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Enhanced osteogenic differentiation of mesenchymal stem cells in ankylosing spondylitis: a study based on a three-dimensional biomimetic environment

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Abstract

The mechanism of pathological osteogenesis in Ankylosing spondylitis (AS) is largely unknown. Our previous studies demonstrated that the imbalance between BMP-2 and Noggin secretion induces abnormal osteogenic differentiation of marrow-derived mesenchymal stem cells (MSCs) from AS patients in a two-dimensional culture environment. In this study, HA/ β -TCP scaffolds were further used as a three-dimensional (3D) biomimetic culture system to mimic the bone microenvironment in vivo to determine the abnormal osteogenic differentiation of AS-MSCs. We demonstrated that when cultured in HA/ β -TCP scaffolds, AS-MSCs had a stronger osteogenic differentiation capacity than that of MSCs from healthy donors (HD-MSCs) in vitro and in vivo. This dysfunction resulted from BMP2 overexpression in AS-MSCs, which excessively activated the Smad1/5/8 and ERK signalling pathways and finally led to enhanced osteogenic differentiation. Both the signalling pathway inhibitors and siRNAs inhibiting BMP2 expression could rectify the enhanced osteogenic differentiation of AS-MSCs. Furthermore, BMP2 expression in ossifying entheses was significantly higher in AS patients. In summary, our study demonstrated that AS-MSCs possess enhanced osteogenic differentiation in HA/ β -TCP scaffolds as a 3D biomimetic microenvironment because of BMP2 overexpression, but not Noggin. These results provide insights into the mechanism of pathological osteogenesis, which can aid in the development of niche-targeting medications for AS.

Introduction

Ankylosing spondylitis (AS) is a common autoimmune disease that affects the axial skeleton, and one of its critical pathogenic features is new bone formation at local sites of entheses¹, which is confined to the periosteal bone compartment, outside the cortical bone lining. In recent

years, many researchers have investigated the mechanism of pathological osteogenesis in AS^{2,3}. However, the concrete details of pathological osteogenesis are still controversial, impeding the development of a specific medication for AS. Furthermore, the lack of a specific therapeutic target for pathological osteogenesis results in a high disability rate in AS patients⁴.

Mesenchymal stem cells (MSCs) are one of the most important kinds of multipotential stem cells and possess strong immunoregulatory and trilineage differentiation abilities⁵. Many studies have determined that MSCs are the major origin of osteoblasts⁶. Nevertheless, dysfunction of the osteogenic differentiation ability of MSCs contributes to bone metabolism disorders in rheumatic diseases⁷. For

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example, although mesenchymal progenitors increased, decreased osteoblast differentiation is progressive with disease development in Interleukin-1 receptor antagonist knock-out mice, which spontaneously develop RA-like disease⁸. The activated NF- κ B pathway in MSCs from Systemic lupus erythematosus (SLE) patients inhibits osteoblastic differentiation through BMP/Smad signaling pathway, which may participate in the pathology of osteoporosis in SLE patients⁹. Several histopathologic studies in AS patients prove that endochondral new bone formation during bone erosion involves a series of events from chondrocyte apoptosis to colonization of preosteoblasts, differentiation of the preosteoblasts to osteoblasts and bone matrix secretion¹⁰. MSCs as progenitor cells may play an important role in pathophysiological processes.

Recently, our research, as well as that of other researchers, demonstrated that MSCs from AS patients (AS-MSCs) outperformed MSCs from healthy donors (HD-MSCs) in osteogenic differentiation in two-dimensional (2D) culture, which could be an important mechanism of pathological osteogenesis in AS^{11,12}. However, whether AS-MSCs exhibit an enhanced osteogenic differentiation ability *in vivo* is still unclear.

We tried to use three-dimensional (3D) biomimetic culture, which mimics the real microenvironment *in vivo*, providing a more suitable *in vitro* culture environment for studying the real properties of these cells¹³. Hydroxyapatite and β -tricalcium phosphate (HA/ β -TCP) scaffolds are frequently used 3D biomimetic culture systems that simulate the bone microenvironment because of their similar properties¹⁴. Many studies have proven that HA/ β -TCP scaffolds act as a bone-like 3D biomimetic microenvironment and promote the osteogenic differentiation of MSCs^{15,16}. In this study, we used an HA/ β -TCP scaffold as a 3D biomimetic microenvironment to gain insights into the osteogenic differentiation ability of AS-MSCs *in vitro* and *in vivo*.

Previously, we found that an imbalance between BMP2 and its antagonist noggin resulted in abnormal osteogenic differentiation of AS-MSCs in 2D culture *in vitro*¹². However, the detailed role of BMP2 and noggin in pathological osteogenesis in AS needs further study. In this study, we cultured HD-MSCs and AS-MSCs in an HA/ β -TCP scaffold and compared their osteogenic differentiation abilities *in vitro* and *in vivo*. Our data demonstrated that under both *in vitro* and *in vivo* conditions, AS-MSCs outperformed HD-MSCs in osteogenic differentiation in a 3D biomimetic microenvironment constructed by an HA/ β -TCP scaffold. This enhanced osteogenic differentiation ability of AS-MSCs was due to BMP2 overexpression, but not noggin. These results may not only provide a more realistic perspective on the pathological osteogenic process in AS, but they may also aid in the identification

of a precise therapeutic target for the pathological osteogenesis associated with AS.

Materials and methods

Cell isolation and culture in HA/ β -TCP scaffolds

This study conformed to the Declaration of Helsinki and was approved by the Ethics Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China. Thirty healthy donors and thirty AS patients were informed of possible risks and signed informed consent forms. All patients were diagnosed according to the New York modified criteria¹⁷. The characteristics of the study subjects are presented in Supplemental Table 1. MSCs were isolated, cultured and identified using flow cytometry as described in our previous research¹² (Supplemental Fig. 1).

The HA/ β -TCP scaffold, a circular cylinder ($\phi = 9$ mm, volume = 127 mm³), was produced as described to mimic the 3D biomimetic microenvironment¹⁸. A total of 1×10^6 MSCs were seeded on the HA/ β -TCP scaffold in a volume of 30 μ l with Dulbecco's modified Eagle's medium (DMEM; Gibco). After co-culture for 1 h, osteogenic differentiation medium consisting of DMEM, 10% foetal bovine serum (FBS; Gibco), 100 IU/ml penicillin, 100 IU/ml streptomycin, 0.1 μ M dexamethasone, 10 mM β -glycerol phosphate and 50 μ M ascorbic acid (Sigma-Aldrich) was added. The medium was replaced every 3 days. For some studies, LDN193189 was added at a concentration of 1 μ M to block the Smad1/5/8 signalling pathway, and SCH-772984 was added separately at a concentration of 0.2 μ M to block the ERK signalling pathway.

Scanning electron microscopy (SEM)

HA/ β -TCP scaffolds were fixed with 3% pentanediol and dehydrated in graded concentrations of ethanol. After being dried in a vacuum, the scaffolds were sputter-coated with gold and observed using a FEI Quanta Scanning Electron Microscope (Thermo Fisher).

GFP fluorescence assay

Lentiviruses (10^9 TU/ml) carrying the *gfp* gene and polybrene (5 μ g/ml) were added to MSCs at a multiplicity of infection (MOI) of 50 and incubated for 24 h. MSCs expressing GFP were seeded in the HA/ β -TCP scaffolds. The morphology and growth states of MSCs in the HA/ β -TCP scaffolds were observed through green fluorescence using an Axio Observer Fluorescence Microscope (Carl Zeiss).

Cell proliferation assay

The proliferation ability of MSCs in the HA/ β -TCP scaffolds was detected using Cell Counting Kit-8 (Dojindo) according to the manufacturer's protocol. The assays were performed from day 1 to day 15. HA/ β -TCP scaffolds without MSCs were used as controls.

Alkaline phosphatase (ALP) assay

For the quantitative assay, MSCs in the HA/ β -TCP scaffolds were washed and lysed in radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors and phosphatase inhibitors (Roche). The lysates were centrifuged, and the supernatants were incubated with reaction buffer (Nanjin Jiancheng Biotech) at 37 °C for 15 min. Colour development was stopped with stop solution (Nanjin Jiancheng Biotech), and the absorbance was measured at 405 nm. The lysate protein concentration was determined using a Pierce BCA protein assay kit (Thermo Fisher). The ALP activity was ultimately expressed as units per gram protein per 15 min (U/gpro/15 min).

For the qualitative assay, MSCs in the HA/ β -TCP scaffolds were fixed with a citrate-acetone-formaldehyde fixative solution and incubated with an alkaline dye solution for 15 min in the dark (Sigma). The stained samples were observed by photography.

Immunofluorescence assay

HA/ β -TCP scaffolds with MSCs were fixed in 4% paraformaldehyde for 20 min, followed by permeabilization with 0.1% Triton X-100 for 10 min. After blocking for 30 min, anti-collagen I antibody (1:500, Abcam34710) was added and incubated at 4 °C overnight. The samples were washed and incubated with a fluorescein secondary antibody (Thermo Fisher) and 4',6-diamidino-2-phenylindole (Thermo Fisher). The samples were examined with a confocal laser scanning microscope (Nikon Ni-E).

Western blotting

Protein from MSCs was extracted and quantified as described above. Equal concentrations of proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The PVDF membranes were blocked and incubated with primary antibodies against GAPDH (CST5417), Smad1 (CST6944), p-Smad1/5/8 (CST13820), ERK (CST4695), p-ERK (CST4370), p38 (CST8690), p-p38 (CST4511), JNK (CST9252), p-JNK (CST4668), β -catenin (CST8480), non-p- β -catenin (CST19807), BMP2 (Abcam14933), BMP4 (Abcam39973), BMP6 (Abcam155963), BMP7 (Abcam56023), BMP9 (Abcam35088), noggin (Abcam16054), Runx2 (Abcam76956) or OPN (Abcam8448; all 1:1000). Specific antibody-antigen complexes were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Small interfering RNA (siRNA) assay

Three siRNAs for BMP2 were constructed, and the most effective siRNAs were identified for experiments

(Supplemental Table 2). MSCs were seeded at a density of 1.5×10^4 cells/cm² in a 12-well plate. Lipofectamine RNAiMAX (Thermo Fisher) and Opti-MEM (Gibco) were mixed and incubated at 37 °C for 5 min, followed by the addition of siRNA and incubation at 37 °C for another 20 min. The mixture was used to treat MSCs for 6 h, and MSCs were seeded in the scaffold for further use.

Bone formation assay

This study was approved by the Animal Ethical and Welfare Committee of Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University, Guangzhou, China. Bone formation assays *in vivo* were performed as described in our previous study¹⁹. HA/ β -TCP scaffolds with MSCs were implanted subcutaneously into the dorsal side of 8-week-old BALB/c-nu/nu female mice (Laboratory Animal Center of Sun Yat-Sen University). The mice were sacrificed, and the implants were obtained at 2, 4 and 8 weeks. The implants obtained in the bone formation assay were successively fixed, decalcified, embedded in paraffin and sliced into sections.

Enteseal biopsy assay

Ossifying entheses from 10 AS patients and 10 non-AS patients (diagnosed with lumbar intervertebral disc herniation) were obtained during lumbar spine surgery. The characteristics of the study subjects are presented in Supplemental Table 3. The obtained tissues were sliced into sections.

Haematoxylin and eosin (H&E) and Masson trichrome staining

The sections were deparaffinized and hydrated. For H&E staining, the sections were incubated with haematoxylin for 5 min. After washing three times, the sections were cleared in 1% HCl in 70% alcohol and further stained with eosin for 3 min. For Masson trichrome staining, the sections were stained using a Masson trichrome staining kit (Sigma-Aldrich) according to the manufacturer's protocol. All sections were dehydrated and observed with a microscope.

Immunohistochemistry assay

The sections were deparaffinized and hydrated, followed by antigen retrieval in citrate buffer. After quenching with 3% H₂O₂/H₂O and blocking in goat serum, the sections were incubated with a human BMP2 antibody (R&D) overnight at 4 °C. Specific labelling was detected using Elivision™ plus Polymer HRP Kits and DAB Plus Kits (Maixin Biotech).

Statistical analysis

All results were determined based on at least three separate experiments that included at least triplicate

samples. All data are expressed as the mean \pm standard deviation. Statistical analysis was performed using *t* tests in SPSS (SPSS, Inc.). The *n* values indicate the number of individuals in each experiment. *P*-values less than 0.05 were considered statistically significant.

Results

HD-MSCs and AS-MSCs were cultured in a 3D biomimetic microenvironment composed of HA/ β -TCP

HD-MSCs and AS-MSCs were seeded in HA/ β -TCP scaffolds, which served as 3D biomimetic microenvironments. Through SEM, we confirmed that the MSCs could adhere to and survive in the HA/ β -TCP scaffolds (black arrow head; Fig. 1a). In addition, the number of MSCs expressing GFP gradually increased from day 3 to day 14 in HA/ β -TCP, indicating that the MSCs could proliferate in this scaffold (white arrow head; Fig. 1b). Moreover, the HD-MSCs and AS-MSCs had similar proliferation rates when cultured in HA/ β -TCP (Fig. 1c).

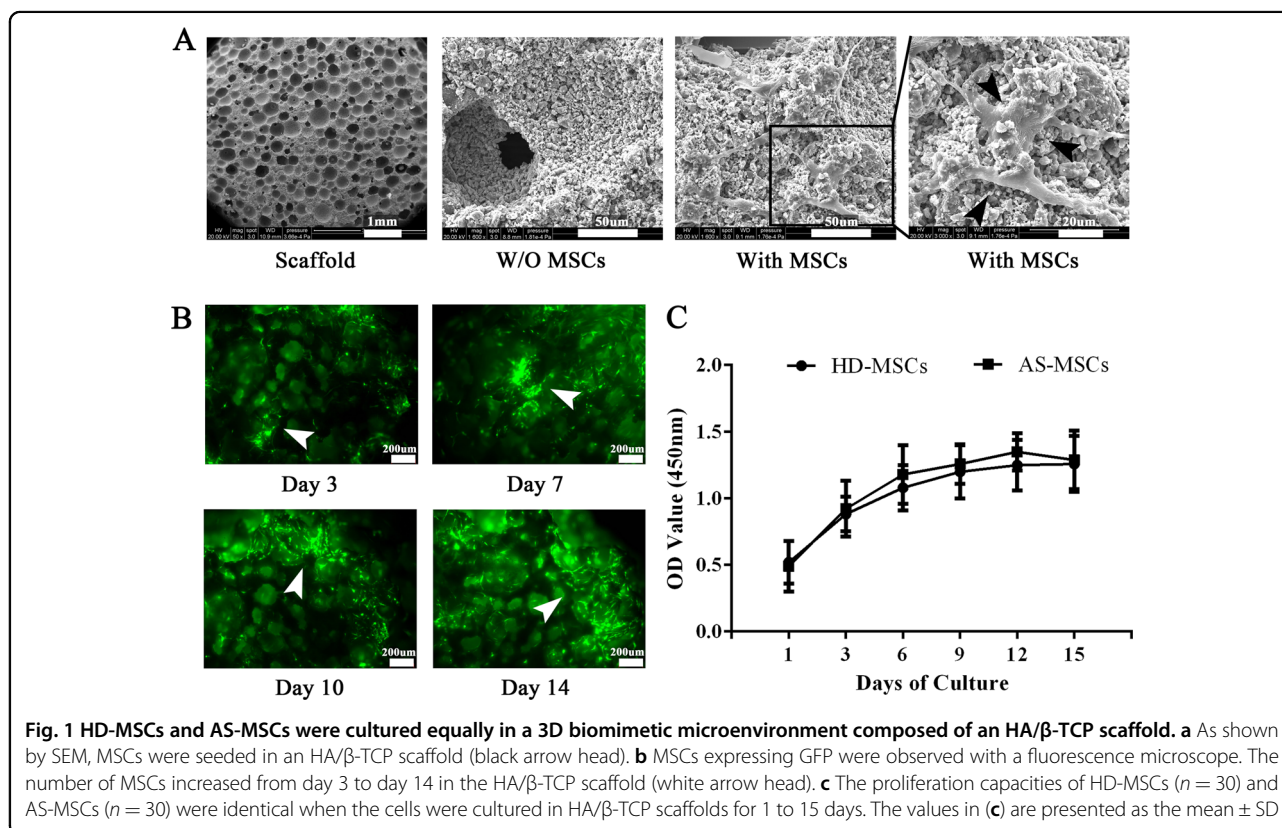
AS-MSCs showed an enhanced osteogenic differentiation capacity in the 3D biomimetic microenvironment

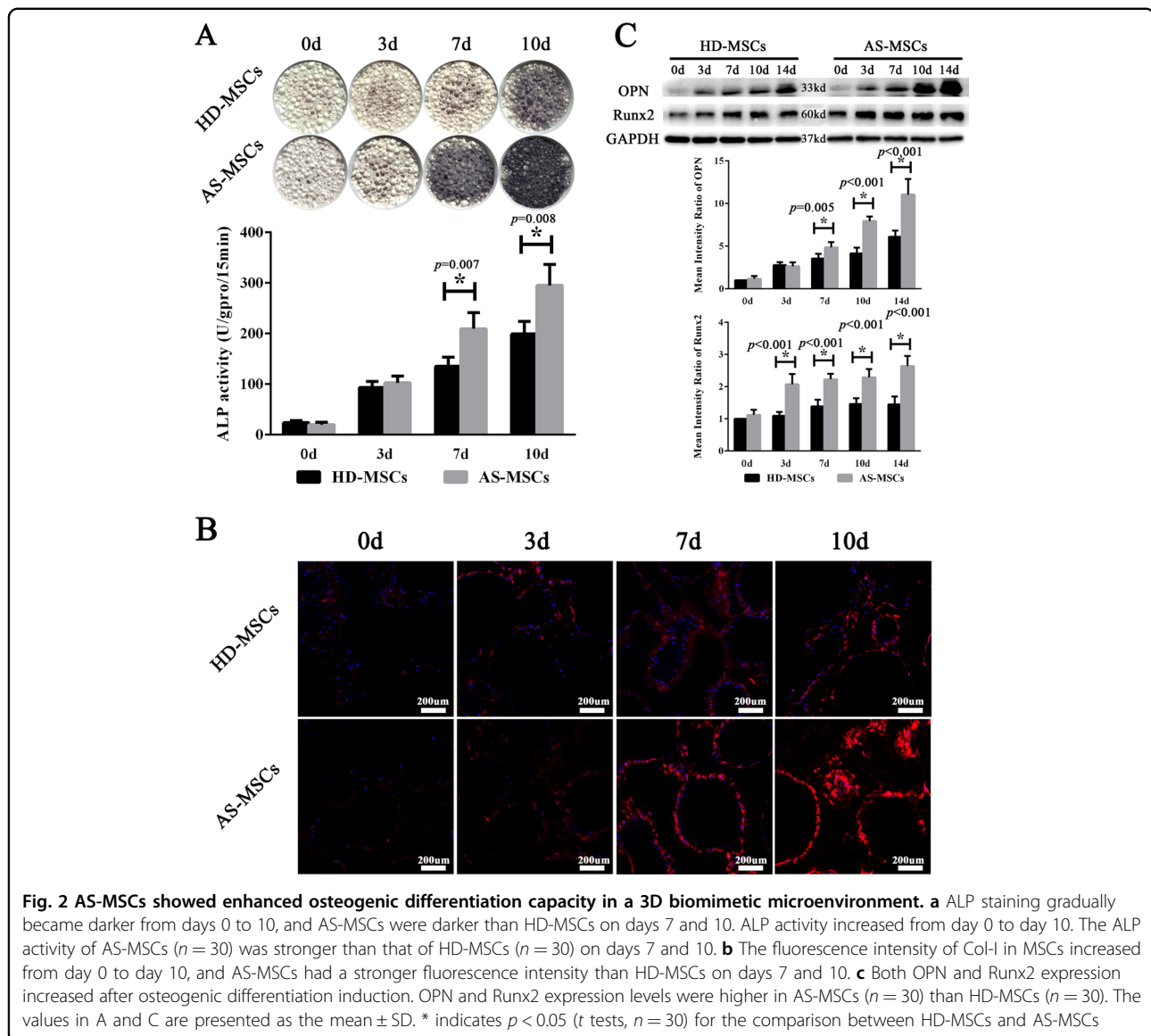
HD-MSCs and AS-MSCs in HA/ β -TCP scaffolds were induced to undergo osteogenic differentiation. The intensity of ALP staining in HA/ β -TCP gradually increased during osteogenic differentiation. On days 7 and

10 of induction, the ALP staining of AS-MSCs was darker than that of HD-MSCs. Consistent with this result, AS-MSCs had higher ALP activity than HD-MSCs on days 7 and 10 of osteogenic differentiation (Fig. 2a). The collagen I expression of HD-MSCs and AS-MSCs in HA/ β -TCP sections, as shown by an immunofluorescence assay, also increased during osteogenic differentiation. The fluorescence intensity of collagen I in AS-MSCs was stronger than that in HD-MSCs on days 7 and 10 of osteogenic differentiation (Fig. 2b). Osteogenesis markers, including Runx2 and OPN, were detected by western blotting. Similarly, the protein expression levels of Runx2 and OPN in AS-MSCs were higher than those in HD-MSCs during differentiation in HA/ β -TCP (Fig. 2c).

The Smad1/5/8 and ERK signalling pathways were excessively activated during osteogenic differentiation of AS-MSCs in the 3D biomimetic microenvironment

To study in depth the mechanism of the enhanced osteogenic differentiation capacity observed in AS-MSCs, we detected the activation levels of several signalling pathways related to MSC osteogenic differentiation. The results showed that the phosphorylation levels of the Smad1/5/8 and ERK signalling pathways in AS-MSCs were significantly higher than those in HD-MSCs during osteogenic differentiation. No significant differences in





the p38, JNK and WNT signalling pathways were found (Fig. 3).

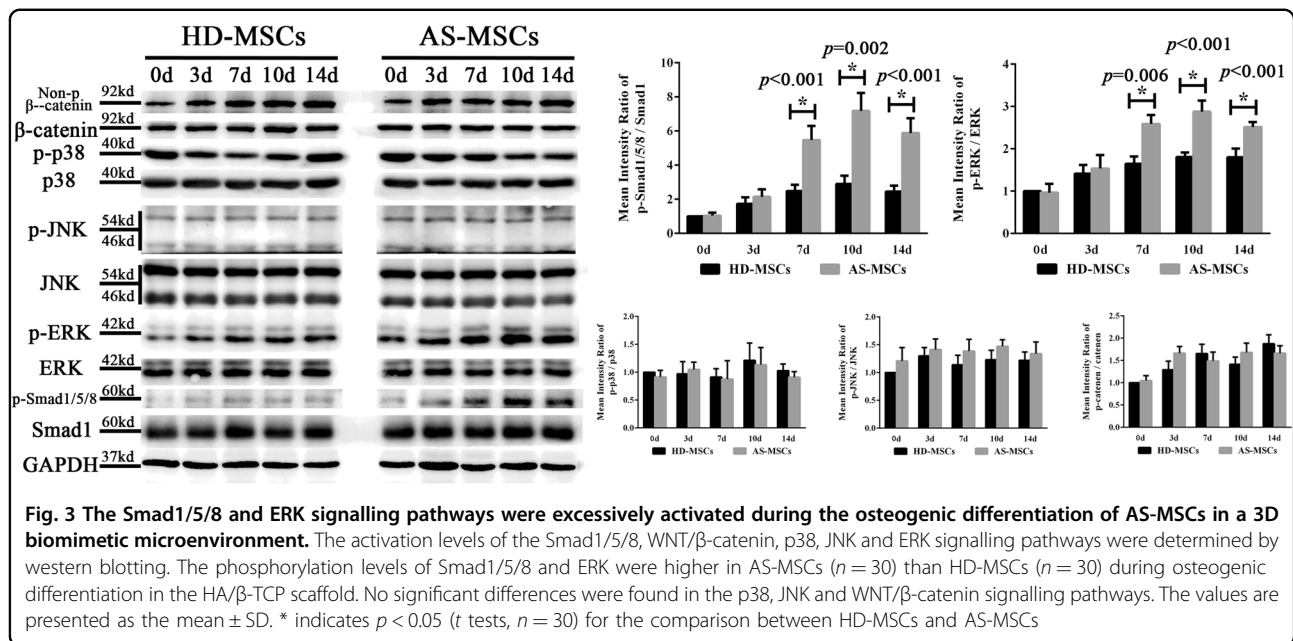
Blocking the Smad1/5/8 and ERK signalling pathways ameliorated the osteogenic differentiation of AS-MSCs in the 3D biomimetic microenvironment

LDN193189 and SCH-772984, which are inhibitors of the Smad1/5/8 and ERK signalling pathways, respectively, were used in this study. The results showed that the activation of the Smad1/5/8 and ERK signalling pathways was inhibited to equal levels by their inhibitors in HD-MSCs and AS-MSCs. In addition, inhibiting one pathway had little effect on the other pathway (Fig. 4a). Adding LDN193189 not only reduced the ALP staining intensity but also decreased the ALP activity of both HD-MSCs and AS-MSCs in HA/ β -TCP, reducing the difference between

these two cells. These effects were also induced by SCH-772984. In addition, LDN193189 showed a slightly stronger inhibitory ability than SCH-772984 (Fig. 4b). The collagen I immunofluorescence assay showed consistent results (Fig. 4c). Moreover, both LDN193189 and SCH-772984 decreased the expression of osteoblastic markers, reducing the Runx2 and OPN expression of HD-MSCs and AS-MSCs (Fig. 4d).

BMP2 overexpression leads to signalling pathway activation and enhanced osteogenic differentiation in AS-MSCs in a 3D biomimetic microenvironment

BMP2 and its antagonist noggin were critical factors in the abnormal osteogenic differentiation of AS-MSCs in 2D culture plates¹². Through western blotting, we demonstrated that AS-MSCs expressed more BMP2 than



HD-MSCs during osteogenic differentiation in HA/β-TCP. Surprisingly, noggin protein levels were identical in HD-MSCs and AS-MSCs (Fig. 5a). The expression of other BMPs did not differ significantly between HD-MSCs and AS-MSCs when cultured in the HA/β-TCP scaffold (Supplemental Fig. 2).

To suppress BMP2 expression, three siRNAs for BMP2 were constructed, and the most efficient siRNA was chosen for further use (Supplement Table 2). This siRNA effectively inhibited BMP2 expression in both HD-MSCs and AS-MSCs in HA/β-TCP (Fig. 5b). The ALP assays showed that inhibiting BMP2 expression could suppress the osteogenic differentiation of MSCs in the HA/β-TCP scaffold, reducing the osteogenic differentiation of AS-MSCs to the normal level observed in HD-MSCs. The significant difference in osteogenic differentiation capacity between HD-MSCs and AS-MSCs was rectified by the BMP2 siRNA (Fig. 5c). Moreover, the BMP2 siRNA also reduced the stronger fluorescence intensity of collagen I in AS-MSCs in HA/β-TCP, eliminating the gap between HD-MSCs and AS-MSCs (Fig. 5d). At the protein level, Runx2 and OPN expression showed results similar to those described for the assays above. Moreover, the excessive activation of the Smad1/5/8 and ERK signalling pathways in AS-MSCs was also reduced and restored to the levels observed in HD-MSCs by the BMP2 siRNA in HA/β-TCP (Fig. 5e).

AS-MSCs outperformed HD-MSCs in osteogenic differentiation in vivo

As we had demonstrated that AS-MSCs had a stronger capacity for osteogenic differentiation than HD-MSCs in

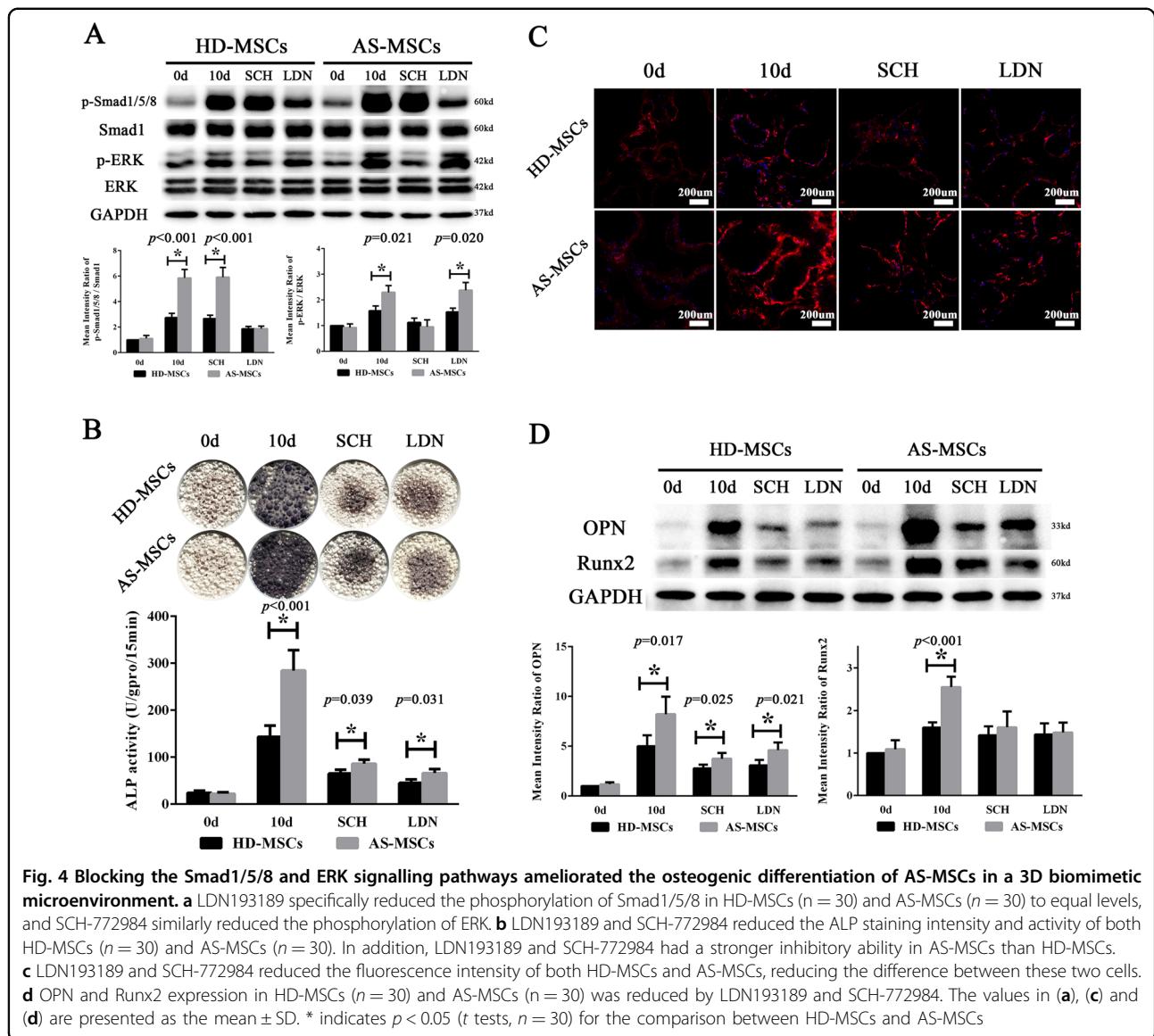
3D culture in vitro, we conducted bone formation assays to study the osteogenic differentiation capacity of AS-MSCs in HA/β-TCP in vivo. As shown in Fig. 6a, MSCs gradually differentiated, and new bone was formed around the HA/β-TCP scaffold from weeks 2 to 8. In week 8 after transplantation, a larger quantity of new bone formation was observed in the AS-MSC group than in the HD-MSC group. Masson staining assays demonstrated that the amount of collagenous fibre in the HA/β-TCP scaffold with AS-MSCs was higher than that in scaffolds with HD-MSCs from week 2 after transplantation, and new bone formation was consistent with the H&E staining results in week 8 (Fig. 6b). Similar to the results of the in vitro study, the BMP2 expression of AS-MSCs in the HA/β-TCP scaffold in vivo was significantly higher than that of HD-MSCs from weeks 4 to 8 (Fig. 6c).

Higher BMP2 expression at local sites of ossifying entheses in AS patients

To detect the expression level of BMP2 at local sites of ossifying entheses, 10 AS patients and 10 matched control patients with lumbar intervertebral disc herniation were recruited. We found that BMP2 expression in osteoblasts at the site of ossifying enthesis was markedly higher in AS patients than in the control group (Fig. 6d), indicating the important role of BMP2 in pathological osteogenesis in AS. However, noggin expression was very low but was identical in these two tissues (Supplemental Fig. 3).

Discussion

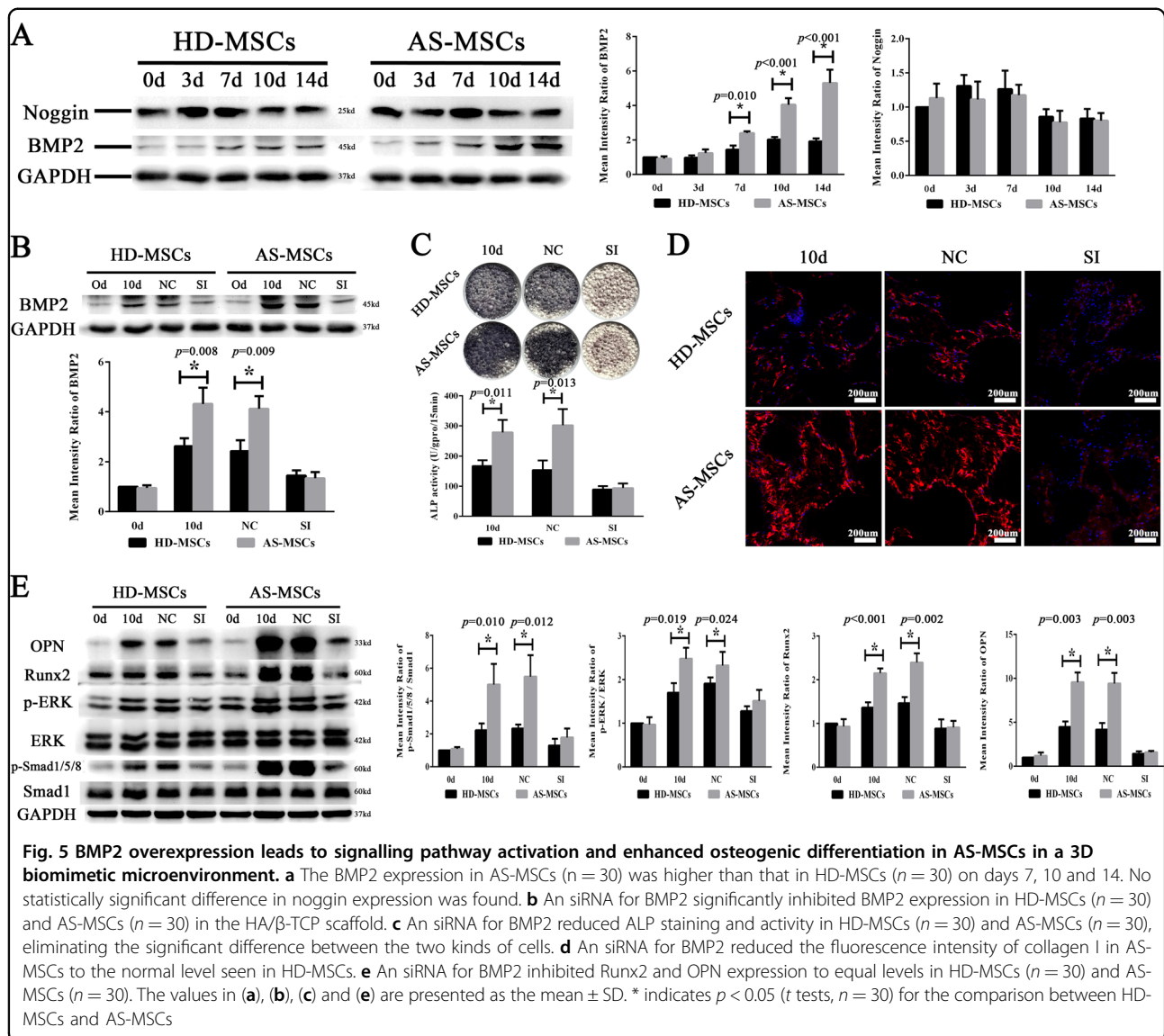
In this study, we demonstrated that AS-MSCs had a stronger capacity for osteogenic differentiation than HD-



MSCs when cultured in an HA/ β -TCP scaffold as a 3D biomimetic microenvironment in vitro or in vivo. This dysfunction was caused by BMP2 overexpression in AS-MSCs, which excessively activated the Smad1/5/8 and ERK signalling pathways and ultimately led to enhanced osteogenic differentiation. Moreover, we determined that BMP2 expression at the site of ossifying enthesis was higher in AS patients.

Bone metabolism depends on the balance between osteoblasts and osteoclasts. When osteoblasts predominate in quantity and activity, new bone formation occurs. However, when osteoclasts dominate, bone resorption occurs²⁰. In autoimmune diseases, the balance between these two cell types is usually broken, which leads to dysfunctional bone metabolism²¹. AS is a common autoimmune disease and has the critical

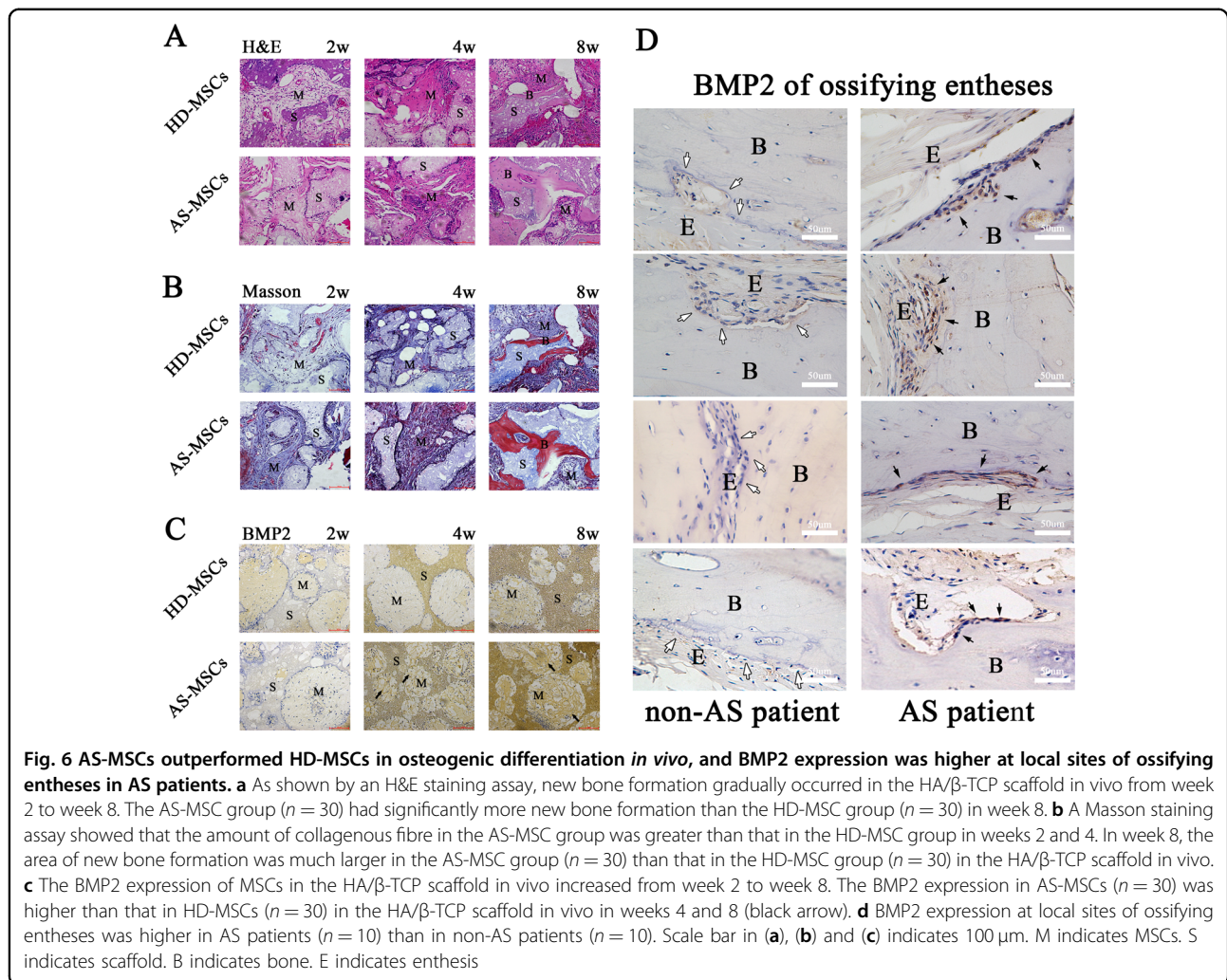
characteristic of pathological osteogenesis²². Recently, impaired osteoclastogenesis of monocytes from AS patients was demonstrated, indicating that dysfunction of osteoclasts contributes to the pathological osteogenesis in AS²³. As the major origin of osteoblasts in vivo, MSCs also play an important role in bone formation and could be the critical cause of pathological osteogenesis in AS. Many studies have determined that the dysfunction of MSCs in osteogenic differentiation contributes to abnormal bone metabolism in autoimmune diseases, such as systemic lupus erythematosus⁹ and rheumatoid arthritis⁸. Our previous study demonstrated for the first time that AS-MSCs outperform HD-MSCs in osteogenic differentiation when cultured in a 2D system in vitro¹². However, some studies reported that the function of cells in 2D culture cannot completely reflect their real capacity



in vivo²⁴. Recently, 3D biomimetic microenvironments with different types of materials have been widely used in cellular function studies²⁵. Due to its better spatial simulation of the in vivo environment, 3D biomimetic culture allows the cells to perform their real functions in vitro by providing a more suitable stereoscopic environment²⁶. Therefore, to further probe the mechanism of pathological osteogenesis in AS, studying the osteogenic differentiation ability of AS-MSCs in a 3D biomimetic microenvironment that mimics the microenvironment in vivo is required.

It is acknowledged that MSCs exist in various types of connective tissue inside and outside of bone²⁷. However, pathological osteogenesis in AS mostly occurs on the surface of bone, as observed in entheses^{28,29}. In addition to other factors, such as the local inflammatory

microenvironment and mechanical strain, we suggest that the attachment medium, specifically the 3D structure and composition of bone, is also a key cause of this phenomenon. Many studies have demonstrated that the structure and composition of bone represents a more suitable environment for MSC osteogenic differentiation than other tissues in vivo^{30,31}. These studies not only partially explain why new bone formation tends to form on the surface of bone but also emphasize the importance of the bone microenvironment in studying the functions of AS-MSCs. To simulate the 3D bone microenvironment in vivo and study the osteogenic differentiation capacity of AS-MSCs in that microenvironment, we chose an HA/ β -TCP scaffold as a 3D biomimetic microenvironment. HA/ β -TCP is a frequently used material composed of hydroxyapatite and β -tricalcium phosphate¹⁴. Due to its



similar molecular composition and construction to bone, HA/ β -TCP has excellent biocompatibility and osteoconduction properties, which allow it to be widely used as a bone substitute in clinical practice³². Moreover, previous studies have found that HA/ β -TCP scaffolds could mimic the 3D microenvironment of bone *in vivo* and accelerate the osteogenic differentiation of MSCs^{15,16}. In this study, we found that both HD-MSCs and AS-MSCs could be cultured in the HA/ β -TCP scaffold. In addition, AS-MSCs have a stronger osteogenic capacity than HD-MSCs when cultured in HA/ β -TCP scaffolds both *in vitro* and *in vivo* in a mouse bone formation model. These results prove that the osteogenic capacity of MSCs from AS patients is intrinsically stronger than that of HD-MSCs. This dysfunction may play an important role in the mechanism of pathological osteogenesis in AS.

The osteogenic differentiation of MSCs is under the control of various kinds of intracellular signalling pathways, including the BMP/Smad signalling pathway, the WNT/ β -catenin signalling pathway and the MAPK

signalling pathway³³. These signalling pathways are also involved in the pathological osteogenesis of AS²². To explore the cause of the enhanced osteogenic differentiation capacity of AS-MSCs in the HA/ β -TCP scaffold, we detected the phosphorylation levels of these signalling pathways. Through this study, we determined that the Smad1/5/8 and ERK signalling pathways were more active in AS-MSCs than HD-MSCs during osteogenic differentiation in HA/ β -TCP scaffolds. Moreover, blocking the Smad1/5/8 signalling pathway with LDN193189 or inhibiting the ERK signalling pathway using SCH-772984 reduced the difference in osteogenic differentiation between HD-MSCs and AS-MSCs. These results more precisely highlight the importance of the Smad1/5/8 and ERK signalling pathways in pathological osteogenesis in AS. Notably, there was no difference in p38 signalling pathway activation between HD-MSCs and AS-MSCs in 3D culture. A previous study found that although the p38 signalling pathway was related to chondrogenesis, inhibiting this pathway could not prevent the progression

of ankylosis in an in vivo model of AS³⁴. This study, as well as our results, suggested that the p38 signalling pathway may not be the crucial mechanism of pathological osteogenesis in AS. In addition, several studies also suggested that the WNT/ β -catenin signalling pathway participates in osteogenic progression in AS³⁵. However, this pathway shows no difference between HD-MSCs and AS-MSCs, either in 2D culture, as reported in our previous study¹², or in a 3D biomimetic microenvironment with the HA/ β -TCP scaffold. We suggest that the WNT/ β -catenin signalling pathway may contribute to AS by other mechanisms.

BMP2 is one of the most important osteogenesis-promoting cytokines³⁶. Secreted by different kinds of cells, especially MSCs, BMP2 has significant effects on bone metabolism in vivo. Under physiological conditions, BMP2 promotes the osteogenic differentiation of MSCs through autocrine and paracrine mechanisms, thereby promoting bone repair and regeneration. In contrast, MSCs secrete excess BMP2, which leads to the enhanced osteogenic differentiation of MSCs and eventually promotes pathological conditions, such as new bone formation^{37,38}. In our study, we found that the BMP2 expression in AS-MSCs, in contrast to the expression of other BMPs, was significantly higher than that of HD-MSCs during osteogenic differentiation in HA/ β -TCP scaffolds both in vitro and in vivo. In addition, inhibiting BMP2 using an siRNA could rescue the overactivation of the Smad1/5/8 and ERK signalling pathways and restore the enhanced osteogenic differentiation of AS-MSCs to the normal level seen in HD-MSCs. These results demonstrated that BMP2 is the key factor that excessively activates the Smad1/5/8 and ERK signalling pathways and ultimately results in the enhanced osteogenic differentiation capacity of AS-MSCs in a 3D biomimetic microenvironment with HA/ β -TCP scaffolds. A previous study showed that BMP2 is highly expressed at the site of ankylosis in an AS mouse model³⁹. In this study, we further demonstrated that the BMP2 expression level at the local site of ossifying entheses was much higher in the AS patient group than in the non-AS control group. Therefore, we suggest that BMP2 is the main cause of pathological osteogenesis in AS. Moreover, these results suggest that BMP2 inhibitors may be therapeutic targets for pathological osteogenesis in AS.

Noggin is a specific extracellular antagonist of BMP2⁴⁰. In our previous study, we found that noggin expression in AS-MSCs was much lower than that in HD-MSCs during osteogenic differentiation in 2D culture¹². However, noggin expression was similar between HD-MSCs and AS-MSCs during osteogenic differentiation in a 3D biomimetic microenvironment with HA/ β -TCP scaffolds. Noggin expression was also unchanged at the site of ossifying entheses in AS patients compared to that in the

non-AS patient group. Several studies have found that the gene and protein expression patterns of cells vary in different microenvironments, such as 2D and 3D culture⁴¹. Considering that 3D culture with HA/ β -TCP scaffolds is a more suitable system for mimicking the 3D microenvironment of bone in vivo, we suggest that noggin may not be the cause of pathological osteogenesis in AS. Nevertheless, as a specific antagonist of BMP2, noggin still has broad application prospects for preventing ankylosis progression in AS.

In this study, we demonstrated that AS-MSCs possess enhanced osteogenic differentiation in HA/ β -TCP scaffolds used as a 3D biomimetic microenvironment because of BMP2 overexpression. These results provide insights into the mechanism of pathological osteogenesis, helping to product niche-targeting medications for AS. However, several questions remain. Is this enhanced osteogenic differentiation capacity coupled to the radiographic progression of AS patients? Why does new bone formation mainly occur in the spine rather than the peripheral joints? How do other factors, such as inflammation and mechanical strain, affect pathological osteogenesis? Several limitations still exist and need to be addressed in future studies.

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Authors' contributions

P.W., Y.W. and H.S. designed the experiments. G.Z., Z.X., J.L., M.L. and S.C. conceived and performed the experiments. S.T., W.L. and G.Y. conceived the experiments and analysed the data. Y.L., S.W., X.W. and H.S. carried out the experiments. All authors were involved in writing the manuscript and approved the submitted and published versions.

Conflict of interest

The authors declare that they have no conflict of interest.

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