Phosphate promotes osteogenic differentiation through non-canonical Wnt signaling pathway in human mesenchymal stem cells

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Abstract

Background Phosphate is indispensable in osteogenesis and mineralization. However, mechanisms by which phosphate enhances osteogenic differentiation are not fully understood. In this study, we studied the effect of phosphate on osteogenic differentiation as well as signaling pathways induced by phosphate in the process.

Method Induced human bone marrow-derived mesenchymal stem cells differentiation into osteoblasts by the change of media containing β-glycerophosphate (GP), 1mM inorganic phosphate, or 3mM inorganic phosphate (Pi). The differentiation of osteoblasts was verified by the expression of osteoblast differentiation markers and calcium deposition. RNA sequencing was performed to assess transcriptome in the early stage of osteogenic differentiation.

Results Osteogenic differentiation and mineralization were promoted in the 3 mM Pi group compared to those in the GP and 1 mM Pi groups on day 7 of culture. RNA sequencing revealed that the gene expressions involved in osteogenesis and the components in the Wnt signaling pathway was increased in 3 mM Pi group compared with those in the GP on day 7. Analysis with qPCR and Western blot suggested upregulation of components in the non-canonical Wnt signaling pathway, including WNT5b and phosphorylated-c-Jun in the 3 mM Pi group on day 7. *WNT11* mRNA expression was increased in the 2 induction groups on day 7. Inhibition of WNT5b by siRNA experiment attenuated the components in non-canonical Wnt signaling expression, including *WNT5b*, *WNT11* and *ROR2* mRNA expression and phosphorylated-c-Jun protein expression. In addition, osteogenic differentiation and mineralization were partly decreased in 3 mM Pi group on day 7 by the inhibition of WNT5b.

Conclusion Pi promoted osteogenic differentiation through the up-regulation of the non-canonical Wnt signaling pathway, including WNT5b, WNT11, p-c-Jun/c-Jun, in the early stage of differentiation. These

findings provide a new perspective into the association of Pi and the non-canonical Wnt signaling pathway during osteogenic differentiation.

Keywords: phosphate, non-canonical Wnt signaling pathway, WNT5b, osteogenic differentiation, human mesenchymal stem cells

1. Introduction

Biomineralization is a complex and lifelong process, and the basis of mineralization research is to elucidate how organic materials (generally protein) and inorganic crystals coordinately interact during this process [1]. During the biomineralization process, organic phosphate in the extracellular milieu is degraded to produce free phosphate (Pi), and Pi is a crucial element for biological mineralization, which combines with calcium ions to form hydroxyapatite (HA). HA is the most important constituent of the extracellular matrix of the skeleton and accounts for approximately 80% of Pi in the whole body [2, 3].

In organism, the mineralization sites of bone tissue could express the tissue-nonspecific alkaline phosphatase (TNAP), which promotes HA crystal formation by hydrolyzing pyrophosphate (PPi), which inhibits mineralization, and producing and enhancing Pi concentration [4, 5]. Pi is known essential for mineralization and osteogenic differentiation [6, 7]. Actually, many studies have shown that Pi enhanced multiple signaling pathways in the osteogenic differentiation process [8, 9]. However, specific mechanism by which Pi promotes osteogenic differentiation remains unclear.

The Wnt signaling pathway is a vital signaling pathways for the development and homeostasis of bone [10]. It has been reported that Pi enhanced osteogenic differentiation through the canonical Wnt and Notch signaling pathways [11]. However, the non-canonical Wnt signaling pathway is not well studied in osteoblastic differentiation, although a few forms of skeletal dysplasia are caused by pathogenic variants of genes encoding it [12, 13].

In this study, we explored the function of Pi in osteogenic differentiation using hMSCs. We have performed RNA sequencing (RNA-seq) to examine changes in the transcriptome, and demonstrated the non-canonical Wnt signaling through WNT5b was enhanced in the early stage of Pi-induced osteogenic differentiation.

2. Materials and methods

2.1 Cell culture and reagents

Human bone marrow-derived mesenchymal stem cells (MSCs, No. 429Z022) were purchased from PromoCell Company (Germany) and were seeded at a density of 5×10^3 cells/cm^2 and routinely cultured in maintenance medium (MM). MM composition: α-minimum essential medium (MEM, No. 135-15175, Wako, Japan), 10% fetal calf serum (FCS, v/v), 1% penicillin/streptomycin (P/S) (Sigma). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2 in air. Cells were detached with Accutase-Solution (C-41310, PromoCell) and then subcultured when they reached approximately 80–90% confluence. To induce osteoblast differentiation, once cells reached confluence, medium was replaced with osteogenic induction medium which contains α-MEM supplemented with 10% FCS, 1% P/S, 100 nM dexamethasone (Wako), 50 μM L-ascorbic acid (Sigma-Aldrich, USA) and a phosphate source. Ten mM β-glycerophosphate (GP) (Sigma-Aldrich, USA), 1 mM Pi or 3 mM Pi sodium phosphate (Na_2HPO_4; Sigma-Aldrich) was used as the phosphate source. The maintenance group was defined to MM group, and each induction with GP, 1 mM Pi and 3 mM Pi was referred to as GP, 1 mM Pi and 3 mM Pi groups, respectively. Medium was refreshed every 48 hours. One hundred nM fibroblast growth factor receptor 1 (FGFR1) inhibitor, PD173034 (219580-11-7, Selleckchem, USA), was used to inhibit FGFR1, and DMSO (Sigma-Aldrich, USA) was used as a control.

2.2 Alizarin red staining and quantification of calcium deposition

Alizarin red staining was used to check calcification condition in cultures. Fixed cells with 10% neutral buffered formalin for 15 min, then washed cells with deionized water. Next 2% Alizarin red S (A5533-25G, Sigma) was used to incubate cells for 30 min at room temperature. Finally, removed alizarin red and washed cells with deionized water to remove excess stain.

To quantify calcium deposition, cells were cultured in 6-well dishes, and each cell well was washed with PBS and harvested by scraping. Added 1mL 0.1M HCl in each well and incubated at room temperature for 1 h on a rotary shaker. Collected cell samples and centrifuged at 10,000×*g* for 15 min, the 500μL supernatants were collected neutralized with NaOH (50μL, 1M). According to the manufacturer's instructions tested the calcium level of the cell layers by a calcium assay kit (CA01M, Metallogenics, Japan).

2.3 Alkaline phosphatase activity assay

Cell samples were collected on days 7, 14, and 21 after the induction of osteogenesis. Cell samples were lysed in 0.05% Triton in 10 mM Tris-HCl (pH 7.4) buffer and then analyzed using an alkaline phosphatase (ALP) test kit (No. 291-58601, Wako) to assess ALP activity. Total protein concentration was measured by a bicinchoninic acid (BCA) assay (Thermo Scientific, USA). ALP activity was calculated and normalized to the amount of total protein.

2.4 Pi and PPi assays

Cells were cultured as described above, rinsed with fresh medium, and then incubated for 24 hours before the collection of medium for analysis. A phosphate test kit (270-49801, Wako) was used for analyze Pi concentrations, pyrophosphate assay kit (ab112155, Abcam, UK) was employed to measure free PPi concentrations according to the manufacturer's protocol. The standard curve was used to calculate PPi concentrations ($r > 0.98$).

2.5 quantitative polymerase chain reaction (qPCR)

Total RNA was extracted by RNeasy Mini Kit (74106, Qiagen, USA) and evaluated the concentration and purity of RNA. Complimentary DNA was synthesized from RNA using ReverTra Ace qPCR RT Master Mix with a gDNA Remover (FSQ-301, Toyobo，Japan). qPCR was performed using

THUNDERBIRD SYBR qPCR Mix (QPS-201, Toyobo). *Glyceraldehyde phosphate dehydrogenase* (*GAPDH*) as the internal reference was used. Every sample was analyzed in triplicate, and data were examined using the $2^{-\triangle\triangle Ct}$ method. Oligonucleotide sequences are listed in supplement file 1 (Table S1).

Supplemental Table 1. Primer list

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ALPL	CCTCGTTGACACCTGGAAGAG	TTCCGTGCGGTTCCAGA
OPN	TTGCAGCCTTCTCAGCCAA	GGAGGCAAAAGCAAATCACTG
OCN	GCAAAGGTGCAGCCTTTGTG	GGCTCCCAGCCATTGATACAG
RUNX2	TAGCAAGGTTCAACGATCTGAG	AGCTTCTGTCTGTGCCTTCTG
COL1A1	GGGATTCCCTGGACCTAAAG	GGAACACCTCGCTCTCCA
ANKH	GAGGTGACAGACATCGTGG	CCTTTAAATCAAGGCCTCTTTCATTAC
ENPP1	AAATATGCAAGCCCTCTTTGT	TTTAGAAGGTGGTTAAGACTTCCATGA
PHOSPHO1	GACGAAAACAGCGACGATTC	CGGAGATGAGAATCACCTCG
WNT5b	AGACTGGCATCAAGGAATGC	GTCTCTCGGCTGCCTATCTG
JUN	CCAAAGGATAGTGCGATGTTT	CTGTCCCTCTCCACTGCAAC
ROR2	CCCCTCATTAACCAGCACAA	TTCCCAAACCGGTCCTCT
MAPK9	GATATTCCAAGGCACTGACCA	TTCCTCACAGTTGGCTGAAGT
WNT11	AGCCAAAGCGATCTACAAAAGG	AAGTCAAAAACATCTGGTAGGCA
WNT2	GGATGCCAGAGCCCTGATGAATC	GCCAGCCAGCATGTCCTGAGAGT
Axin2	GCCAAGTTTTCCGATGCTCC	GACACCCCATGGCACTTACA
WISP2	CATGCAGAACACCAATATTAAC	TAGGCAGTGAGTTAGAGGAAAG
β-Catenin	TGTTAAATTCTTGGCTATTACGACA	CCACCACTAGCCAGTATGATGA
LRP5	GCCAAGACAGACAAGATCGAG	CAGCGTGAACCCAAAAATG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

2.6 RNA-sequencing and data processing

Total RNA was extracted by RNeasy Mini Kit (74106, Qiagen, USA) on day 7 and RNA samples were subjected to an RNA-seq analysis. Each group was analyzed in triplicate. After performing quality control (QC), qualified RNA samples were used for library construction. The constructed library was then sequenced using 2×101-bp paired end reads on the HiSeqX platform (Illumina, USA). Sequencing data were converted into FASTQ files utilizing Illumina software. Raw data were FASTQ files, and adapter trimming was performed to generate files after QC. The quasi-mapping method was performed

to analyze data, and the same reference transcriptome was employed for all workflows (GRCh38, only protein coding). After obtaining quantitative data, we conducted principal component analysis (PCA) with regularized log transformation and differential gene expression (DGE) analysis among each sample set using DESeq2. A pathway analysis using Gene Ontology (GO, http://geneontology.org/docs/ontology-documentation/), Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www.genome.jp/kegg/pathway.html) was conducted on DGE genes in each group using a cluster Profiler.

2.7 Western blot analysis

Total protein was extracted by RIPA Buffer (PTH4492, Wako), incubated for 20 min on ice, centrifuged at 15000 r/min at 4℃ for 15 min, followed by the collection of supernatants. Tested total protein concentration by a BCA assay (Thermo Scientific). Ten micrograms of protein were dissolved in 2 × sodium dodecyl sulfate loading buffer, boiled at 95°C for 15 min. Proteins were separated and transferred by the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories, USA). And membrane was blocked with Blocking One-P buffer (05999-84, Nacalai Tesque, Inc., Japan) for 1 h at room temperature, then probed at 4°C overnight with diluted rabbit antibodies against WNT5b (2530, 1:1000, Cell Signaling Technology, USA), c-Jun (9165, 1:1000, Cell Signaling Technology), phosphor-c-Jun (Ser73) (3270, 1:1000, Cell Signaling Technology), β-Catenin (total, 8480, 1:1000, Cell Signaling Technology), phosphor-β-Catenin (Ser33/37/Thr41) (inactive, 9561, 1:1000, Cell Signaling Technology), non-phosphor-β-Catenin (active, 8841, 1:1000, Cell Signaling Technology), phosphor-GSK-3β（Ser9）(5558, 1:1000, Cell Signaling Technology), alpha-Tubulin (ab15246, 1:1000, Abcam), p44/42 MAPK (ERK1/2) (4695, 1:1000, Cell Signaling Technology) and P-p44/42 MAPK (ERK1/2) (4370, 1:1000, Cell Signaling Technology). After washing 3 times with TBST buffer, then membrane was re-probed

with horseradish peroxidase-labeled secondary antibody immunoglobulin G (IgG) (1:5000) for 1 h. Immunocomplexes on the membrane were visualized using Clarity™ Western ECL Substrate (170-5060, Bio-Rad Laboratories) chemiluminescence reagents, and photographed using the Bio-Rad image analysis system (Bio-Rad Laboratorie). Quantity One v4.6.2 software was used to quantification of the band intensities. Relative protein expression was reflected by the ratio of the gray value of the target band to alpha-tubulin.

2.8 Silencing of WNT5b by siRNA

The WNT5b siRNA (Qiagen, USA) is a 21-bp duplex oligoribonucleotide and corresponding to human WNT5b mRNA sequence: 5′-CTCCTGGTGGTCATTAGCTTT-3′. The manufacturer provided four different sequences, logarithmically growing cells were seeded at a density of 5×10^3 cells/cm^2 and with the transfection of 5 nM WNT5b siRNA (si-WNT5b) using HiPerFect HTS reagent (Cat. No. 301704, Qiagen). AllStars Negative Control siRNA (Cat, No. 1027415, Qiagen) was used as negative control. Efficiency of the siRNA was tested by qPCR 48h after transfection. Since the batch of si-WNT5b-3 suppressed the expression level of Wnt5b most efficiently between the four batches (Fig.S1), si-WNT5b-3 was used to inhibit WNT5b expression in the following experiments.

Transfection experiment was carried out as above stated, and after 48h of transfection, the medium was changed into osteogenic induction media or MM. Five experiment groups: MM (maintenance group without si-RNA), GP+si-Cont (GP group transfected with 5 nM si-Control), GP+si-WNT5b (GP group transfected with 5 nM si-WNT5b), 3 mMPi+si-Cont (3 mM Pi group transfected with 5 nM si-Control), 3 mMPi+si-WNT5b (3 mM Pi group transfected with 5 nM si-WNT5b). Protein and mRNA were collected from samples on day 7 of osteogenic differentiation induction.

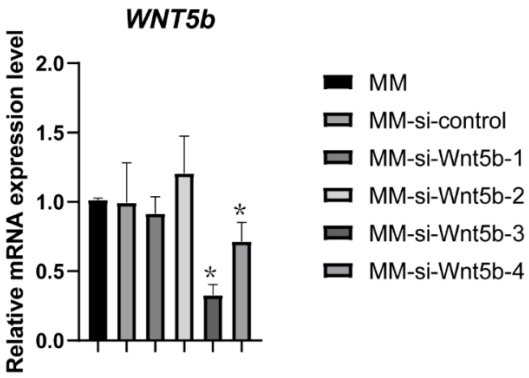

Fig.S1 The efficiency of si-WNT5b

The mRNA expression of WNT5b after transfection of si-RNA were detected by qPCR. *, $P<0.05$, significantly different from the MM+si-Cont group. MM, maintenance medium. Data are shown as the mean ± SEM (N = 3).

2.9 Statistical analysis

All experiments were independently repeated at least three times. Data are expressed as the mean ± the standard error of the mean (SEM). SPSS 19.0 (SPSS, Inc., USA) was used to do statistical analyses. The comparisons between two groups analyzed by *t*-test. The comparisons among multiple groups analyzed by a one-way analysis of variance (ANOVA), and then the Turkey significant difference test was performed for pairwise comparisons. *P* was a bilateral test, P value < 0.05 was considered to be significant.

3. Results

3.1 Pi concentration influences on MSC osteogenic differentiation and mineralization

Sodium Pi buffer is a direct source of phosphate and can be used to induce osteogenic differentiation in many studies [4, 14], GP was then replaced with sodium Pi to test effects of Pi concentrations on osteogenic differentiation. Three groups containing 10 mM GP, 1 and 3mM Pi in media and one MM control group were examined in the study. Alizarin red staining and the calcium deposition level showed that mineral deposition was significantly enhanced in the 3 mM Pi group compared with those in the 1

mM Pi and GP groups on day 7 (Fig. 1A and B). On days 14 and day 21, mineralization was similar between the GP and 3 mM Pi groups. TNAP activity was significantly increased in the 3 induction groups on day 7, 14 and 21 (Fig. 1C). Next, Pi concentrations were increased continued in the GP group compared with those in the MM group during the culture, whereas those in the 1 mM Pi and 3 mM Pi groups remained stable and were higher than those in the MM group. Of note, on day 7, the highest Pi concentration was observed in the 3 mM Pi group among all the 4 groups (Fig. 1D). PPi concentrations of MM group was increased compared with than those in the 3 induction groups (Fig. 1E), while the Pi/PPi ratio was markedly decreased in the comparison of MM group and 3 induction groups (Fig. 1F). Then, the expression levels of genes involved in osteogenesis and mineralization was investigated (Fig. 1G-N). The mRNA expression levels of *RUNX2*, *COL1A1*, *OPN*, *OCN*, *ANKH*, and *ENPP1* were increased in the 3 mM Pi group on day 7 than those in the GP, 1 mM Pi, and MM groups. However, with the extension of culture times, gene expression levels in the GP and 1mM Pi groups reached those in the 3 mM Pi group. Above results suggested that 3 mM Pi promoted osteogenesis and mineralization compared to those in the GP and 1 mM Pi groups.

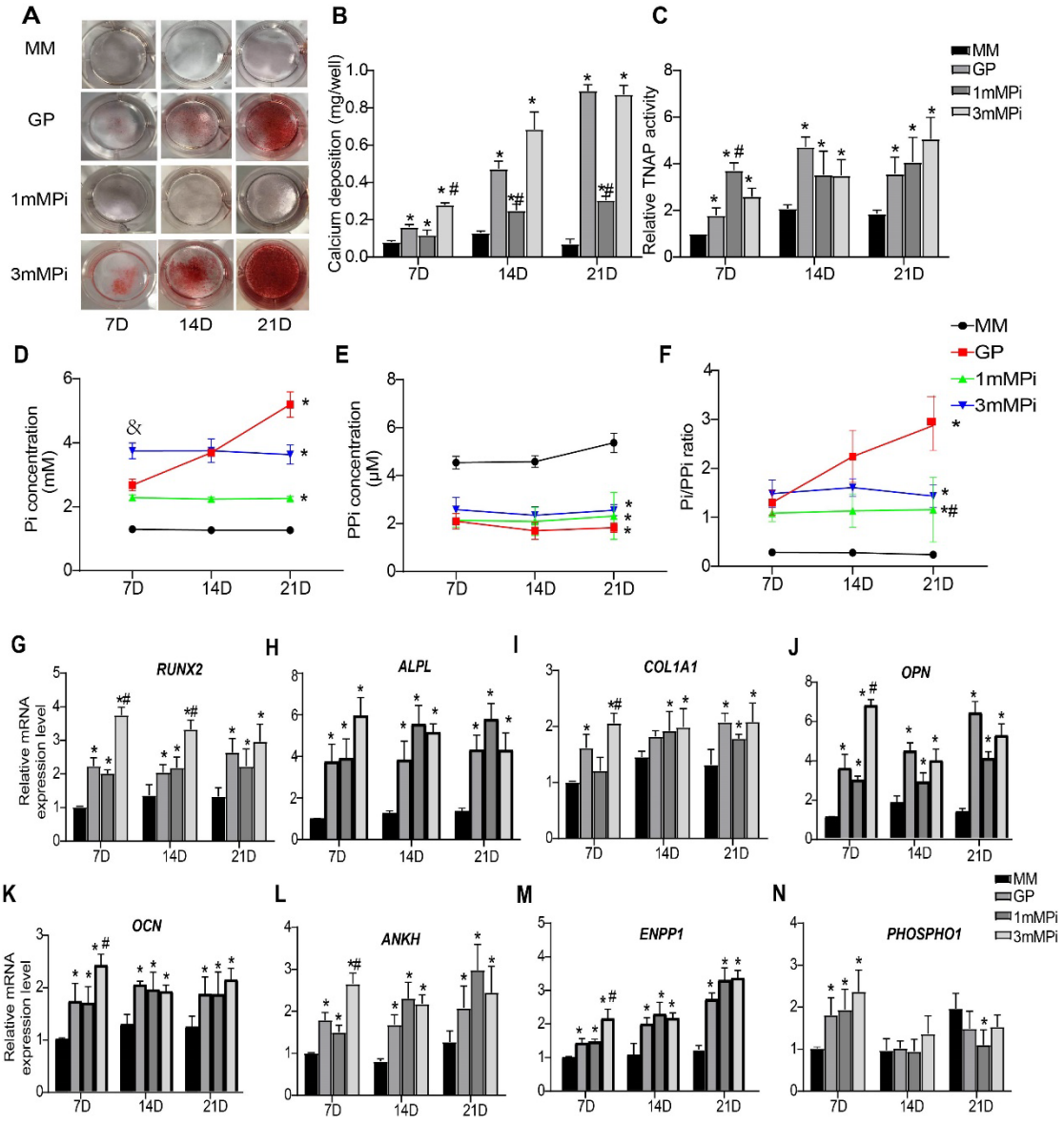

Fig. 1. Pi concentration influences on MSC osteogenic differentiation and mineralization.

A, Alizarin red staining on days 7, 14, and 21. B, The quantification of calcium level in MSC culture on day 7, day 14, and day 21. C, Relative TNAP activity in MSCs on days 7, 14, and 21. D, E, F, Pi and PPi concentrations and the ratio of Pi/PPi in MSC culture medium days 7, 14, and 21. G-N, The mRNA expression of genes involved in osteogenic differentiation and mineralization from MSCs were detected with qPCR. D-N, *, $P < 0.05$ vs the MM group; #, $P < 0.05$ vs the GP group; & $P < 0.05$ vs the GP and 1 mM groups on day 7. MM, maintenance medium; GP, β-glycerophosphate; 1 and 3mM Pi, differentiation medium with 1 and 3mM sodium phosphate (Pi), respectively, instead of β-

glycerophosphate; PPi, pyrophosphate. Data are shown as the mean ± SEM (N = 3).

3.2 RNA-seq analysis to assess effects of Pi concentrations on the early stage of osteoblast differentiation from MSCs

The MSCs culture demonstrated that 3 mM Pi promoted osteogenic differentiation and mineralization on day 7 of culture. Then, we performed RNA-seq to examine changes in the transcriptome on day 7 of culture to investigate gene expression comprehensively.

Principal Component Analysis (PCA) can explain the differences between groups of samples. The first principal component mainly explained the variance of gene expression levels among the MM, GP, and 3 mM Pi groups and showed a significant difference in mRNA expression between the MM group and the other 2. The second principal component also showed a marked difference between the GP and 3 mM Pi groups (Fig. S2A). In DGE analysis between the groups, 2581 up-regulated genes and 2202 down-regulated genes were found in the GP group compared to those in the MM group, and 2524 up-regulated genes and 2090 down-regulated genes in the 3 mM Pi group (Fig. S2B). In addition, only 138 genes were up-regulated and 78 were down-regulated in the comparison of 3 mM Pi and GP groups. Since the differential expressed genes between the 3 mM Pi and GP groups may contribute to the quick response to Pi during the osteoblast differentiation, we applied the K-means clustering method to DGEs in the MM, GP, and 3 mM Pi groups to obtain gene expression patterns in detail. The analysis showed that 67 genes in the cluster 2 had a gradual increase in the order of the MM, GP and 3 mM Pi groups (Fig. S2C). The volcano plot indicated the top 20 genes from the 138 up-regulated genes in the comparison of 3 mM Pi and GP groups (Fig. 2A). Therefore, we focused on the 67 genes of the cluster 2, which were up-regulated in the 3 mM Pi group compared to those in the GP group, in the biological

process analysis of GO analysis. The analysis showed that these genes were involved in many different cellular biological activities, including ossification and osteoblast differentiation (Fig. 2B). The gene-gene interaction plot showed the condition of genes involved in biological processes (Fig. 2C). A KEGG enrichment analysis revealed that *NKD2*, *CCN4*, *SERPINF1*, *WNT5B*, and *CTNNB1*, which are the components of Wnt signaling pathway, were up-regulated in the comparison of 3 mM Pi and GP groups (Table S2). The Wnt signaling pathway is indispensable for the development and homeostasis of bone tissue. Collectively, the RNA-seq analysis demonstrated that genes related with osteoblast differentiation, ossification and Wnt signaling pathway were up-regulated in the 3 mM Pi group compared to the GP group in the early stages of osteoblast differentiation from MSCs.

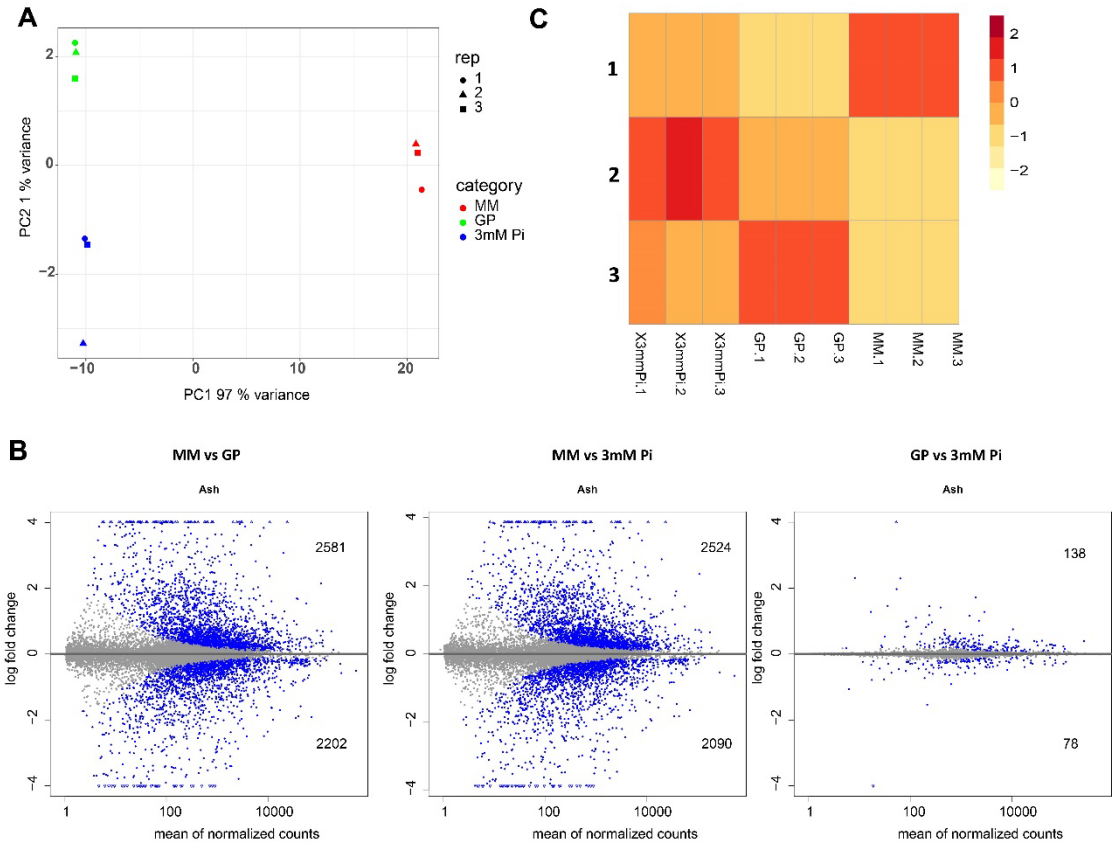

Fig. S2. RNA sequencing analysis

A, The results of sample clustering by PCA based on the gene expression levels. The scatter plots of PC1 and PC2 show the percentages of the variance explained by each PC. B, DGE analysis between each

sample group. Blue dots are genes over the threshold (Padj < 0.01, Padj is the corrected *P* value by the Benjamin-Hochberg method). The points above baseline 0 are up-regulated genes, while the points below 0 are down-regulated genes. C. K-means clustering result based on the gene expression levels. Heatmap shows genes expression levels in each group, and 1, 2, 3 in the left side are different gene clusters. MM, maintenance medium; GP, β-glycerophosphate; PCA, principal component analysis; DGE, differential gene expression. N = 3.

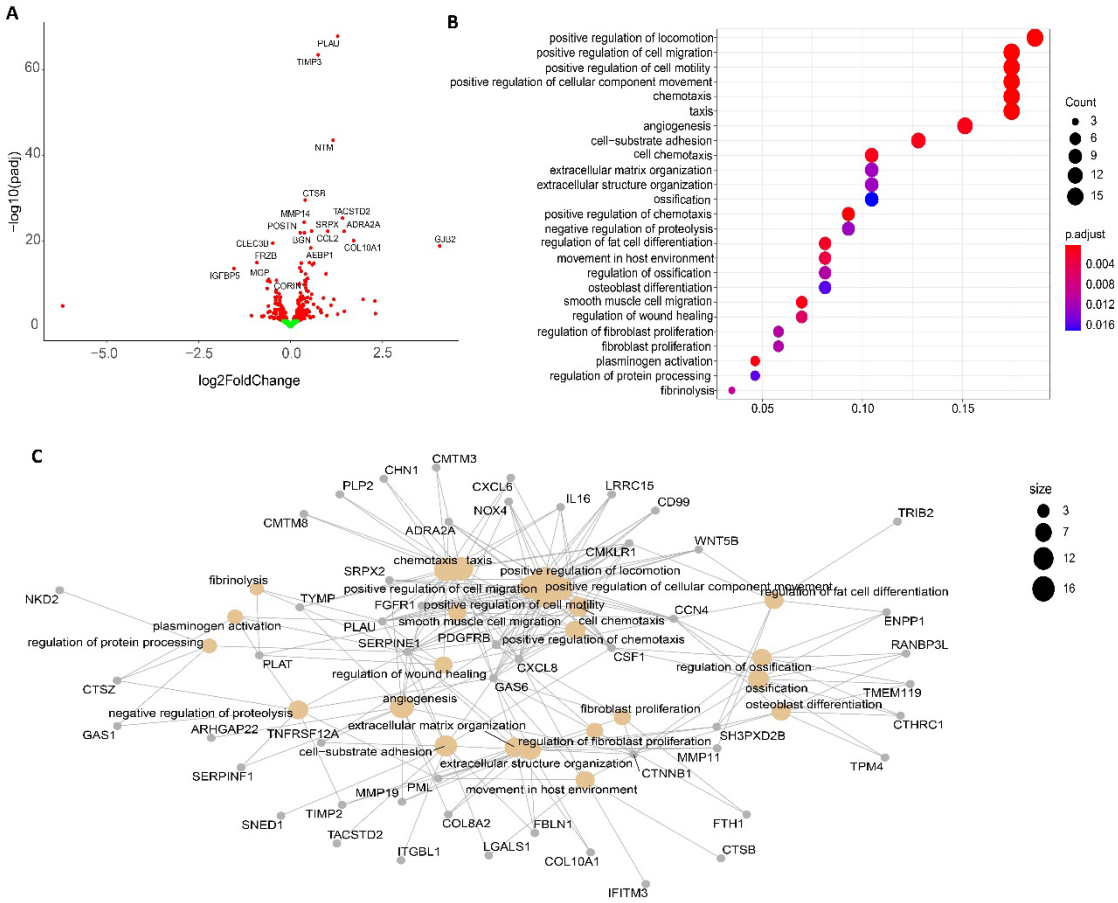

Fig. 2. RNA sequencing analysis

A, Volcano plot showing the top 20 significantly up-regulated genes in the 3 mM Pi group compared to the GP group. D, K-means clustering result based on the gene expression levels. Heatmap shows genes expression levels in each group, and 1, 2, 3 in the left side are different gene clusters. B, Gene ontology

analysis and biological process analysis of the genes in cluster 2. C, Gene-gene interaction plot to capture various relationships in the genes in cluster 2. The size of the points indicates the importance of genes in the network. N = 3.

Table S2 Results of the KEGG analysis

ID	Description	*P* value	gene	Count
hsa04310	Wnt signaling pathway	0.0008793427	*NKD2/CCN4/SERPINF1/WNT5B/ CTNNB1*	5

3.3 High Pi concentrations promote the activation of the non-canonical and canonical Wnt signaling pathways

Because RNA-seq analysis revealed that Wnt signaling pathway is up-regulated by 3 mM Pi, we examined expressions of components in the Wnt signaling pathway by qPCR. Among them, *WNT5b, WNT11, JUN, ROR2*, and *MAPK9* were the components in the non-canonical Wnt signaling, while *Axin2, β-Catenin*, *WISP2, WNT2* and *LRP5* were components in the canonical Wnt signaling. The qPCR results showed that the expression levels of *WNT5b, JUN* and *ROR2* in the 3 mM Pi group were increased in the comparison with MM and GP groups on day 7. However, mRNA expression levels of these genes did not have a significant difference between two induction groups on day 14 (Fig. 3A, B). These results were consistent with the findings that the Pi concentration and osteogenic differentiation gene expression were significantly increased in the 3mM Pi group on day 7, but not on day 14, compared to those in the GP group (Fig. 1D-N). Nevertheless, the mRNA expression levels of *Axin2*, *β-Catenin*, and *LRP5* were increased in the GP and 3 mM Pi groups compared with the MM group on day 7; *Axin2*, *WISP2, WNT2* and *LRP5* were increased in the GP and 3 mM Pi groups compared with the MM group on day 14, whereas those levels were comparable in the GP and 3 mM Pi groups (Fig. 3C, D). These mRNA

expression studies suggested that the components in the non-canonical Wnt signaling responded to the higher Pi concentration at the early stage of osteogenic differentiation, but not the canonical Wnt signaling. Western blot was performed to confirm enhanced non-canonical Wnt signaling pathway on day 7 of culture and revealed that the protein expressions of WNT5b and phosphor-c-Jun (p-c-Jun) to c-Jun ratio were significantly increased in the 3 mM Pi group compared with those in the MM and GP groups on day 7 (Fig.3E–G). In contrast, protein levels of total β-Catenin, active β-Catenin, p-β-Catenin (an inactive form) and p-GSK-3β (an inactive form) were not different between the 3 mM Pi and GP groups on days 7 and 14 (Fig.3H–J). On the other hand, p-GSK-3β and p-β-Catenin protein levels were increased and decreased, respectively, in both 3 mM Pi and GP groups compared to those in the MM group on day 7. On day 14, protein levels of β-Catenin, active β-Catenin, p-β-Catenin and p-GSK-3β were changed in the 2 induction groups compared with those in the MM group. Taken together, our experiment results indicated that not only the canonical Wnt signaling but non-canonical Wnt signaling pathways were also involved in Pi-induced osteoblast differentiation from MSCs, and that the non-canonical Wnt signaling pathway, at least WNT5b/c-Jun signaling pathway, was activated quickly in the high Pi group at the early stage of osteogenic differentiation.

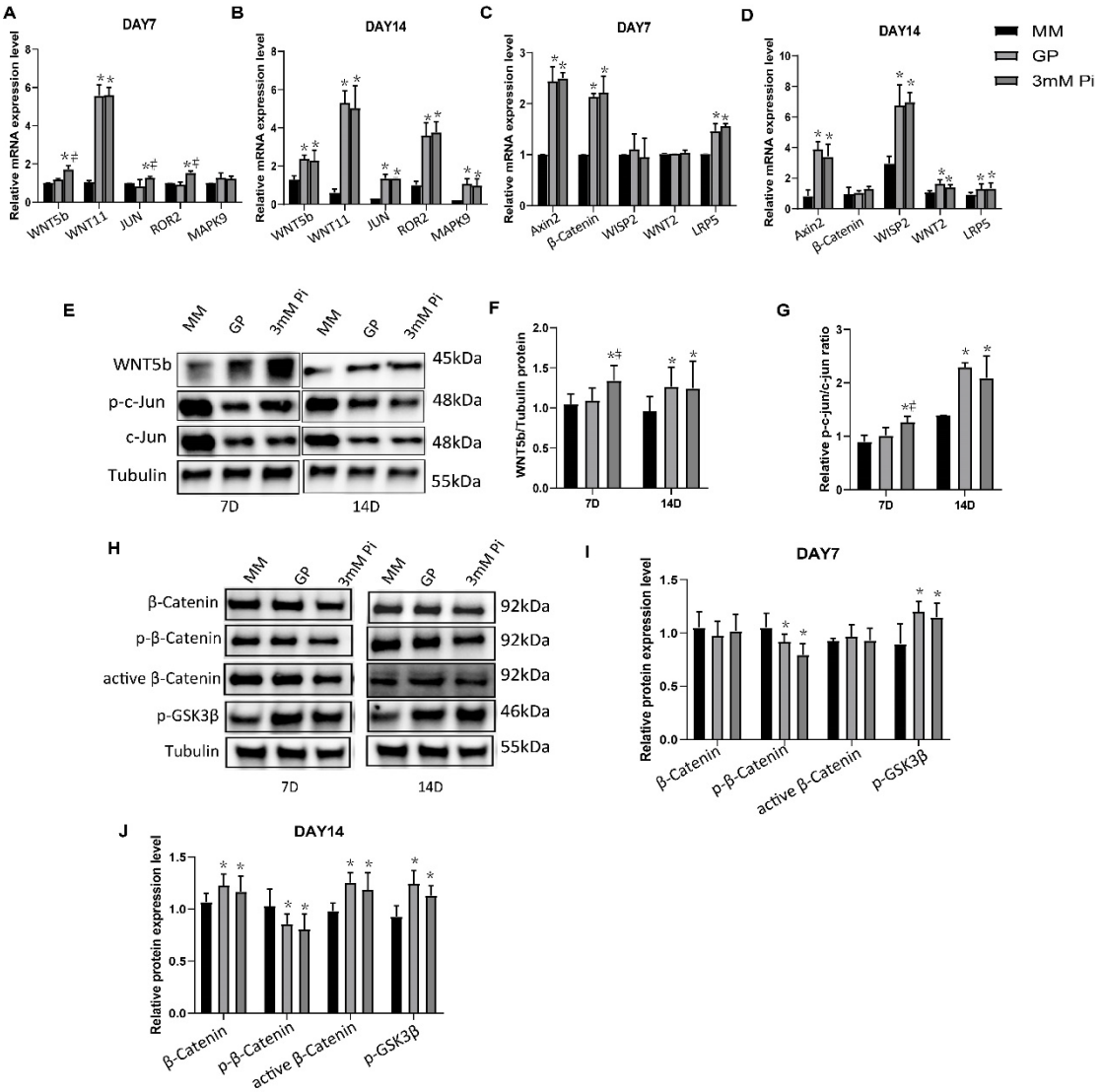

Fig. 3. High Pi concentrations promote the activation of the non-canonical and canonical Wnt signaling pathways

A-D, q-PCR showed mRNA expression levels of components in the Wnt signaling pathway on days 7 and 14. E, Representative images of WNT5b, phosphorylated c-Jun (p-c-Jun), c-Jun and tubulin protein expression on days 7 and 14 detected using a Western blot analysis. Tubulin is an internal control. F, G, Quantification the relative expression level of WNT5b protein and p-c-Jun/c-Jun ratios on days 7 and 14. H, Representative images of β-Catenin, inactive phosphorylated β-Catenin (p-β-Catenin), active-β-Catenin, phosphorylated GSK-3β (p-GSK3β) and tubulin protein expression on days 7 and 14 detected using a Western blot analysis. Tubulin is an internal control. I, J, Quantification of β-Catenin, p-β-Catenin,

active-β-Catenin, p-GSK3β relative protein expression levels on days 7 and 14. Tubulin is an internal control. *, $P < 0.05$ vs the MM group; #, $P < 0.05$ vs the GP group. MM, maintenance medium; GP, β-glycerophosphate. Data are shown as the mean ± SEM (N = 3).

3.4 WNT5b induced by Pi mediated further induction of Wnt signaling pathway

The qPCR and immunoblotting showed high Pi concentration activated non-canonical Wnt signaling pathway, including WNT5b, on day 7 after osteogenic differentiation induction. Then, we examined whether inhibiting the expression of WNT5b would further affect non-canonical and canonical Wnt signaling pathways induced by Pi treatment. The process involved transfection of si-WNT5b into cells on day 7 and day 14 after osteogenic induction.

As expected, the suppression of *WNT5b* mRNA expression on days 7 and 14 led to suppression of WNT5b protein level (Fig. 4A, B). As for the non-canonical Wnt signaling, *WNT11* mRNA expression level was also decreased in both 3 mM Pi and GP groups with transfection of si-WNT5b. *JUN* and *ROR2* mRNA expression levels were, however, reduced in the 3 mM Pi group in the knockdown of WNT5b, while in the GP group did not show significant difference after transfection with si-WNT5b on day 7 of osteogenic induction. No difference was observed in *JUN* and *ROR2* expression in the GP group between the transfections of si-WNT5b and si-Cont on day 7. However, on day 14, *JUN* and *ROR2* mRNA expression levels were decreased in the GP groups with si-WNT5b compared to those with si-Cont, but not in the 3 mM Pi group. Their expression levels were increased in all the 4 induction groups compared to those in the MM group on day 14. In other side, *Axin2, β-Catenin, WISP2, WNT2* and *LRP5* mRNA expression levels in the canonical Wnt signaling were not different in the 3 mM Pi and GP groups between si-WNT5b and si-Cont on days 7 and 14. *Axin2, β-Catenin*, and

LRP5 expression levels were increased in 4 induction groups compared with those in the MM group on days 7 and 14. We then investigated protein expression level (Fig. 4C, 4F). p-c-Jun to c-Jun ratio on day 7 was obviously decreased in the 3 mM Pi group with si-WNT5b versus with si-Cont but not in the GP group. However, on day 14, p-c-Jun to c-Jun ratio was reduced in both 3 mM Pi and GP groups transfected with si-WNT5b (Fig. 4C-E). Regarding the canonical Wnt signaling pathway, compared to the MM group on day 7, total β-Catenin and active β-Catenin expressions were unchanged for the 3 mM Pi and GP groups in the treatment of si-WNT5b and si-Cont, while expressions of p-GSK3β increased and p-β-Catenin decreased in all the induction groups with si-WNT5b and si-Cont (Fig. 4F, G). On day 14 of osteogenic induction, total β-Catenin, active β-Catenin (except p-β-Catenin), and p-GSK3β expressions were increased in all the induction groups relative to the MM group. No difference was observed in expression levels of total β-Catenin, p-β-Catenin, active β-Catenin and p-GSK3β between the transfections of si-WNT5b and si-Cont. Collectively, these results indicated that Pi-induced p-c-Jun protein and *JUN*, *ROR2*, and *WNT11* mRNA expression levels were mediated, at least in part, by WNT5b.

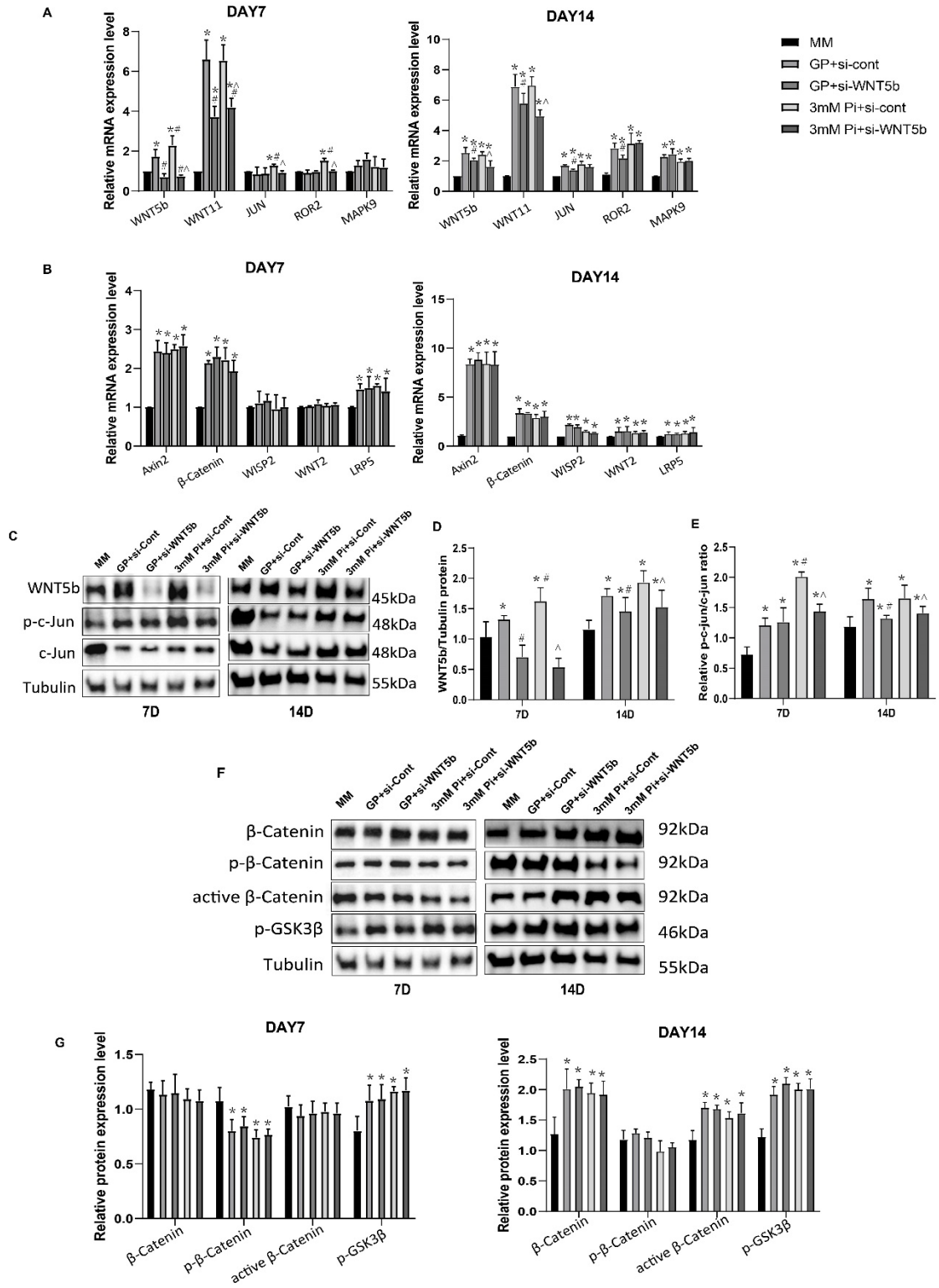

Fig. 4. WNT5b induced by Pi mediated further induction of Wnt signaling pathway

A, B, q-PCR showed mRNA expression levels of components in Wnt signaling pathway on days 7 and 14. C, Representative images of WNT5b, phosphorylated c-Jun (p-c-Jun), c-Jun and tubulin protein

expression on days 7 and 14 detected using a Western blot analysis. Tubulin is an internal control. D, E, Quantification of relative expression level of WNT5b protein and p-c-Jun/c-Jun ratios on days 7 and 14. F, Representative images of total β-Catenin, inactive phosphorylated β-Catenin (p-β-Catenin), active β-Catenin, phosphorylated GSK-3β (p-GSK3β) and tubulin protein expression on days 7 and 14 detected using a Western blot analysis. Tubulin is an internal control. G, Quantification of β-Catenin, p-β-Catenin, active β-Catenin, p-GSK3β relative protein expression levels on days 7 and 14. Tubulin is an internal control *, $P < 0.05$ vs the MM group; #, $P < 0.05$ vs the GP+si-Cont group; ^, $P < 0.05$ vs 3 mM Pi +si-Cont. MM, maintenance medium; GP, β-glycerophosphate; si-WNT5b, siRNA of WNT5b; si-Cont, control of siRNA. Data are shown as the mean ± SEM (N = 3).

3.5 The inhibition of WNT5b expression attenuated osteogenic differentiation

We then tested the effect of the inhibition of WNT5b expression on osteogenic differentiation from MSCs and mineralization induced by Pi on day 7 of culture. Firstly, we tested mRNA expression levels (Fig. 5A-H). *RUNX2* and *ALPL* mRNA expression levels were reduced in the 3 mM Pi group transfected with si-WNT5b compared to those in the 3 mM Pi group with si-Cont group, but not in the GP group. *OPN* mRNA expression levels were increased in both GP and 3 mM Pi groups with si-WNT5b compared to those with si-Cont. mRNA expression levels of other genes were not affected after transfection of si-WNT5b in both 3 mM Pi and GP groups compared to those of si-Cont. Next, the TNAP activity and mineralization level were decreased in both 3 mM Pi and GP groups with transfection of si-WNT5b compared to those with si-Cont (Fig. 5I-K). Finally, we investigated Pi and PPi concentrations and the ratio of Pi/PPi (Fig. 5L-N). The Pi/PPi ratio was decreased in both 3 mM Pi and GP groups with transfection of si-WNT5b compared to that with si-Cont although Pi and PPi

concentrations were not different in both 3 mM Pi and GP groups between treatment of si-RNA and si-Cont. These results indicated that inhibiting WNT5b affected osteogenic differentiation, TNAP activity and mineralization promoted by Pi. In addition, considering the results of *RUNX2, ALPL, JUN* and *ROR2* mRNA levels and p-c-Jun protein levels in the knockdown of WNT5b, WNT5b-mediated non-canonical Wnt signaling pathway is thought to be involved in Pi-induced early osteogenic differentiation.

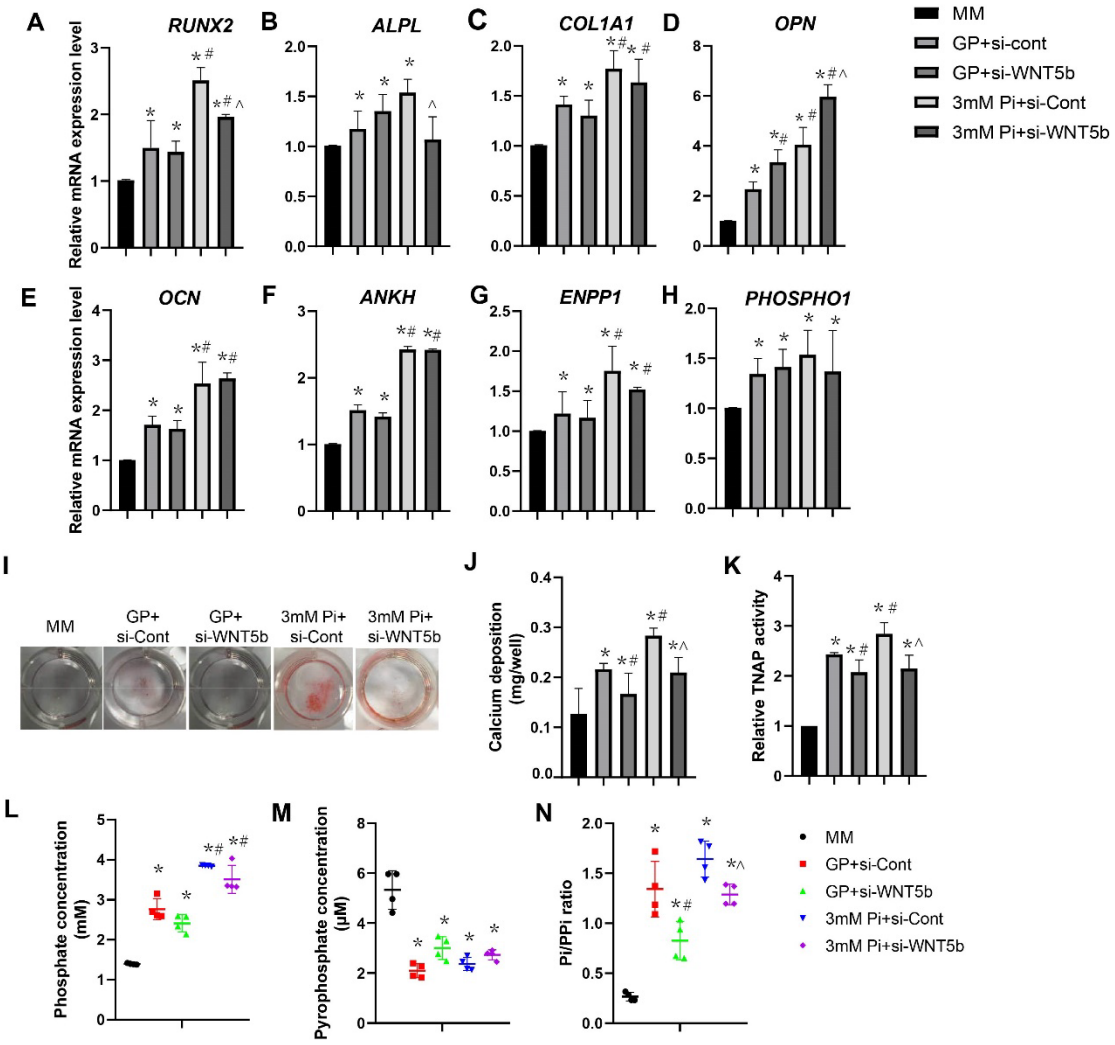

Fig. 5. The inhibition of WNT5b expression by siRNA effect on osteogenic differentiation

A-H, q-PCR showed the mRNA expression level of osteogenesis differentiation genes on day 7 of culture. I, Alizarin red staining revealed mineralization on day 7. J, The quantification of calcium deposition on day 7. K, Relative TNAP activity on day 7. L-N, Pi and PPi concentrations and the ratio of Pi/PPi on day

7. *, $P < 0.05$ vs the MM group; #, $P < 0.05$ vs the GP+si-Cont group; ^, $P < 0.05$ vs 3mM Pi +si-Cont. MM, maintenance medium; GP, β-glycerophosphate; si-WNT5b, siRNA of WNT5b; si-Cont, control of siRNA; Pi, inorganic phosphate; PPi, pyrophosphate. Data are shown as the mean ± SEM (N = 3).

3.6 Inhibition of FGFR1 attenuated the non-canonical Wnt signaling expression

Since it has been shown that FGFR1 is involved in Pi homeostasis in the body and osteogenic lineage cells respond to extracellular Pi via FGFR1[15], we investigated whether inhibition of FGFR1 using an FGFR1 inhibitor, PD173074, influenced the activation of the non-canonical and canonical Wnt signaling enhanced by high Pi. Western blot analysis showed that p-ERK expression was significantly decreased in the groups treated with PD173074 on day 7 of osteoblast differentiation culture compared to DMSO (Fig6A). Then, we examined the effect of PD173074 on expressions of components in the Wnt signaling pathway by qPCR and Western blotting (Fig6B-G). As for the non-canonical Wnt signaling, *WNT5b*, *WNT11* mRNA and WNT5b protein expression level were suppressed on day 7 in both 3 mM Pi and GP groups with PD173074, and *ROR2* mRNA expression level was attenuated in 3 mM Pi group with PD173074. p-c-Jun to c-Jun ratio had a tendency to be decreased in the 3 mM Pi group treated with PD173074 compared to the 3 mM group treated with DMSO. On the other side, *Axin2, WISP2, WNT2* and *LRP5* mRNA expression levels in the canonical Wnt signaling did not show a significant difference in the 3 mM Pi and GP groups between the presence and absence of PD173074, although *β-Catenin* mRNA expression level was decreased in the groups treated with PD173074. While total β-Catenin, active β-Catenin, p-β-Catenin and p-GSK3β expression did not show a significant difference in four induction groups between the presence and absence of PD173074. These results indicate that inhibition of FGFR1 attenuated the non-canonical Wnt signaling expression.

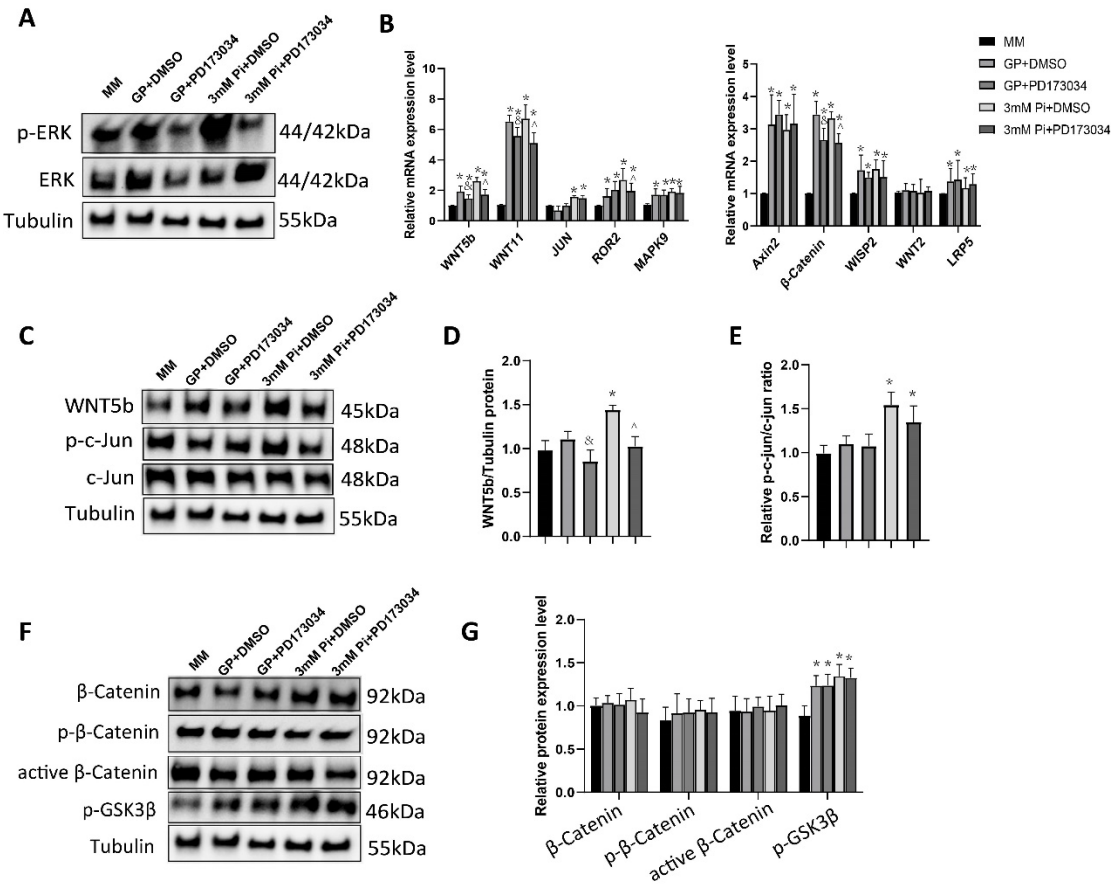

Fig. 6. Inhibition of FGFR1 attenuated the non-canonical Wnt signaling expression

A, Western blot showed p-ERK and ERK expression on day 7 of culture. B, q-PCR showed mRNA expression levels of components in Wnt signaling pathway on day 7. C, Representative images of WNT5b, phosphorylated c-Jun (p-c-Jun), c-Jun and tubulin protein expression on day 7 detected using a Western blot analysis. Tubulin is an internal control. D, E, Quantification of relative expression level of WNT5b protein and p-c-Jun/c-Jun ratios on day 7. F, Representative images of total β-Catenin, inactive phosphorylated β-Catenin (p-β-Catenin), active β-Catenin, phosphorylated GSK-3β (p-GSK3β) and tubulin protein expression on day 7 detected using a Western blot analysis. Tubulin is an internal control. G, Quantification of β-Catenin, p-β-Catenin, active β-Catenin, p-GSK3β relative protein expression levels on day 7. Tubulin is an internal control *, $P < 0.05$ vs the MM group; &, $P < 0.05$ vs the GP+DMSO group; ^, $P < 0.05$ vs 3 mM Pi +DMSO. MM, maintenance medium; GP, β-

glycerophosphate. Data are shown as the mean ± SEM (N = 3).

4. Discussion

In this study, we have suggested that the non-canonical Wnt signaling pathway via WNT5b is involved in the osteogenic differentiation and mineralization in response to 3 mM Pi at the early stage of osteogenic differentiation using human MSCs.

GP is generally used as a sauce of Pi in the osteogenic induction culture. TNAP is needed for the conversion of GP into Pi and it takes time during the process of osteogenic induction. The relative TNAP activity had increased in all of the induction groups since day 7 of culture, which contributed to increase Pi concentration of GP group. Indeed, we demonstrated that extracellular Pi concentration of 3 mM Pi group was significantly higher compared to that in the 10mM GP group on day 7, while the concentration was similar between both groups on day 14. Taking advantage of the difference in Pi concentration, we then examined mRNA expression level of osteogenic differentiation marker genes and found that *RUNX2, COLIA1, OPN, OCN, ANKH*, and *ENPP1* expressions were significantly enhanced in the 3 mM Pi group compared with those in the GP group on day 7. In this experimental condition, we performed the RNA-seq of cells in the MM, 3mM Pi and GP groups and found 138 up-regulated genes in the comparison of the 3 mM Pi group and GP group.

The bioinformatics analysis indicated that Wnt signaling pathways were enhanced in the early stage of osteoblast differentiation induced by Pi from MSCs. While the canonical Wnt signaling plays a significant role in osteogenesis [16], it has been recognized in recent years the non-canonical Wnt signaling is promoted in the process of osteogenesis. The non-canonical Wnt signaling pathway has been indicated to regulate the bone-resorbing activity of osteoclasts [17] and to affect osteoblast differentiation,

maturation, and function [18]. Zhao et al. have found that WNT5b expression was increased in bone tissue during fracture healing [19], which has illustrated that WNT5b is enhanced during bone formation process. Liu et al. have shown that the up-regulation of WNT5b and p-c-Jun influenced the heterotopic ossification of tendon tissue subjected to excessive tension [20]. We showed the expressions of non-canonical Wnt signaling components, including WNT5b, p-c-Jun and ROR2, and mineralization were increased in the 3 mM Pi group with high Pi concentrations on day 7 compared to those in the GP group. Mozar et al. have shown that the range of 1.5–4.5 mM Pi induced a weak activation of JNK, an upstream of c-Jun [21]. Furthermore, It has been suggested that FGFR1 work as a Pi-sensing receptor in the regulation of serum Pi level [15] FGFR1 has been shown to interact with the WNT signaling pathway [22, 23]. In our study, the expressions of the non-canonical Wnt signaling components, including W*NT5b, WNT11* and *ROR2* mRNA and WNT5b protein, induced by high Pi concentrations were suppressed in the treatment with a FGFR1 inhibitor, PD173074. These results indicate that at the early stage of osteogenesis, FGFR1 mediates the non-canonical Wnt signaling induced by high Pi concentration.

In our research, inhibition of WNT5b suppressed expressions of *WNT11*, *ROR2* and *JUN* mRNA expressions and p-c-Jun to c-Jun ratio expressions in the 3 mM Pi+si-WNT5b group compared with that in the 3 mM Pi+si-Cont group on day 7 of culture. Boyan et al. have shown that WNT11 is important for osteoblast maturation and is regulated primary bone formation during osseous integration [24]. In addition, WNT5b and WNT11 expressions have been reported to overlap in chondrogenesis [25] and in the areas of human aortic valve calcification [26]. Also, siRNA experiment of WNT5b in the present study suggested that *ROR2* is involved in Pi-induced WNT5b-mediated osteogenic differentiation. ROR2 is the receptor for WNT5a/WNT5b, and the physiological interaction of WNT5b/ROR2 has been also reported [27]. Menck et al. have shown that WNT11 is also a ligand of ROR2 [28], and that ROR2

overexpression has upregulated WNT11. In addition, *JUN* mRNA and p-c-Jun protein expressions were decreased in the 3 mM Pi+si-WNT5b compared to those in the 3 mM Pi+si-Cont group on day 7. Liu et al. have demonstrated that shRNA knockdown of WNT5a and WNT5b expression in rat tendon-derived stem cells reduced the expression of p-JNK and RUNX2 [20]. Seo et al. have found that WNT11 itself is a downstream target of the JNK cascade in the non-canonical Wnt pathway [29]. Collectively, these findings suggest that WNT5b may mediate *ROR2* and *JUN* expression and interact with *WNT11* through *ROR2* and *JUN* in Pi-induced osteoblast differentiation. WNT11 activates both canonical and non-canonical Wnt signaling pathways because Wnt11 promotes osteoblast maturation through β-Catenin and R-spondin 2 [30]. WNT11 is likely to be an intersection for the canonical and non-canonical Wnt signaling pathways. In addition, we found suppressed WNT5b would attenuate mineralization and osteogenic differentiation on day 7 of the culture. Silencing of WNT5b suppressed the expression of *RUNX2* and *ALPL* mRNA in 3 mM Pi group after transfected si-WNT5b, while *OPN, OCN, ANKH* and *ENPP1* expression did not affected by si-WNT5b. Several studies have showed that *OPN* and *OCN* expressions respond to the canonical Wnt/β-catenin signaling [31, 32]. *ANKH* expression is also promoted by WNT1, a ligand of the Wnt/β-catenin signaling [33]. In this study, we found that the Wnt/β-catenin signaling was not affected by si-RNA of WNT5b and that the non-canonical Wnt signaling was down-regulated by the si-RNA of WNT5b. These results suggest that the non-canonical Wnt signaling induced by high Pi concentration may not be involved in *OPN*, *OCN* and *ANKH* expression.

The limitation of this research is that only *in vitro* experiments were conducted in the present study. *In vivo* study is necessary to confirm that the involvement of non-canonical Wnt in Pi-induced osteogenic differentiation and mineralization in the body. In addition, siRNA of WNT5b could not completely block

WNT5b expression although siRNA of WNT5b led to a reduction in WNT5b to a considerable extent and components of the non-canonical Wnt signaling and an impairment of osteogenic differentiation. Knockout of WNT5b using the CRISPER/Cas9 system will augment the results of the siRNA experiment of WNT5b in the non-canonical Wnt signaling and osteogenic differentiation. We will continue to explore the role of the non-canonical Wnt signaling on differentiation from MSCs to osteoblasts. However, this is the first time, to the best of my knowledge, to illustrate the non-canonical WNT5b/c-Jun signaling pathway mediates osteogenesis and mineralization induced by high Pi and responds earlier than the canonical Wnt signaling.

In conclusion, this study indicated an increase in Pi concentration promoted osteogenic differentiation and mineralization, at least in part, via the non-canonical Wnt signaling through Wnt5b/ROR2/c-Jun on the early stage of osteogenic differentiation from MSCs. Our findings provide new perspective into the association of Pi with the non-canonical Wnt signaling in osteogenesis differentiation.

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Credit authorship contribution statement

Shumin Rui: Experiments, Statistical analysis, Formal analysis, Investigation, Writing-Original Draft. **Takuo Kubota**: Conceptualization, Methodology, Writing-Review and Editing, Supervision, Project

administration, Funding acquisition.

Yasuhisa Ohata: Methodology, Supervision, Writing-Review and Editing.

Kenichi Yamamoto: Methodology, Supervision, Writing-Review and Editing.

Makoto Fujiwara: Methodology, Supervision, Writing-Review and Editing.

Shinji Takeyari: Methodology, Supervision, Writing-Review and Editing.

Keiichi Ozono: Conceptualization, Methodology, Writing-Review and Editing, Supervision, Project administration.

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