WNT16 Elevation Induced Cell Senescence of Osteoblasts in Ankylosing Spondylitis

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Abstract

Background: WNT16 is critical for bone homeostasis, but the effect of WNT16 in ankylosing spondylitis (AS) is still unknown. Here, we investigated whether WNT16 influences bone formation and pathophysiological changes of AS in an in vitro model.

Methods: Bone tissue from facet joints was obtained from five disease control and six AS patients. Primary osteoprogenitor cells of the facet joints were isolated using an outgrowth method. Isolated osteoprogenitor cells from both control and AS tissues were analysed by microarray, RT-qPCR, immunoblotting, and immunohistochemistry. Bone-forming activity of osteoprogenitor cells was assessed by various in vitro assays. β -galactosidase staining and senescence-associated secretory phenotype (SASP) using RT-qPCR were used to assess cell senescence.

Results: In microarray analysis, WNT16 expression was significantly elevated in AS osteoprogenitor cells compared to the control. We also validated that WNT16 expression was elevated in AS-osteoprogenitor cells and human AS-bone tissues. WNT16 treatment inhibited bone formation in AS-osteoprogenitor cells but not in the control. Intriguingly, β -galactosidase for cell senescence was strongly stained in AS-osteoprogenitor cells treated with WNT16. Furthermore, in an H_2O_2 stress-induced premature senescence condition, WNT16 treatment increased cell senescence in AS-osteoprogenitor cells and WNT16 treatment under the H_2O_2 stress condition showed an increase in p21 protein and SASP mRNA expression. The WNT16-induced SASP expression in AS-osteoprogenitor cells was reduced in WNT16 knockdown cultures.

Conclusion: WNT16 is highly expressed in AS and WNT16 treatment facilitated cell senescence in AS-osteoprogenitor cells during osteoblast differentiation accompanied by suppression of bone formation. The identified role of WNT16 in AS could reflect the bone loss in AS patients.

Introduction

Ankylosing spondylitis (AS) is defined by paradoxical features that are clinically characterized by an increase in new bone formation and bone loss [1-6]. Pathological bone changes in AS have been associated with the WNT signalling pathway [7-9] and, therefore, there is an increasing interest in the molecular partners of WNT signalling and bone changes in AS. Among the WNT molecules, many studies have clearly shown that WNT16 signalling increases bone formation while WNT16 inhibition leads to decreased bone formation [10-12]. Although the role of WNT16 signalling and bone formation has been studied using various genetic murine models, the conclusions in human AS are still elusive.

The main cause of bone loss in patients with AS is unclear. It has been reported that the prevalence of AS patients with bone loss is about 25% and low vertebral bone mineral density in AS is closely associated with high modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS), and C-reactive protein (CRP) [13, 14]. Chronic inflammation and immobilization have been speculated as risk factors in bone loss in AS patients. A recent study reported increased cellular senescence in

mesenchymal stem cells (MSCs) derived from the bone marrow of patients with AS [15]. In this paper, oxidative stress (OS) facilitated senescence by an increase in the release of reactive oxygen species (ROS) and mitochondrial deregulation. Clinically, oxidative stress status and associated serum proteins were higher in AS and associated with low bone mineral density in patients with AS [16-18]. However, whether the senescence influences bone-forming activity in AS is still unknown.

Elevated expression of WNT/ β -catenin signalling is known to be involved in AS pathophysiology, but little is known about the association of WNT16 with osteoporotic bone loss in human AS. Thus, in this study we investigated the functional roles of WNT16 on bone loss in AS.

Materials And Methods

Human facet joint bone tissue

The present study was conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of Hanyang University, Seoul (IRB 2014-05-002) and Guri Hospital (IRB 2014-05-001). We obtained facet joints from six AS patients who met modified New York criteria [19] and underwent spinal surgery and from five patients with non-inflammatory disease as the control.

Isolation of osteoprogenitor cells

The protocol for the isolation of osteoprogenitor cells was previously reported [20-23]. Briefly, osteoprogenitor cells were isolated until the fourth passage using the outgrowth method for one month in Dulbecco's Modified Eagle Medium-high glucose (DMEM-HG; Hyclone, SH30243.01) containing 10% fetal bovine serum (FBS; Gibco, 16000-044) and 1% antibiotics (penicillin/streptomycin; Gibco, 1540-122). All isolated osteoprogenitor cells were checked for mycoplasma using a PCR-based method (Takara, 6601) before long-term storage in liquid nitrogen.

In vitro bone formation

The protocol for the use of osteoprogenitor cells for assessing bone-forming activity was previously reported [20-23]. Briefly, osteoprogenitor cells were seeded in 96-well plates at 1×10^4 cells/well and incubated for 24 h. These cells were changed to osteogenic medium containing DMEM-HG (Hyclone, SH30243.01) containing 10% fetal bovine serum (FBS; Gibco, 16000-044), 1% antibiotics (penicillin/streptomycin; Gibco, 1540-122), 50 μ M ascorbic acid (Sigma, A4544), 10 mM β -glycerol phosphate (Santa Cruz Biotechnology, sc-220452A;), and 100 nM dexamethasone (Sigma, D2915). The medium was changed every three or four days and bone-forming activity was assessed at the indicated days. The matrix maturation of bone formation was assessed by alkaline phosphatase (ALP) activity (BioVision, K412-500), ALP (Sigma, 85L2), and collagen using Picro-Sirius Red (Abcam, ab150681) staining. The matrix mineralization of bone formation was assessed using Alizarin Red S (ARS; ScienCell, 8678), hydroxyapatite (HA; Lonza, PA-1503), and Von Kossa (5% silver nitrate solution; SAMCHUN Chemical, S0228) staining. Stained wells were imaged via microscopy (Nikon eclipse Ti-U, Nikon).

Cell viability

The water-soluble tetrazolium salt (WST) assay was performed using the EZ-CYTOX kit (Dogen Bio, EZ-1000) according to manufacturer instructions. Primary osteoprogenitor cells were seeded into 96-well plates at 1×10^3 cells/well and treated with WNT16 at the indicated dose and time point (days). At the indicated day, 10 μ l WST solution was added to the cells and incubated for 1 h. Absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Microarray analysis

Isolated primary control and AS-osteoprogenitor cells were screened using the Affymetrix GeneChip Human Gene 2.0 ST array. For microarray analysis, background adjustment, normalization, and median polish summarization of CEL files were performed with the robust multi-average method using Partek Genomics Suite software version 7.0. Differential expression was calculated from the normalized data using ANOVA and visualized as a heatmap for WNT genes.

RT-qPCR

Primary osteoprogenitor cells were lysed using TRIzol and 1.0 μ g of total RNA was subjected to reverse transcription using a cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA). Relative gene transcript levels were determined by RT-qPCR using SYBR Green and qPCR was performed in triplicate for each sample. The mean expression level of each gene was normalized to GAPDH. The primers used in study are listed in Table 1.

Immunoblot assays

Stimulated osteoprogenitor cells were lysed with 1X RIPA buffer containing phosphatase (5870S, Cell Signaling Technology, USA) and protease (535140, Calbiochem, USA) inhibitors. Proteins were quantified using the Bradford assay. Total cell protein (10-30 μ g) was subjected to immunoblotting using the following antibodies: β -catenin (9562S), active β -catenin (19807S), phos-AKT (Ser473) (4060), total-AKT (4691), phos-ERK (9101), total-ERK (9102), and GAPDH (2118) from Cell Signaling Technology (Danvers, MA, USA). CDKN1A (p21; A19094) and WNT16 (sc271897) antibodies were purchased from Abclonal and Santa Cruz Biotechnology, respectively. Goat anti-rabbit IgG (111-035-003) and goat anti-mouse IgG (115-035-003) secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Immunohistochemistry

The detailed materials and methods for immunohistochemistry (IHC) analysis were previously described [20]. Briefly, facet joint tissues were fixed in 10% formalin for a week, decalcified with 10% formic acid for a week, and embedded in paraffin. Tissue slides (5 μ m) were treated with Neo-Clear for deparaffination, followed by dehydration with ethanol solutions (100% to 50% ethanol), permeabilization with 0.25% Triton X-100, and blocking with BLOXALL (Vector Lab, SP-6000) for 1 h. The slides were then incubated with primary antibodies (1:100 ratio in antibody diluent) overnight, followed by washing with

PBS-T, incubation with biotinylated secondary antibodies for 1 h, washing with PBS-T, incubation with ABC kit components as specified by the manufacturer (Vector Lab, PK-6102), and incubation using a DAB substrate kit (Vector Lab, sk4100) for 1 to 5 min. The slides were counterstained with haematoxylin (Merck, 1.05174.0500) for 10 sec, dehydrated through ethanol and Neo-Clear, and coverslipped using a permanent mounting medium (Vector Lab, H-5000). Microscopy was used to visualize and image stain cells (Leica).

Human WNT16 serum level measurements

Sera were collected from 30 healthy male donors and 36 patients with AS who met the modified New York criteria [19]. Serum collection methods and storage conditions were previously described [20]. We measured WNT16 serum level using a human WNT16 ELISA kit (CUSABIO, CSB-EL026132HU) according to manufacturer instructions.

Promoter assays

SaOS2 cells were seeded in 60 mm plates at 2×10^5 cells/well and co-transfected with each ALP, BSP, OSE, OCN, TOP, or FOP promoter DNA (3 μg /well) and *Renilla* luciferase (0.3 μg /well) using Lipofectamine 3000 (Thermo Fisher, L3000015). A day after transfection, the cells were reseeded in 12-well plates at 5×10^4 cells/well and incubated for 24 h. The next day, cells were treated with WNT16 (50 ng/mL) for 24 h and lysed to measure luciferase activity (Promega, E1500), according to manufacturer instructions. The luciferase activity data were normalized to *Renilla* luciferase activity.

Small interference RNA (siRNA) and knockdown efficiency testing

Five siRNA oligos against human WNT16 were designed and generated by GENOLUTION Inc. (Seoul, Korea). Oligo sequences for siRNA are listed in Table 2. To test WNT16 knockdown efficiency, FOB cells were seeded in 6-well plates and transfected with one of the five siRNA oligos against WNT16 using Lipofectamine 3000 (Thermo Fisher, L3000015). After 48 h, the transfected FOB cells were lysed and analysed by RT-PCR. Selected siRNA oligos were used for further investigation.

Senescence staining with β -galactosidase

To test the effect of WNT16 on cell senescence, AS-osteoprogenitor cells were seeded in 96-well plates at 4×10^3 cells/well and incubated for 24 h. The next day, the cells were stimulated H_2O_2 (200 μM) for 2 h, washed with 1X PBS, and suspended in new growth medium with WNT16 (50 ng/mL) or vehicle. After three days, the senescence effect of WNT16 was assessed using a senescence detection kit (BioVision, K320), according to manufacturer instructions. After staining, stained wells were imaged by microscopy (Nikon eclipse Ti-U). For senescence quantification, stained images were analysed by counting senescence positive cells.

Statistical analysis

Data images were generated using GraphPad Prism 6 software (GraphPad Software, Inc.). Two-tailed Student's *t*-tests were used to compare data between two unpaired groups. All data are expressed as the mean $\pm$ SEM ($n \geq 3$). A $p < 0.05$ was considered statistically significant.

Results

WNT16 is highly expressed in AS

We performed microarray experiments with control and AS-osteoprogenitor cells and analysed the data to attain molecular insights among the WNT genes in those samples. Differential expression gene (DEG) analysis revealed that WNT1, WNT3, and WNT16 were highly expressed in AS-osteoprogenitor cells (Figure 1A, red arrows). We validated these data using RT-qPCR, and showed that WNT1 was not detected, but WNT3, WNT5, and WNT16 were significantly increased in AS (Figure 1B). Since the functional role of WNT3 and WNT5 in AS had been reported elsewhere [24], we focused the role of WNT16 in AS-osteoprogenitor cells. WNT16 protein was found to be higher in AS-osteoprogenitor cells relative to the control, which was revealed by immunoblotting and its quantification (Figure 1C). We confirmed that there was no significant difference in WNT16 serum levels between healthy and AS groups (data now shown), but we consistently found increased WNT16 expression in bone-lining cells and in facet joint periosteum tissue in AS-osteoprogenitor cells compared with the control. Collectively, WNT16 was significantly elevated in AS-osteoprogenitor cells and AS tissues.

WNT16 treatment decreased matrix mineralization during osteoblast differentiation and increased senescence in AS

We next investigated whether WNT16 treatment influences matrix maturation and mineralization of osteoprogenitor cells during osteogenic differentiation. Two doses (25 and 50 ng/ml) of WNT16 did not affect the proliferation rate of AS-osteoprogenitor cells (Supp. Figure 1), but treatment with 50 ng/mL WNT16 inhibited ALP activity and staining status in AS-osteoprogenitor cells. To investigate the effect of WNT16, both control and AS-osteoprogenitor cells were treated with 50 ng/mL WNT16 during osteoblast differentiation. As shown in Figure 2A-F, treatment with WNT16 significantly inhibited bone matrix maturation (ALP staining and activity) and matrix mineralization (ARS, VON, and HA staining) during osteogenic differentiation. These inhibitory effects by WNT16 were also pronounced in AS-osteoprogenitor cells. WNT16 treatment also reduced bone formation-related gene promoter activity in alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OCN), as revealed by luciferase activity (Figure 2G). Intriguingly, SA- β -gal was strongly stained in AS-osteoprogenitor cells at 28 days of differentiation when compared with the control (Figure 2H). WNT16 treatment also showed stronger SA- β -gal staining results in AS (Figure 2I), although the cell staining rates were not significantly different. These findings show that treatment with WNT16 inhibits matrix maturation and mineralization during osteogenic differentiation and promotes cell senescence in AS-osteoprogenitor cells.

WNT16 treatment induces AS-osteoprogenitor cellular senescence

We next sought to investigate AS without osteogenic differentiation and designed an experimental condition using H₂O₂-induced senescence (Figure 3A). WNT16 treatment in AS-osteoprogenitor cells exhibited a marked increase in SA-β-gal staining, SA-β-gal positive cells rates, and hydrogen peroxidase activity (Figure 3B, 3C, and 3D). In these conditions, WNT16 treatment increased active β-catenin, β-catenin, and p21 protein expression in AS-osteoprogenitor cells and decreased phos-AKT without changes in phos-p38 or phos-ERK protein levels (Figure 3E). Moreover, WNT16 treatment significantly increased p21 mRNA expression and senescence-associated secretory phenotype (SASP)-related genes such as IL-1α, IL-1β, IL-6, and IL-8 (Figure 3F) in AS-osteoprogenitor cells. WNT16 treatment activated the β-catenin functional motif, as confirmed by promoter activity assays (Figure 3G). In addition, WNT16 knockdown using siRNA attenuated the increased SASP mRNA expression by H₂O₂ stimulation (Figure 3H and 3I). Taken together, WNT16 treatment facilitated cell senescence of AS-osteoprogenitor cells under H₂O₂ stimulation.

Discussion

In this study, WNT16 was significantly increased in AS-osteoprogenitor cells and was observed in AS facet joint tissues. During osteoblast differentiation, WNT16 treatment decreased matrix maturation and mineralization and increased cell senescence, particularly in AS-osteoprogenitor cells. Moreover, WNT16 treatment strikingly facilitated cell senescence, p21 protein, and SASP expression in AS-osteoprogenitor cells under H₂O₂-induced stimulation, while WNT16 knockdown attenuated this senescence effect. Collectively, high WNT16 expression in AS-osteoprogenitor cells may reflect the bone loss in patients with AS by inducing cell senescence.

In general, WNT3 is well-known to play a positive role in bone formation through β-catenin activation. Li et al. showed that WNT molecule serum levels (WNT3a, WNT4, WNT5a, WNT7b, and WNT10) were increased dependent on the progression status of spinal ankylosis. In particular, the results showed that WNT3 and WNT5 were associated with hyperosteoblastic activity and spinal ectopic new bone formation, and their expression was in response to TNF stimulation [24]. In this paper, we showed that WNT3 expression was highly expressed in AS-osteoprogenitor cells (Figure 1A and 1B). However, reports of WNT3 serum level in AS/SpA groups have been inconsistent [24-26], suggesting that the functional and pathophysiological changes need further investigation and a constructive approach.

Many studies have indicated that WNT16 transgenic mice show an increase in bone mineral density and mass whereas WNT16 knockout mice show a decrease in cortical and cancellous bone mass [10-12]. Here, we suggest that human WNT16 plays a paradoxical role in bone formation in AS. Consistent with our finding, Jiang et al. reported that WNT16 overexpression without DKK1 expression in osteoblasts reduced calcified nodule formation relative to the control [27], indicating that the functional role of WNT16 in mineralization may be different depending upon DKK1 expression. Also, many studies have reported low levels of DKK1 in AS [28], and high WNT16 expression in AS is considered to lead to restrained bone mineralization.

Many studies have reported that WNT16 expression is strongly associated with bone mineral density, suggesting that WNT16 is a therapeutic target for bone loss-related diseases such as osteopenia or osteoporosis. While these data are mainly from murine models, there are a few human study results. It is a questionable issue whether WNT16 plays a functional role in humans. Since cell senescence in old mice exhibits a decline in bone mass accompanied by Osterix protein decrease [29], we analysed whether WNT16 treatment affects Osterix expression. However, we found no change in Osterix mRNA expression (data now shown).

WNT16 genome-wide association study (GWSA) meta-analyses have shown that polymorphisms of the WNT16 locus are strongly associated with cortical bone thickness, BMD, and risk of osteoporosis fracture in humans [11, 30-34]. Therefore, these studies indicate that WNT16 is an important determinant of cortical bone mass and risk of fracture. As discussed above, the functional relevance of WNT16 in the regulation of bone and mineralization has been confirmed in both in vitro and in vivo studies. Importantly, there is a lack of information on the functional role of WNT16 in AS-associated bone loss; therefore, further study on the relationship between bone loss and WNT16 locus polymorphisms in patients with AS is needed.

We measured WNT16 levels in collected serum and confirmed that there was no significant difference between the control and AS group (data now shown). Our serum results are consistent with previous reports [24], but WNT16 expression was high in AS-osteoprogenitor cells and AS bone tissue compared to the control. Therefore, the difference in WNT16 level between serum and pathological tissue can be interpreted as the distinction between systematic and local disease.

A limitation of our paper is that we did not provide the direct regulatory mechanisms on WNT16-induced p21 expression in AS-osteoprogenitor cells. Second, we did not explore that how WNT16 increases oxidative stress and related protein expression in AS-osteoprogenitor cells. Third, further experiments are needed to explore the effect of WNT16 treatment on the reduction of bone formation-related genes.

Conclusion

Our present study shows that WNT16 is highly expressed in AS-osteoprogenitor cells and in destructive bone tissue. WNT16 treatment in AS-osteoprogenitor cells inhibits mineralization of osteoblast differentiation accompanied by promoting cell senescence. Therefore, elevated WNT16 might be a putative indicator for bone loss in AS.

List Of Abbreviations

AS: Ankylosing spondylitis; HC: Healthy controls; ALP: Alkaline phosphatase; VON: Von Kossa; HA: Hydroxyapatite; BSP: bone sialoprotein; OCN: osteocalcin; SASP: senescence-associated secretory phenotype; mSASSS: modified Stoke Ankylosing Spondylitis Spinal Score (mSASSS); CRP: C-reactive protein; OS: oxidative stress; ROS: reactive oxygen species; MSC: mesenchymal stem cells

Declarations

Ethics approval and consent to participate: The present study was conducted in accordance with the Helsinki Declaration and approved by the Institutional Review Board of Hanyang University, Seoul (IRB 2014-05-002) and Guri Hospital (IRB 2014-05-001). The written informed consent was obtained from all subjects.

Consent for publication: Not applicable.

Availability of data and material: Not applicable

Competing interest: All authors declare that they have no competing interests.

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Authors' contributions: SJ designed and performed the experiments. SW assisted with the laboratory work. M-AJ and HK analyzed microarray data analysis. BL and T-JK evaluated the human sera. Y-SP provided and evaluated the human bone samples. SJ and T-HK wrote the manuscript. T-HK conceived the project and was responsible for the overall design and oversight of the project.

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References

1. Klingberg E, Lorentzon M, Mellstrom D, Geijer M, Gothlin J, Hilme E, Hedberg M, Carlsten H, Forsblad-d'Elia H. Osteoporosis in ankylosing spondylitis - prevalence, risk factors and methods of assessment. *Arthritis Res Ther*. 2012;14(3):R108.
2. van der Weijden MA, Claushuis TA, Nazari T, Lems WF, Dijkmans BA, van der Horst-Bruinsma IE. High prevalence of low bone mineral density in patients within 10 years of onset of ankylosing spondylitis: a systematic review. *Clin Rheumatol*. 2012;31(11):1529-1535.
3. Nigil Haroon N, Szabo E, Raboud JM, McDonald-Blumer H, Fung L, Josse RG, Inman RD, Cheung AM. Alterations of bone mineral density, bone microarchitecture and strength in patients with ankylosing spondylitis: a cross-sectional study using high-resolution peripheral quantitative computerized tomography and finite element analysis. *Arthritis Res Ther*. 2015;17:377.
4. Inman RD. Axial Spondyloarthritis: Current Advances, Future Challenges. *Journal of Rheumatic Diseases*. 2021;28(2):55-59.
5. Akgol G, Kamanli A, Ozgocmen S. Evidence for inflammation-induced bone loss in non-radiographic axial spondyloarthritis. *Rheumatology (Oxford)*. 2014;53(3):497-501.

6. Davey-Ranasinghe N, Deodhar A. Osteoporosis and vertebral fractures in ankylosing spondylitis. *Curr Opin Rheumatol*. 2013;25(4):509-516.
7. Lories RJ, Luyten FP, de Vlam K. Progress in spondylarthritis. Mechanisms of new bone formation in spondyloarthritis. *Arthritis Res Ther*. 2009;11(2):221.
8. Xie W, Zhou L, Li S, Hui T, Chen D. Wnt/beta-catenin signaling plays a key role in the development of spondyloarthritis. *Ann N Y Acad Sci*. 2016;1364:25-31.
9. Lories RJ, Corr M, Lane NE. To Wnt or not to Wnt: the bone and joint health dilemma. *Nat Rev Rheumatol*. 2013;9(6):328-339.
10. Tornqvist AE, Grahnmö L, Nilsson KH, Funck-Brentano T, Ohlsson C, Moverare-Skrtic S. Wnt16 Overexpression in Osteoblasts Increases the Subchondral Bone Mass but has no Impact on Osteoarthritis in Young Adult Female Mice. *Calcif Tissue Int*. 2020;107(1):31-40.
11. Zheng HF, Tobias JH, Duncan E, Evans DM, Eriksson J, Paternoster L, Yerges-Armstrong LM, Lehtimäki T, Bergström U, Kahonen M *et al*. WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. *PLoS Genet*. 2012;8(7):e1002745.
12. Moverare-Skrtic S, Henning P, Liu X, Nagano K, Saito H, Borjesson AE, Sjögren K, Windahl SH, Farman H, Kindlund B *et al*. Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. *Nat Med*. 2014;20(11):1279-1288.
13. Ghozlan I, Ghazi M, Nouijai A, Mounach A, Rezqi A, Achemlal L, Bezza A, El Maghraoui A. Prevalence and risk factors of osteoporosis and vertebral fractures in patients with ankylosing spondylitis. *Bone*. 2009;44(5):772-776.
14. Kim JW, Chung MK, Lee J, Kwok SK, Kim WU, Park SH, Ju JH. Low bone mineral density of vertebral lateral projections can predict spinal radiographic damage in patients with ankylosing spondylitis. *Clin Rheumatol*. 2019;38(12):3567-3574.
15. Ye G, Xie Z, Zeng H, Wang P, Li J, Zheng G, Wang S, Cao Q, Li M, Liu W *et al*. Oxidative stress-mediated mitochondrial dysfunction facilitates mesenchymal stem cell senescence in ankylosing spondylitis. *Cell Death Dis*. 2020;11(9):775.
16. Wang L, Gao L, Jin D, Wang P, Yang B, Deng W, Xie Z, Tang Y, Wu Y, Shen H. The Relationship of Bone Mineral Density to Oxidant/Antioxidant Status and Inflammatory and Bone Turnover Markers in a Multicenter Cross-Sectional Study of Young Men with Ankylosing Spondylitis. *Calcif Tissue Int*. 2015;97(1):12-22.
17. Yazici C, Kose K, Calis M, Kuzuguden S, Kirnap M. Protein oxidation status in patients with ankylosing spondylitis. *Rheumatology (Oxford)*. 2004;43(10):1235-1239.

18. Karakoc M, Altindag O, Keles H, Soran N, Selek S. Serum oxidative-antioxidative status in patients with ankylosing spondylitis. *Rheumatol Int.* 2007;27(12):1131-1134.
19. van der Linden S, Valkenburg HA, Cats A. Evaluation of diagnostic criteria for ankylosing spondylitis. A proposal for modification of the New York criteria. *Arthritis Rheum.* 1984;27(4):361-368.
20. Jo S, Wang SE, Lee YL, Kang S, Lee B, Han J, Sung IH, Park YS, Bae SC, Kim TH. IL-17A induces osteoblast differentiation by activating JAK2/STAT3 in ankylosing spondylitis. *Arthritis Res Ther.* 2018;20(1):115.
21. Jo S, Lee EJ, Nam B, Kang J, Lee S, Youn J, Park YS, Kim YG, Kim TH. Effects of dihydrotestosterone on osteoblast activity in curdlan-administered SKG mice and osteoprogenitor cells in patients with ankylosing spondylitis. *Arthritis Res Ther.* 2020;22(1):121.
22. Park P-R, Jo S, Jin S-H, Kim T-J. MicroRNA-10b Plays a Role in Bone Formation by Suppressing Interleukin-22 in Ankylosing Spondylitis. *Journal of Rheumatic Diseases.* 2020;27(1).
23. Jo S, Won EJ, Kim MJ, Lee YJ, Jin SH, Park PR, Song HC, Kim J, Choi YD, Kim JY *et al.* STAT3 Phosphorylation Inhibition for Treating Inflammation and New Bone Formation in Ankylosing Spondylitis. *Rheumatology (Oxford).* 2020, 10.1093/rheumatology/keaa846.
24. Li X, Wang J, Zhan Z, Li S, Zheng Z, Wang T, Zhang K, Pan H, Li Z, Zhang N *et al.* Inflammation Intensity-Dependent Expression of Osteoinductive Wnt Proteins Is Critical for Ectopic New Bone Formation in Ankylosing Spondylitis. *Arthritis Rheumatol.* 2018;70(7):1056-1070.
25. Iaremenko O, Shynkaruk I, Fedkov D, Iaremenko K, Petelytska L. Bone turnover biomarkers, disease activity, and MRI changes of sacroiliac joints in patients with spondyloarthritis. *Rheumatol Int.* 2020;40(12):2057-2063.
26. Klingberg E, Nurkkala M, Carlsten H, Forsblad-d'Elia H. Biomarkers of bone metabolism in ankylosing spondylitis in relation to osteoproliferation and osteoporosis. *J Rheumatol.* 2014;41(7):1349-1356.
27. Jiang Z, Von den Hoff JW, Torensma R, Meng L, Bian Z. Wnt16 is involved in intramembranous ossification and suppresses osteoblast differentiation through the Wnt/beta-catenin pathway. *J Cell Physiol.* 2014;229(3):384-392.
28. Zhang L, Ouyang H, Xie Z, Liang ZH, Wu XW. Serum DKK-1 level in the development of ankylosing spondylitis and rheumatic arthritis: a meta-analysis. *Exp Mol Med.* 2016;48:e228.
29. Kim HN, Chang J, Shao L, Han L, Iyer S, Manolagas SC, O'Brien CA, Jilka RL, Zhou D, Almeida M. DNA damage and senescence in osteoprogenitors expressing *Osx1* may cause their decrease with age. *Aging Cell.* 2017;16(4):693-703.

30. Garcia-Ibarbia C, Perez-Nunez MI, Olmos JM, Valero C, Perez-Aguilar MD, Hernandez JL, Zarrabeitia MT, Gonzalez-Macias J, Riancho JA. Missense polymorphisms of the WNT16 gene are associated with bone mass, hip geometry and fractures. *Osteoporos Int.* 2013;24(9):2449-2454.

31. Hendrickx G, Boudin E, Fijalkowski I, Nielsen TL, Andersen M, Brixen K, Van Hul W. Variation in the Kozak sequence of WNT16 results in an increased translation and is associated with osteoporosis related parameters. *Bone.* 2014;59:57-65.

32. Kemp JP, Medina-Gomez C, Estrada K, St Pourcain B, Heppe DH, Warrington NM, Oei L, Ring SM, Kruithof CJ, Timpson NJ *et al.* Phenotypic dissection of bone mineral density reveals skeletal site specificity and facilitates the identification of novel loci in the genetic regulation of bone mass attainment. *PLoS Genet.* 2014;10(6):e1004423.

33. Koller DL, Zheng HF, Karasik D, Yerges-Armstrong L, Liu CT, McGuigan F, Kemp JP, Giroux S, Lai D, Edenberg HJ *et al.* Meta-analysis of genome-wide studies identifies WNT16 and ESR1 SNPs associated with bone mineral density in premenopausal women. *J Bone Miner Res.* 2013;28(3):547-558.

34. Medina-Gomez C, Kemp JP, Estrada K, Eriksson J, Liu J, Reppe S, Evans DM, Heppe DH, Vandenput L, Herrera L *et al.* Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. *PLoS Genet.* 2012;8(7):e1002718.

Tables

Table 1. RT-qPCR primers

Gene	5´-Forward-3´	3´-Reverse-5´
GAPDH	CAAGATCATCAGCAATGCC	CTGTGGTCATGAGTCCTTCC
WNT16	CTACGGAGCCCAAGGAAACT	CTCTCGTGTCTGAACTGGCT
p53	ACCTATGGAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
p21	GCAGAGGAAGACCATGTGGA	CGGCGTTTGGAGTGGTAGAA
IL1α	TGGTAGTAGCAACCAACGGG	GGTGCTGACCTAGGCTTGAT
IL-1β	CAGAAGTACCTGAGCTCGCC	AGATTCGTAGCTGGATGCCG
IL-6	ATGAACTCCTTCTCCACAAGCG	CTCCTTTCTCAGGGCTGAG
IL-8	TTCTGCAGCTCTGTGTGAAGG	TAATTTCTGTGTTGGCGCAGTG

Table 2. siRNA oligos against WNT16

siRNA	Sequence (5'-3')	
Negative Control	Sense	CCUCGUGCCGUUCCAUCAGGUAGUU
	Antisense	CUACCUGAUGGAACGGCACGAGGUU
WNT16 #1	Sense	CCAAGUUGAUGUCAGUAGAUU
	Antisense	UCUACUGACAUCAACUUGGUU
WNT16 #2	Sense	GGAUGAUCUGCUCUAUGUUUU
	Antisense	AACAUAGAGCAGAUCAUCCUU
WNT16 #3	Sense	CCAACUACUGUGUAGAAGAUU
	Antisense	UCUUCUACACAGUAGUUGGUU
WNT16 #4	Sense	CUGAUCAACCCAUCAAUCAUU
	Antisense	UGAUUGAUGGGUUGAUCAGUU
WNT16 #5	Sense	CUGACUUACCCUUUCAUGUUU
	Antisense	ACAUGAAAGGGUAAGUCAGUU

Figures

Figure 1

WNT16 is highly expressed in AS. (A) Heatmap diagram of differentially expressed probes mapped to WNT genes in AS and control osteoprogenitor cells. In AS osteoprogenitor cells, WNT16 showed significant upregulated expression with a p-value < 0.05 and fold change > 2 compared to the control (n=3 per each group). (B) Verification of mRNA level using qPCR of (A) for the WNT family (n=6 per each group). (C) Immunoblotting for WNT16 protein level in control and AS-osteoprogenitor cells (n=5 per each

group). (D) Immunohistochemistry (IHC) staining of WNT16 in spinal facet joints tissue from control or AS patients (n=3 per each group). Data are presented as the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

Figure 2

WNT16 treatment inhibited mineralization and promoted cell senescence of AS-osteoprogenitor cells during osteoblast differentiation. Both control and AS-osteoprogenitor cells were differentiated with osteogenic medium in the presence of vehicle or WNT16. Analysis of (A) ALP staining, (B) intercellular

ALP activity, (C) ARS staining and Von Kossa staining at the indicated days. (D) Quantitation of ARS and Von Kossa staining. (E) Analysis of HA staining at the indicated days and (F) quantitation of HA staining. (G) SaOS2 cells were co-transfected with 3 μ g ALP, BSP, OSE, or OCN promoter plasmid along with β -gal plasmid for 48 h followed by and WNT16 treatment for 24 h, and then analysed by luciferase assay. (H) SA- β -gal staining was performed after osteoblast differentiation of control and AS-osteoprogenitor cells. (I) Counting results of Figure 3H. Scale bar = 200 μ m. Data are presented as the mean $\pm$ SEM. * p <0.05.

Figure 3

WNT16 treatment facilitated cell senescence of AS-osteoprogenitor cells under H2O2-stimulation. (A) Experimental design for cell senescence treated with WNT16. AS-osteoprogenitor cells were exposed to H2O2 for 2 h and then treated with vehicle or WNT16 for 70 h. Analysis of (B) SA- β -gal staining, (C) counting results of SA- β -gal positive cells, (D) assessment of osteoprogenitor cell lysates using hydrogen

peroxidase assay, (E) immunoblotting for protein level, and (G) qPCR for mRNA level. (F) SaOS2 cells were transfected with TOP or FOP-flash in the presence β -galactosidase for 24 h, treated WNT16 for 24 h, and then analysed via luciferase assays. AS-osteoprogenitor cells were transfected with 100 nM WNT16 siRNA #2 for 48 h, exposed to H₂O₂ for 2 h, and followed by analysis of (H) immunoblotting for WNT16 protein level and (I) qPCR for mRNA level. Data are presented as the mean $\pm$ SEM. *p<0.05, **p<0.01. Representative images are shown. Scale bar = 200 μ m.

Supplementary Files

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