (Figure S1A,B, $p = 0.87$). Moreover, in both osteoblasts and BMSCs, phosphorylated HSP27 levels were not different between male and female AIS patients (Figure 1F, $p = 0.86$, and $p = 0.79$).

3.2 | Heat shock treatment increases phosphorylated HSP27 levels and promotes bone formation in the osteoblasts from AIS patients

To determine the function of heat shock treatment, P2 generation osteoblasts were divided into four groups. The AIS and control with heat shock (HS) groups underwent a 4-day heat shock treatment (41°C for 1 h once a day). The AIS and control with no heated (NH) groups were kept at 37°C. The cell viability analysis results showed that heat shock exerted no lethal effect on the cells (Figure S2). The HS group had more calcium mineral deposits than the NH group in both the control and AIS groups (Figure 2A,B, $p < 0.05$ and $p < 0.0001$).

For the mRNA, the levels of *HSP27* and *SPP1* in the AIS with HS group were 4.5 ± 1.3 times and 1.5 ± 0.4 times higher than those in the AIS with NH group (Figure 2D, $p < 0.0001$ and $p < 0.01$). There

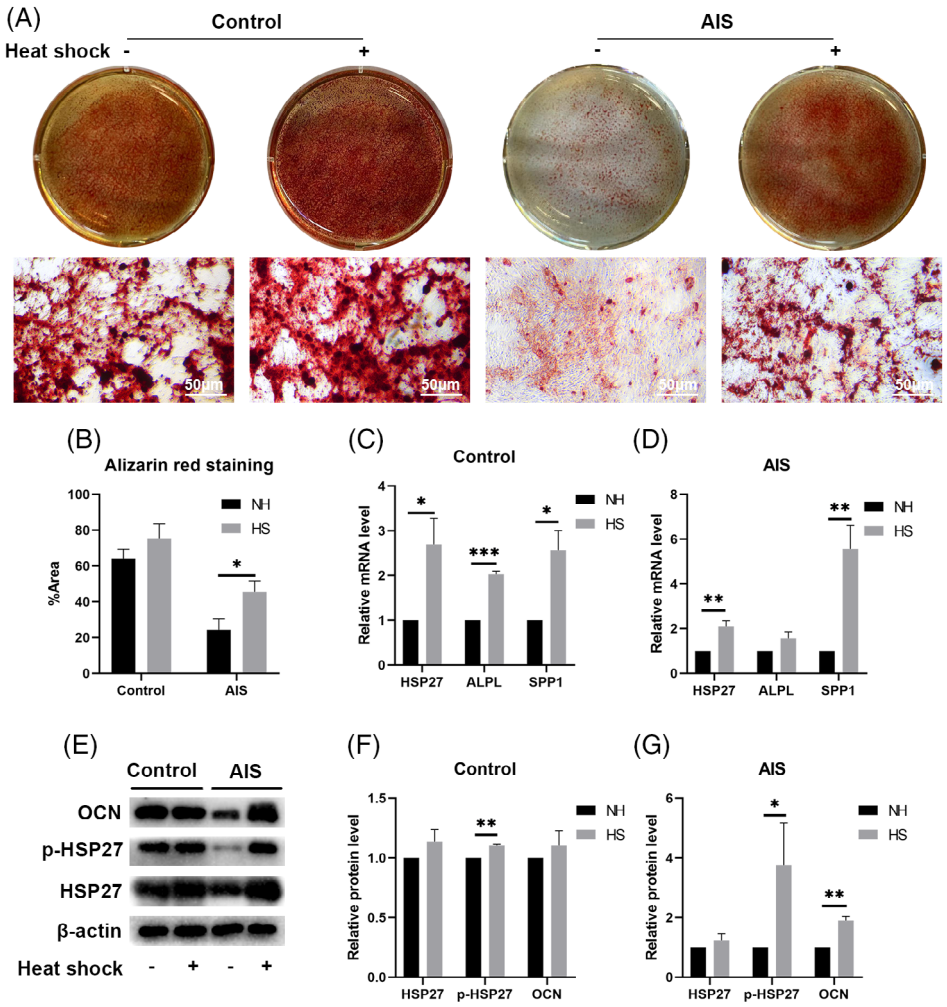

FIGURE 3 Heat shock treatment increases the phosphorylated HSP27 and promotes the osteogenic differentiation of BMSCs. (A) Alizarin red staining for osteogenic differentiation of BMSCs (controls, $n = 3$, AIS, $n = 3$). (B) The percentage of Alizarin red staining area. (C) The mRNA level of *HSP27*, *ALPL* and *SPP1* between the Control BMSCs with heat shock (HS) and the control BMSCs not heated (NH) (controls, $n = 3$, AIS, $n = 3$). (D) The mRNA level of *HSP27*, *ALPL* and *SPP1* between the AIS BMSCs with HS and the AIS BMSCs with NH (controls, $n = 3$, AIS, $n = 3$). (E–G) The protein levels of OCN, p-HSP27, HSP27 in control BMSCs with/without heat shock and AIS BMSCs with/without heat shock (controls, $n = 3$, AIS, $n = 3$). AIS, adolescent idiopathic scoliosis; BMSC, bone marrow mesenchymal stem cell; HSP27, heat shock protein 27; OB, osteoblast; p-HSP27, phosphorylated HSP27 (Ser78). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

was no significant difference in *HSP27* and *SPP1* between the control with HS group and the control with NH group (Figure 2C, $p = 0.22$ and $p = 0.67$).

At the protein level, phosphorylated HSP27 levels were 2.4 ± 0.5 times higher in the AIS with HS group than that in the AIS with NH group (Figure 2G, $p < 0.05$). OCN was 1.2 ± 0.01 times higher in the AIS with HS group than that in the AIS with NH group (Figure 2G, $p < 0.001$). Among the control samples, the HS group and NH group had no significant difference in phosphorylated HSP27 and OCN levels (Figure 2F, $p = 0.06$ and $p = 0.15$).

3.3 | Heat shock treatment increases phosphorylated HSP27 and promotes the osteogenic differentiation of BMSCs

Osteoblasts are derived from BMSCs, so we decided to determine the function of heat shock treatment in BMSCs from AIS patients and controls. As described before, the HS group underwent a 10-day heat shock treatment (41°C for 1 h once a day) with osteogenic differentiation medium. The cell viability analysis results showed that heat shock exerted no lethal effect on the cells (Figure S2). Among the AIS samples, the HS group had more calcium mineral deposits than the NH group. (Figure 3A,B, $p < 0.05$).

According to the mRNA analysis, in the control group, the levels of *HSP27*, *ALPL*, and *SPP1* were 2.7 ± 0.6 times, 2.0 ± 0.1 times, and 2.6 ± 0.4 times higher, respectively, during heat shock stimulation (Figure 3C, $p < 0.05$, $p < 0.001$, and $p < 0.05$). In the AIS group, the levels of *HSP27* and *SPP1* were 2.1 ± 0.3 times and 5.6 ± 1.1 times higher, respectively, during heat shock stimulation (Figure 3D, $p < 0.01$ and $p < 0.01$). There was no significant difference in the *ALPL* in levels of AIS samples between the heat shock and NH groups (Figure 3D, $p = 0.05$).

Regarding protein expression, in the control group, the level of phosphorylated HSP27 was 1.1 ± 0.01 times higher during heat shock (Figure 3F, $p < 0.01$), but the difference in OCN was not significantly different (Figure 3F, $p = 0.29$). In the AIS group, the levels of phosphorylated HSP27 and OCN were 3.8 ± 1.4 times and 1.9 ± 0.1 times higher, respectively, after a heat shock (Figure 3G, $p < 0.05$ and $p < 0.01$). There was no significant difference in HSP27 between the heat shock and NH groups of the control and AIS samples. (Figure 3F,G, $p = 0.13$ and $p = 0.16$).

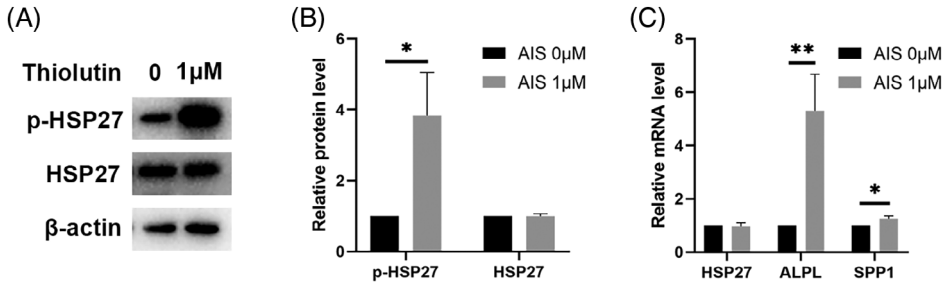

FIGURE 4 Increased the phosphorylated HSP27 levels increase the mRNA levels of *SPP1* and *ALPL* in the osteoblasts from AIS patients. (A,B) The protein levels of p-HSP27 and HSP27 in AIS osteoblasts with/without Thiolutin (controls, $n = 3$, AIS, $n = 3$). (C) The mRNA levels of *HSP27*, *ALPL*, *SPP1* in AIS osteoblasts with/without Thiolutin (controls, $n = 3$, AIS, $n = 3$). AIS, adolescent idiopathic scoliosis; HSP27, heat shock protein 27; OB, osteoblast; p-HSP27, phosphorylated HSP27 (Ser78). $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4 | Increased the phosphorylated HSP27 levels increases the mRNA levels of *SPP1* and *ALPL* in the osteoblasts from AIS patients

Mild heat shock causes in a variety of protein changes in the cell.[23] We wanted to prove that the elevated mRNA levels of *SPP1* and bone formation capacity were improved by the increase in phosphorylated HSP27 rather than changes in other proteins. Therefore, we finally added a rapid HSP27-specific phosphorylation-inducing reagent named thiolutin to osteoblast cell cultures. After phosphorylation-induction for 1 h, the phosphorylated HSP27 protein level in the AIS with 1 μM thiolutin was 3.8 ± 1.2 times greater than that in the AIS samples without thiolutin (Figure 4B, $p < 0.05$). There was no significant difference in HSP27 expression after AIS osteoblasts were treated with thiolutin (Figure 4B, $p = 0.93$). The *ALPL* and *SPP1* mRNA levels in AIS with 1 μM thiolutin were 5.3 ± 1.4 times and 1.3 ± 0.1 times higher than those in the AIS cells treated without thiolutin (Figure 4C, $p < 0.01$ and $p < 0.05$). The *HSP27* mRNA level in the AIS with 1 μM thiolutin and without thiolutin was not significantly different (Figure 4C, $p = 0.67$).

4 | DISCUSSION

Studies have shown that a large number of AIS patients have abnormal bone metabolism.[3–7] Abnormal bone metabolism may lead to a larger curve[8–10] and reduced ability to exercise after surgery in adulthood.[11,12] Currently, an increasing number of studies have confirmed the relationship between abnormal bone metabolism and AIS. In the serum of AIS patients, the receptor activator of nuclear factor-kB ligand (RANKL) and the RANKL/OPG ratio increased,[31] the bone formation marker P1NP increased,[32] 25-OH-D3 and calcitonin decreased[33] and approximately 60% of AIS patients have high tartrate-resistant acid phosphatase 5b (TRAP5b) values.[34] In human mesenchymal stem cells from AIS patients, MAPK7 was decreased.[17] Wang et al. found that the expression of *Runx2* mRNA and protein was significantly reduced in the cancellous bone from AIS patients with low bone density compared with AIS patients with normal bone density.[35] Obviously, abnormal bone metabolism is not negligible in AIS.

However, the cause of abnormal bone metabolism in patients with AIS is still unclear. Published studies have suggested that many factors, such as genes and hormones, may be involved in abnormal bone metabolism in patients with AIS. In terms of genes, overexpression of *miR-151a-3p*,[36] exon gene *GPR126-exon6i*,[37] and expression of long noncoding RNA *lncAIS*[38] can all cause abnormal bone metabolism in patients with AIS. In terms of hormones, published studies have found that ghrelin,[28] melatonin,[39,40] adiponectin,[41] leptin,[42] estrogen[43] and other hormones can affect abnormal bone metabolism in AIS patients. In summary, the abnormal bone metabolism in patients with AIS is caused by a variety of factors, and it is not clear whether there are other factors involved.

In this research, we found that both primary osteoblasts and BMSCs from AIS patients have lower mineralization ability (Figure 1), which is similar to previous article.[7] We think osteoblast and BMSC dysfunction may be an important cause of abnormal bone metabolism in AIS.

HSP27 plays an important role in bone metabolism. Sayed et al. incubated osteoblast-like cells at 41°C for periodic heat shock and found that the bone-related genes *ALP*, *OPN*, *OCN*, *COL10*, and *Runx2* were increased, and the expression of *HSP70* and *HSP27* was significantly upregulated.[15] Omagari et al. found that forced expression of *HSP27* in prelipoid-like 3 T3-L1 cells significantly increased the mRNA expression of the osteogenic genes *ALP* and *BSP* without affecting the expression of *Runx2* and *OSX* mRNA.[24] Kenji Kato et al. found that nonphosphorylated HSP27 has an inhibitory effect on the expression of OCN in osteoblasts, and phosphorylated HSP27 is conducive to higher bone formation abilities.[25] Moreover, a study showed that HSP27 was decreased in the BMSCs of AIS patients.[26] Unfortunately, this article used proteomics and did not observe the abnormal protein phosphorylation.

Then, we found that phosphorylated HSP27 was significantly decreased in both the osteoblasts and BMSCs from patients with AIS (Figure 1). And according to the specific role of HSP27 and its phosphorylation in bone metabolism, these results likely indicate that phosphorylated HSP27 plays a role in the abnormal bone metabolism of AIS.

Articles have shown that the phosphorylation of HSP27 was increased as temperature increases,[44] so we then tried to increase the

phosphorylated HSP27 levels in osteoblasts and BMSCs derived from AIS patients by mild heat shock stimulation. In both osteoblasts and BMSCs, we found that heat shock stimulation increased the phosphorylated HSP27 levels and upregulated the *SPP1* mRNA, OCN expression, and mineralization ability (Figures 2 and 3). The *SPP1* gene produces osteopontin (OPN), which increases proliferation and calcification in osteoblasts.[45] OCN is a marker of bone formation in mature osteoblasts.[46,47] In this part, our experiments demonstrated that for both osteoblasts and BMSCs from patients with AIS, heat shock stimulation increased phosphorylated HSP27 and promoted bone formation at the cellular level.

Mild heat shock changes a variety of proteins in the cell.[23] We finally wanted to prove that the mRNA levels of *SPP1* and bone formation ability were improved by the increase in phosphorylated HSP27 rather than changes in other proteins. Therefore, we decided to analyze the function of phosphorylated HSP27 in osteoblasts from AIS patients without heat shock. A rapid phosphorylation-inducing reagent specific for HSP27 named thiolutin transiently increased the level of phosphorylated HSP27 in AIS patient osteoblasts and increased the osteogenic-related gene mRNA levels of *ALPL* and *SPP1* (Figure 4). The *ALPL* gene encodes a membrane-bound glycosylated enzyme that plays a role in bone mineralization.[47] However, long-term use of thiolutin caused a large amount of cell death. After short-term use, the level of phosphorylated HSP27 decreased to a normal level after a period of increase, so it is difficult to observe the effect of thiolutin on downstream proteins and mineralization capacity.

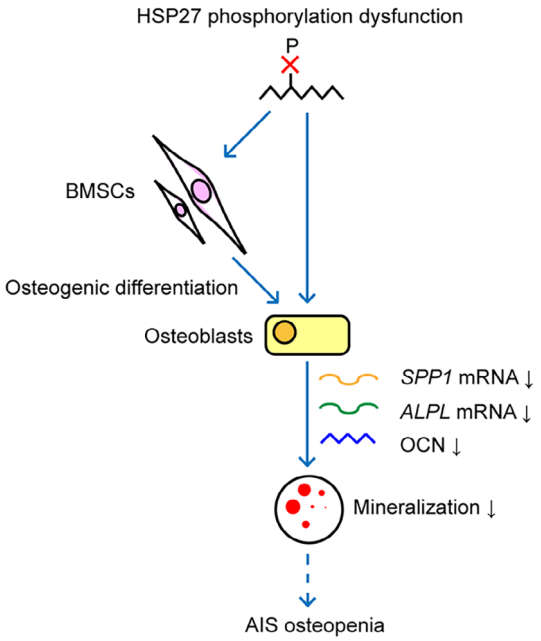

FIGURE 5 The function of phosphorylated HSP27 in AIS osteopenia. HSP27 phosphorylation dysfunction leads to a negative effect on bone metabolism by decreasing *ALPL SPP1* mRNA and OCN in primary osteoblasts and BMSCs in AIS osteopenia. AIS, adolescent idiopathic scoliosis; BMSC, bone marrow mesenchymal stem cell; HSP27, heat shock protein 27; OB, osteoblast.

Our experimental results demonstrate for the first time that the osteoblasts and BMSCs from AIS patients with lower phosphorylated HSP27 levels express lower levels of bone formation markers, such as *ALPL* mRNA, *SPP1* mRNA, and OCN, eventually leading to a low capacity for bone mineralization. This process, to a certain extent, results in bone metabolism disorder and osteopenia in AIS patients (Figure 5). Osteoblasts and BMSCs from AIS patients have the potential to recruit the HSP27 pathway, but phosphorylated HSP27 is expressed at low levels. It seems likely that the HSP27 upstream phosphorylation function is defective. However, the exact cause of decreased HSP27 phosphorylation levels in AIS patients has not been identified. We hold the opinion that in addition to abnormal phosphorylation function, protein modification may play a role in AIS osteopenia. The mechanism underlying the association between phosphorylated HSP27 and AIS deserves further study.

This research also suggests that mild heat shock treatment after posterior spinal fusion surgery may be beneficial to enhance bone formation and shorten the bone recovery time and postoperative hospital stay. However, HSP27 is only a member of the heat shock family, and the effect of heat stimulation treatment on the overall heat shock family is unknown. It is unclear whether there will be any adverse systemic effects on the human body. In addition, there are few clinical reports about the use of heat therapy for improving bone formation, and how to use heat shock treatment for AIS therapy needs more clinical trials.

Our results have certain limitations. First, because there are no complete AIS animal models, we cannot observe how HSP27 and its phosphorylation work in vivo. Our work was conducted in primary cells from AIS patients, but it only represents some of the actual situations in vivo. Second, some AIS patients did not meet the standard of surgical treatment due to insignificant symptoms, and because of small group sizes, the experimental samples could not represent all AIS patients.

5 | CONCLUSION

In this study, we found that phosphorylated HSP27 levels were significantly decreased in both the osteoblasts and BMSCs from patients with AIS. Heat shock treatment and thiolutin increase the phosphorylated HSP27 levels and upregulate bone formation markers in the osteoblasts and BMSCs from AIS patients at the cellular level. This finding suggests a relationship between phosphorylated HSP27 and abnormal bone metabolism in AIS patients. In addition to abnormal phosphorylation function, protein modification may play a role in AIS osteopenia.

AUTHOR CONTRIBUTIONS

Sihan He conceived of the work. Sihan He, Gang Xiang, Guanteng Yang, and Lige Xiao collected and cultured the primary cells and the acquired data. Jiong Li, Yunjia Wang, Mingxing Tang and Hongqi Zhang designed work and revised for important intellectual content and editing. All the authors analyzed and interpreted the data for the work, wrote the original draft, and final approved of the version to be published.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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