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Roles of brain and muscle ARNT-like 1 and Wnt antagonist Dkk1 during osteogenesis of bone marrow stromal cells

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Abstract

Objectives: Many studies have demonstrated that the clock gene, brain and muscle *ARNT-like 1* (*Bmal1*), is directly related to bone ageing by affecting age-related changes to mesenchymal stem cells (MSCs). As a main developmental signal, Wnt may play an important role in this process. Here, we have aimed to elucidate whether *Bmal1* positively regulates osteogenesi *via* Wnt pathways.

Materials and methods: Bone marrow stromal cells were cultured in basic and in osteo-induction medium with Wnt signalling inhibitor Dkk1 and *Bmal1* transfection. Proliferation and osteogenesis of MSCs, expression of *Bmal1* and activation of Wnt signalling were investigated by flow cytometry, senescence-associated β-galactosidase (SA-β-gal) staining, real-time quantitative PCR and western blot analysis.

Results: Expression of *Bmal1* (specially after 7 days osteo-induction), activation of Wnt signalling and osteo-related factors fell significantly during osteo-induction after Dkk1 addition. When cellular *Bmal1* was increased by transfection, osteogenesis inhibition by Dkk1 was rescued to a certain extent with activation of Wnt signalling. However, Dkk1 did not significantly affect proliferation or senescence of MSCs during early periods of culture.

Conclusion: These findings demonstrated that *Bmal1* and Wnt signalling may have a synergistic effect at a particular stage of osteogenesis. Inhibition of Wnt signalling did not greatly affect ageing of MSCs through early passages.

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Introduction

Some studies have shown that reduction in capability of self-healing and regeneration is the primary cause of ageing (1). Such changes may be due to reduction in stem-cell proliferation and differentiation. Osteoporosis is a major symptom of mammalian ageing (2) and is an important part of the process, closely related to bone marrow stromal cells (BMSCs) (3). Proliferation and differentiation abilities of mesenchymal stem cells (MSCs) decline with ageing (4), specially osteogenetic ability (5). As osteoblast progenitors, changes in BMSCs inevitably result in osteoporosis, reduced bone mass and further complications.

However, numbers of experiments have demonstrated that changes in circadian gene expression are probably internal reasons for cell senescence, specially core circadian component brain and muscle ARNT-like 1 (*Bmal1* gene). Researchers have found that mice lacking *Bmal1* (*Bmal1*−/−) show early ageing symptoms compared to wild-type littermates (6). These signs include shorter lifespan, arthropathy, osteoporosis and other ageing precursors. *Bmal1*−/− mice 40 weeks of age have obvious weight loss, as occurs in osteoporosis. After rescuing *Bmal1* expression in *Bmal1*−/− mice, long-term survival has been restored, and early ageing mitigated (7).

Study of gene function should be traced to changing processes of metabolic transduction. Wnt signalling, specially the canonical Wnt pathway (Wnt/β-catenin signalling), plays a significant role in cell proliferation and differentiation. Although the exact role of Wnt signalling in senescence of cells and organs remains controversial, most studies support the idea that its activity can

delay the ageing process (8–10), however, recent studies have provided contradictory results (11,12). Such controversial findings may be attributed to differences in Wnt signal intensity, cell culture conditions *in vitro*, and cell manipulation (13). Nevertheless, importance of Wnt signalling in cell ageing been confirmed (11,14). The Wnt/β-catenin pathway involves the ligand (Wnt), transmembrane receptor (Frizzled), cytoplasmic protein (β-catenin), nuclear transcription factor (TCF) and further components (15,16). When Wnt protein level is low, β-catenin is hydrolysed by phosphorylation-dependent proteolytic enzymes induced by degradation of the glycogen synthase kinase 3β complex, Axin, and adenomatous polyposis coli. Thus, β-catenin level is kept low, which inhibits transcription mediated by T-cell factor/lymphoid enhancer factor. When the Wnt ligand interacts with its transmembrane receptor, degradation of the complex is inhibited, β-cateninfirst accumulates in the cytoplasm then enters the nucleus to activate transcription of target genes mediated by TCF (17).

Dkk1, a protein inhibitor of Wnt signalling, is a physiological protein secreted by MSCs and multiple myeloma cells (18). Dkk1 can bind to a receptor or interact with transmembrane receptor Kremen to induce rapid endocytosis and downregulate expression of low-density lipoprotein receptor-related protein 5/6 (LRP-5/6), which blocks intracellular delivery of Wnt signalling (19,20). Related experiments have indicated that heterozygous Dkk1-deficient mice have increased bone formation and a high bone mass phenotype. Moreover, number of osteoblasts, mineral apposition and bone formation rate were all increased in these mice (21). In contrast, experiments on human fracture non-union stromal cells found that senescent cells secreted more Dkk1 (22). Whether higher secretion of Dkk1 in senescent cells is the result of senescence or its cause, has not been clearly explained. With regard to dose effect of Dkk1, from 25 ng/ml it inhibited non-phosphorylated β-catenin, with maximal inhibition at 50 ng/ml in C2C12 (18). Furthermore, 50 ng/ml is the physiological secretion dose (22).

Thus, we hypothesized that Wnt signalling may be the link in processes by which *Bmal1* affects in changes in MSC ageing. Our preliminary studies demonstrated that in aged mice, reduction in proliferation and osteogenic ability were positively related to reduction in *Bmal1* expression (23). In a further piece of research, Zhang *et al.* showed that activation of Wnt signalling in osteoblasts, specially expression level of β-catenin (cytoplasmic protein of the Wnt signalling pathway), reduces with age (24); furthermore, cell proliferation and expression of β-catenin increase by enhancing *Bmal1* expression (25). However, effects of Wnt signalling on expression of *Bmal1* have remained unknown. To clarify the relationship between *Bmal1* and Wnt signalling in osteo-induction, we have explored the underlying mechanism of *Bmal1* expression in osteo-differentiation during ageing, using Wnt signalling inhibitor Dkk1 and *Bmal1* transfection.

Materials and methods

Cell culture

Four week old C57/BL6 male mice with good BMSC activity and no interference by oestrogen, obtained from the Animal Centre of Sichuan University, were used in this study; all protocols used were approved by the University Bioethics Committee. The mice were sacrificed, and metaphyses from both ends of femora and tibias were obtained and marrow cavities were flushed with sterile medium, using a #25-gauge needle. After washing in phosphate-buffered saline (PBS) by centrifugation, cells were cultured in α-MEM (Gibco, Life Technologies, Carlsbad, CA, USA) with 100 U/ml penicillin, 10% foetal bovine serum (Hyclone, Thermo Fisher Scientific, Waltham, MA, USA) and 100 mg/ml streptomycin (as basic medium). They were then maintained at 37 °C in a humidified atmosphere with 5% CO_2. After 36 h, non-adherent cells were removed by gentle rinsing in sterile PBS prewarmed to 37 °C. Until confluence, cells were maintained in basic medium, which was changed two to three times per week. When confluent, MSCs were detached with 0.25% trypsin in 0.01% ethylenediaminetetraacetic acid then passaged. MSCs were identified by immunohistochemistry as CD29(+), Stro-1(+), CD45(−) and CD34(−), as suggested in other studies (4). Osteo-induction medium (OS medium) used was basic medium supplemented with 10 mM β-glycerophosphate, 10 nM dexamethasone, and 50 mg/l L-ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA). Dkk1 protein powder from recombinant mouse Dkk1 (R&D Systems, Inc., Minneapolis, MN, USA) was added into the α-MEM medium, final concentration 50 ng/ml (26).

Flow cytometry of MSCs after addition of Dkk1

Mesenchymal stem cells in basic medium with and without Dkk1 were passaged. At passage 4, when cells reached 70% confluence, they were detached with trypsin and ethylenediaminetetraacetic acid; after washing twice in PBS and centrifuging at 500 ***g*** for 5 min, they were fixed in 70% alcohol and frozen to below −20 °C. Two hours before detection, cells were centrifuged to remove alcohol and were washed in PBS. After incubating in RNase for 30 min, propidium iodide (PI) was infused into them. Cell density was approximately 1×10^6 cells/ml for detection. One tube was incubated

in steam buffer only as control, to eliminate interference of residual green fluorescent protein (GFP) fluorescence. DNA content of cells was detected by flow cytometry (Beckman Coulter, Brea, CA, USA), S-phase fraction and DNA proliferation index of total cells in each sample was calculated according to the formula:

$$\mathrm{SPF}(\%) = \mathrm{S}(\mathrm{G}_0/\mathrm{G}_1 + \mathrm{G}_2/\mathrm{M}) \times 100\%$$

$$\text{DNA proliferation index}(\%) = (\mathrm{S} + \mathrm{G}_2/\mathrm{M})/(\mathrm{G}_0/\mathrm{G}_1 + \mathrm{S} + \mathrm{G}_2/\mathrm{M}) \times 100\%$$

Assessment of senescence-associated β-galactosidase (SA-β-gal) staining

Passage 4 MSCs were digested and seeded into six-well plates, 1×10^5 cells/well. After 24 h culture in a humidified atmosphere with 5% CO_2, medium was discarded and cells were washed once in PBS. Then, 1 ml fixative per well was added for 15 min, and cells were rinsed three times. They were then incubated in 1 ml per well working solution of *β*-galactosidase with X-Gal, overnight, at 37 °C (SA-β-gal staining kit obtained from Beyotime, Chengdu, China). Senescent cells were observed using an optical microscope and counted in three random fields of vision (200 cells per field used to calculate level of positivity).

Transfection of overexpression lentiviral vector for Bmal1

Passage 2 MSCs were cultured in 12-well plastic plates in Dkk1-conditioned medium (Dkk1 final concentration 50 ng/ml) at initial density of 5×10^4 cells/well. In the order of 48 h after plating, cells were transfected using *Bmal1* overexpressed lentiviral vector (synthesised by Genechem, Shanghai, China) at multiplicity of infection (MOI) MOI = 20; this is called the Bmal group, with the lentiviral vector expressing EGFP as positive control group. Expression of GFP was observed using an inverted fluorescence microscope 2 days later (Fig. S1a); transfection efficiency was calculated. Western blotting assay was used to test transfection effect of *Bmal1* (Fig. S1b). As rhythmic expression of *Bmal1* should not be induced without serum shock *in vitro*, no rigorous time point was followed (27).

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Passage 2 MSCs were divided into three groups for culture, one in OS medium without Dkk1 (control group), one in OS medium with Dkk1 (final concentration Dkk1 50 ng/ml) – Dkk1 group, and the final group with *Bmal1* transfection in Dkk1-conditioned OS medium as the Dkk1 + *Bmal1* group. Cells were sampled and maintained in RNA preservation solution (RNA safeguard; Keygen Biotech, Nanjing, China) after 7 days and after 14 days culture. A simple P total RNA extraction kit (Bioer, Hangzhou, China) was used to extract total RNA; this was quantified by spectrophotometry at absorbance (A) of 260 nm. RNA samples had A260:A280 ratio of 2.0 to ensure high purity., Each real-time PCR was performed in triplicate using ABI PRISM 7300 real-time PCR system in 20-μl reaction mixture. The PCR program was conducted according to the manufacturer's instruction manual. Starting copy numbers of unknown samples were calculated by 7300 System SDS Software (Applied Biosystems, Foster City, CA, USA) from their standard curve. Table 1 shows primers used for RT-PCR analysis.

Quantitative alkaline phosphatase (ALP) and protein assays

Passage 2 MSCs were grouped as described for RT-PCR analysis. For quantitative analysis of ALP activities, after 7 days and after 14 days osteo-induction, cells were washed in PBS, according to instructions of the ALP assay kit (Merit Choice, China). Cells were lysed in 10 mM Tris-HCl and 0.1% Triton X-100 (pH 7.4) and subjected to repeat freeze-thaw cycles three times. After sonication, lysed cells were assayed in microtitre plates with *p*-nitrophenyl phosphate as substrate. Absorbance of *p*-nitrophenol formed by hydrolysis of p-nitrophenyl phosphate, and catalysed by ALP, was measured at 405 nm using a microplate reader. Total protein content was measured using the Bradford method at 595 nm and calculated according to bovine gamma globulin standards. ALP activity was expressed as U/g protein.

Western blot assay for Bmal1and β-catenin protein

Passage 2 MSCs were grouped as described for RT-PCR analysis, and glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) was used as internal reference. To obtain whole-cell protein extracts after 7 days and after 14 days culture, cells were washed twice in ice-cold PBS then lysed in lysis buffer (Keygen total protein extraction kit; Keygen Biotech). Supernatants were collected after centrifugation at 14 000 ***g*** at 4 °C for 15 min, and quantitatively assayed using the BCA method. Total protein extracts were conducted using standard sodium dodecylsulphonate-polyacrylate gel electrophoresis procedures and subsequently transferred to polyvinylidene fluoride membranes. After blocking, membranes were probed with anti-Bmal1 protein primary antibodies (Abcam, Cambridge, UK) or anti-β-

Table 1. Primers used for real-time PCR analysis

Target gene	Primers	Fragment size (bp)
P-actin	F:5′-GGGCTGTATTCCCCTCCATCG-3′ R:5′-GCAGCTCATTGTAGAAGGTGTGGTG-3′	201
Osx	F:5′-TATGCTCCGACCTCCTCAAC-3′ R:5′-AATAGGATTGGGAAGCAGAAA-3′	120
Ocn	F:5′-AAGCAGGAGGGCAATAAGGT-3′ R:5′-CCGTAGATGCGTTTGTAGGC-3′	167
Bmall	F:5′-AACCTTCCCGCAGCTAACAG-3′ R:5′-AGTCCTCTTTGGGCCACCTT-3′	79
Wnt3a	F:5′-CTTAGTGCTCTGCAGCCTGA-3′ R:5′-GAGTGCTCAGAGAGGAGTACTGG-3′	92
P-catenin	F:5′-GGGTCCTCTGTGAACTTGCTC-3′ R:5′-TGTAATCCTGTGGCTTGTCCTC-3′	165
Tcfl	F:5′-CTACAGCGACGAGCACTTTTCTC-3′ R:5′-GTAGAAGGTGGGGATTTCAGGAG-3′	115

catenin primary antibodies (Cell Signaling, Danvers, MA, USA) followed by addition of 1:5000 horseradish peroxidise-conjugated secondary antibody. Immunoreactive proteins were visualized employing a chemiluminescence kit (Millipore, Billerica, MA, USA) and band intensities were determined using the ChemiDoc XRS Gel documentation system and Quantity One software (Bio-Rad, Berkeley, CA, USA).

Statistical analysis

Measurements were expressed as mean ± SD. Statistical comparisons were conducted using factorial analysis of variance (ANOVA), followed by multiple comparisons utilizing the Student–Newman–Keuls test. Values of $P < 0.05$ were considered statistically significant.

Results

MSC proliferation after Dkk1 addition

Proliferation of passage 4 MSCs was detected by flow cytometry, to determine effects of Dkk1 during the process. However, no obvious differences were found between results in the control group and those of the Dkk1 group (Fig. 1). PI and S-phase fraction indices were detected but differences also were not statistically significant ($P > 0.1$). These data indicate that addition of Dkk1 had no effect on MSC proliferation during early passages. As culture-induced senescence begins at passage 4–6 cells *in vitro*, no additional cells were cultured after passage 4 (19).

SA-β-gal staining results of MSCs

As indicated above, the exact role of Wnt signalling in cell senescence remains unclear but as the main antagonists of Wnt, the role of Dkk1 in senescence needs to be understood. Due to complex variations in ageing cells, many interference factors are difficult to control. Thus, we used a physiological dose of Dkk1 in passage 4 MSCs and expected to simulate its effects in physiological senility (22). In the order of 50 ng/ml Dkk1 was added to the osteo-induction medium as Dkk1-conditioned OS medium. Results of SA-β-gal staining conducted on passage 4 MSCs are shown in Fig. 2. Groups showed no obvious differences in ageing, demonstrating that the physiological dose of Dkk1 did not affect MSC senescence during the early passages.

Effects of MSC osteogenesis in Dkk1-conditioned osteo-induction medium after Bmal1 transfection

Considering that Wnt signalling plays an important role in MSC osteogenesis, we anticipated that its inhibition would affects MSC osteogenesis. Our data showed that successive stages of MSC osteogenesis were markedly suppressed by Dkk1. Meanwhile, overexpression of *Bmal1* rescued the reduction in osteogenesis by Dkk1 to some extent (Fig. 3). Moreover, MSC osteogenesis with *Bmal1* overexpression was slightly enhanced (Fig. S2). Osteogenic markers were detected as follows: osterix (Osx), marker of osteoblast progenitors; ALP activity level, an early osteoblast marker; and osteocalcin (Ocn), a late osteogenic marker.

Cells were observed using light microscopy on the 14th day after osteo-induction. Results showed that the control group was clearly calcified and Dkk1 + *Bmal1* group exhibited dotted calcification; however, Dkk1 group had no sign of calcification (Fig. 3a–d). Meanwhile, mRNA levels of osteo-factors with RT-PCR provided more significant results. mRNA expression of Osx and Ocn was detected after 7 days and after 14 days

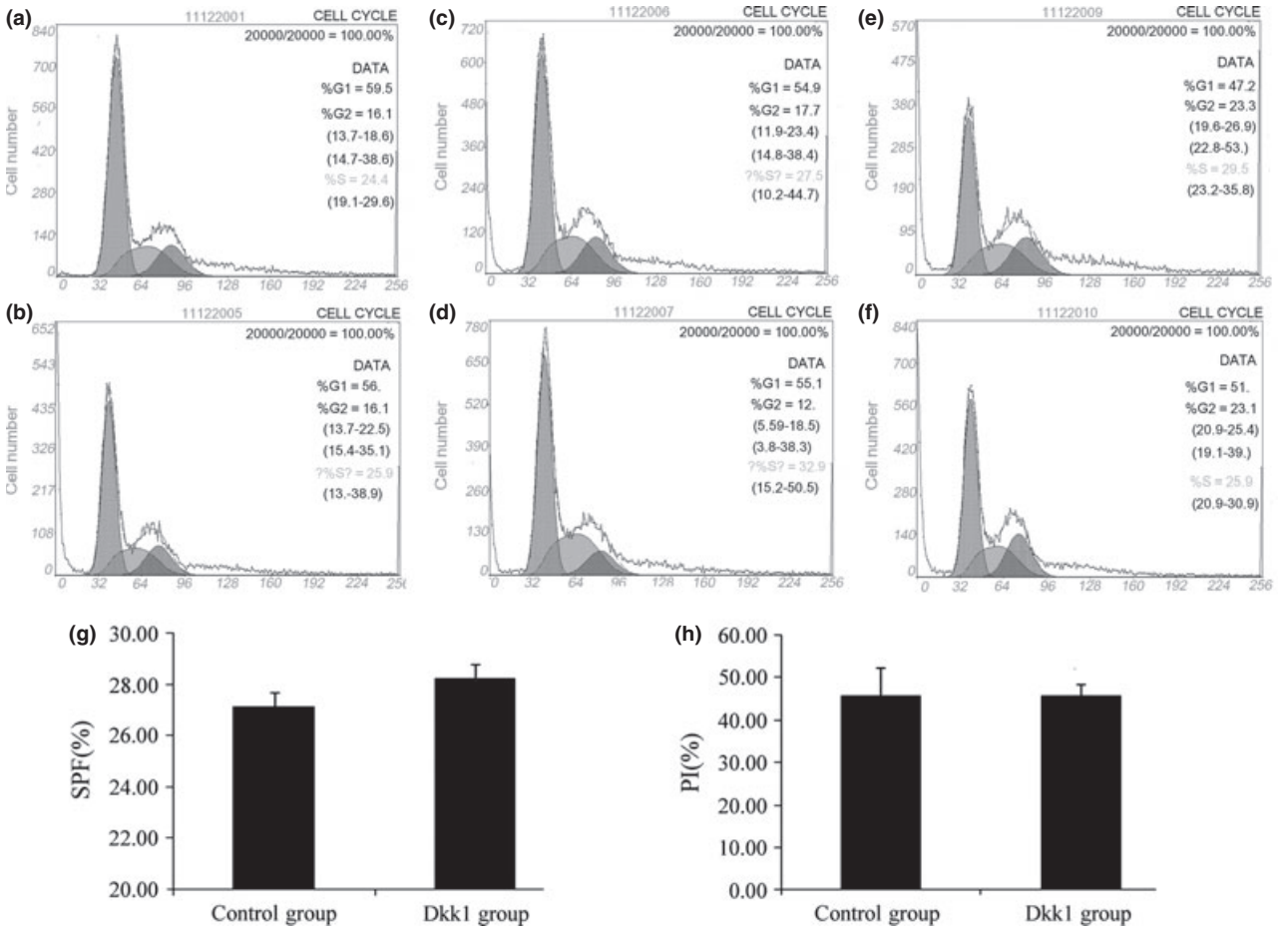

Figure 1. Proliferation of mesenchymal stem cells (MSCs) after addition of Dkk1. (a, c, e) Cell cycle of control group by flow cytometric assay; (b, d, f) cell cycle of Dkk1 group; a and b, c and d, e and f obtained under the same conditions. No clear differences could be seen. (g) Propidium iodide (PI) of two groups and (h) S-phase fraction (SPF). Data represent mean ± SD (n = 3).

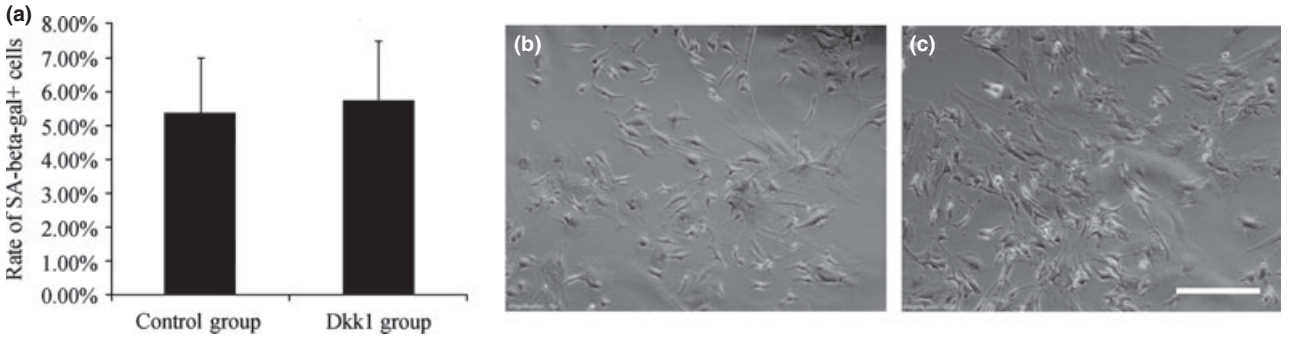

Figure 2. Images of SA-β-gal staining detected in passage 4 mesenchymal stem cells (MSCs). (a) SA-β-ga+ of the two groups. Data were not statistically significant; cells were counted from three different fields to compose the average. (b) Control group was cultured in basic medium, (c) Dkk1 group (50 ng/ml), 200× scale bar = 150 μm.

osteo-induction. Compared to the control group, Osx and Ocn mRNA was obviously lower in the Dkk1 group. In particular, Ocn mRNA decreased by 6% compared to the control group after 14 days osteo-induction. However, in the Dkk1 + *Bmal1* group, more moderate decline of Osx and Ocn mRNA was observed, with Ocn mRNA reaching 23% of control group level by 14 days of osteo-induction (Fig. 3e). As shown in Fig. 3f, ALP

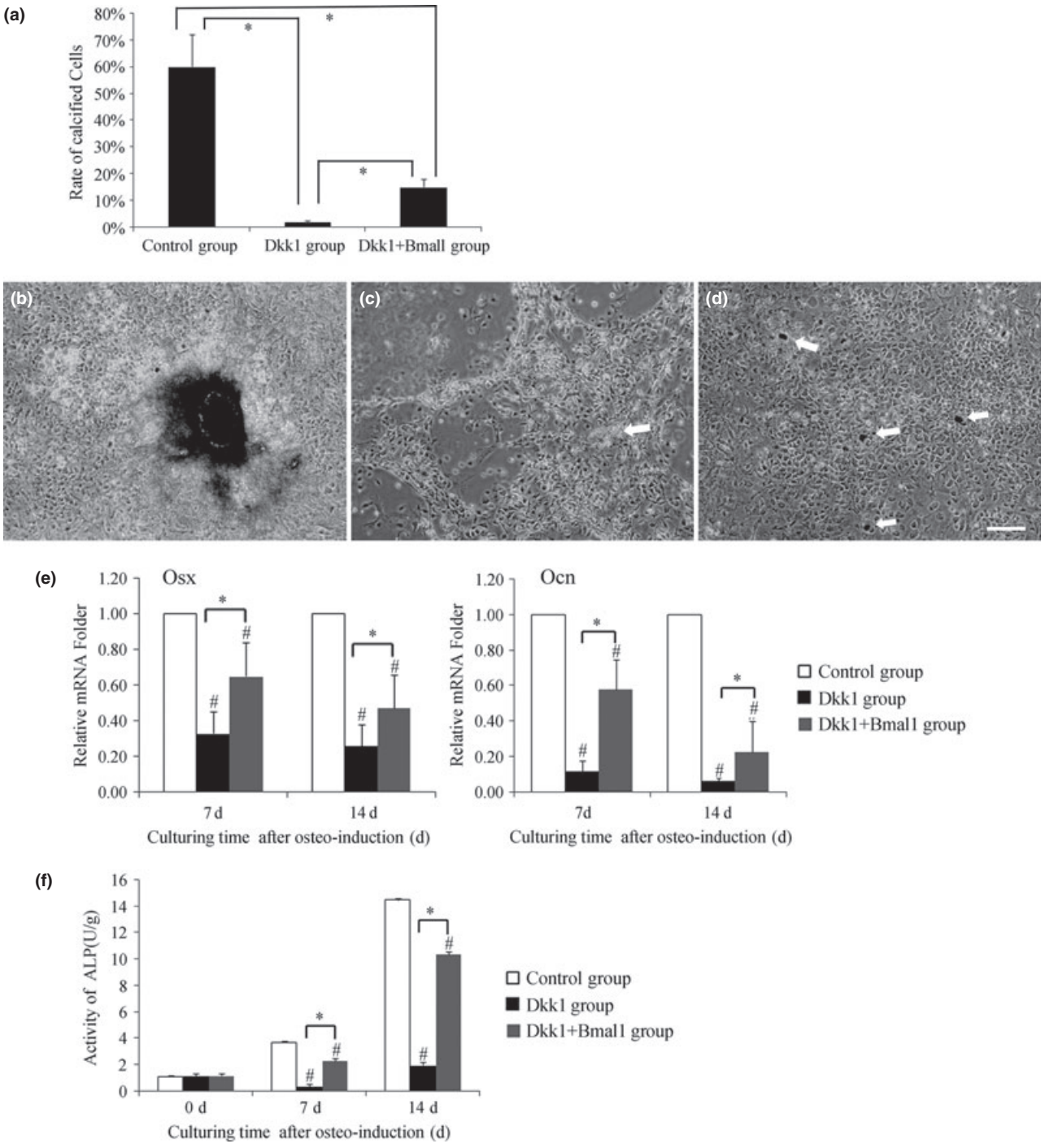

Figure 3. Effect of mesenchymal stem cells (MSCs) osteogenesis in Dkk1-conditioned osteo-induction medium after *Bmal1* transfection. (a) Level of calcified cells among the three groups. Calcified cells were counted using light microscopy from three different fields, to obtain the average. (b) The control group had clear calcifications. (c) Cell shape in the Dkk1 group slightly changed and a necrotic mass (arrow) occurred. (d) Dotted calcification (arrow) could be seen in the Dkk1 + *Bmal1* group. (b, c, d) = 100×, scale bar = 150 μm. (e) Osx and Ocn mRNA expression among the groups during osteo- induction. (f) Alkaline phosphatase (ALP) activity among the groups after 7 and 14 days induction. Data represent mean ± SD ($n = 3$), *$P < 0.05$ indicates significant difference between them; #$P < 0.05$ compared to control group.

activity was markedly suppressed in the Dkk1 group below levels of pre-induction, but increased slightly by 14 days induction. However, was still much lower than level of the control group. Comparatively speaking, the declining trend was not very remarkable in the Dkk1 + *Bmal1* group.

Transcriptional change in Bmal1 and Wnt signalling after overexpression of Bmal1 in Dkk1-conditioned medium during osteo-induction

To investigate whether Wnt signalling is related to the effect of *Bmal1* on MSC ageing, Wnt signalling was inhibited by Dkk1 to detect any changes in *Bmal1* level. Synchronously, effects of Wnt signalling were detected after overexpression of *Bmal1*, by transfection, in osteogenesis with added Dkk1.

After 7 days osteo-induction, mRNA level of Wnt 3a, β-catenin and TCF1 in the Dkk1 group decreased, and continued to decrease over 14 days osteogenesis. Interestingly, at low levels after 7 days osteo-induction, mRNA level of Bmal1 in the Dkk1 group increased by 40% ± 40% (although not statistically significant) compared to the control group after 14 days osteo-induction (Fig. 4a). On the other hand, with *Bmal1* overexpression, mRNA levels of Wnt 3a, β-catenin and TCF1 in the Dkk1 + *Bmal1* group had a certain increase compared with to the Dkk1 group under the same conditions. Among them, increased expression of β-catenin was most significant (β-catenin protein level shown in Fig. 4b,c). Bmal1 protein level exhibited a similarly changing trend to that of Bmal1 mRNA. Western blotting, after 14 days osteo-induction, exhibited a reduction in Bmal1 protein level in the Dkk1 group, although also not statistically significant. Furthermore, in Dkk1 + *Bmal1* group, increase in Bmal1 protein after 14 days osteo-induction was more obvious than after 7 days (Fig.4d,e). These data demonstrate that expression of Bmal1 changed with inhibition of Wnt signalling in osteogenesis, but the change in trend was not synchronous.

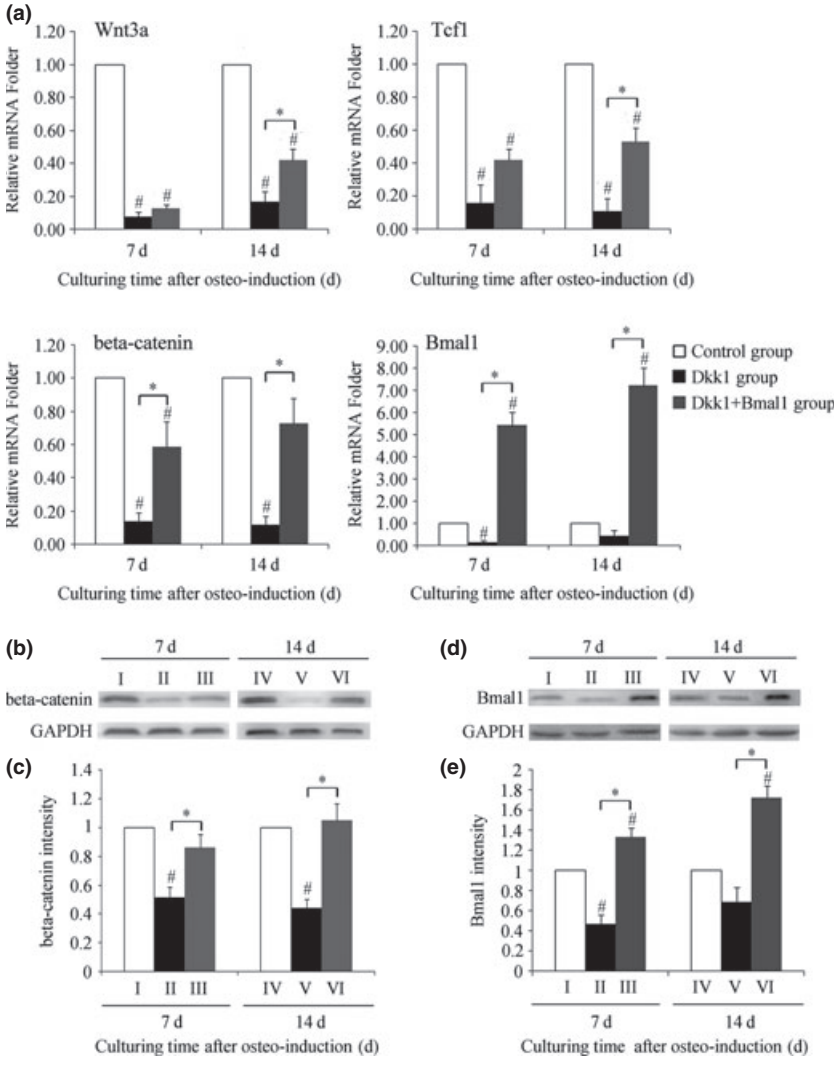

Figure 4. Transcriptional change in Wnt signalling and *Bmal1* after transfection in Dkk1-conditioned medium during osteo-induction. (a) Difference in Wnt 3a, TCF1, β-catenin and Bmal1 mRNA levels after 7 and 14 days osteo-induction among the groups. (b) Western blotting result of β-catenin protein changes among the groups during osteo-induction. (c) Quantified using densitometry by Quantity One software, ratio of β-catenin relative to GAPDH expressed as relative content of β-catenin protein. (d) Western blotting result of Bmal1 protein changes among the groups during osteo-induction. (e) Quantified using densitometry by Quantity One software, ratio of Bmal1 relative to GAPDH is expressed as relative content of Bmal1 protein. I: 7-day induction of control group, II: 7-day induction of Dkk1 group, III: 7-day induction of Dkk1 + *Bmal1* group, IV: 14-day induction of control group, V: 14-day induction of Dkk1 group, VI: 14-day induction of Dkk1 + *Bmal1* group. Data represent mean ± SD ($n = 3$), *$P < 0.05$ indicates significant difference between them; #$P < 0.05$ compared to control group.

Discussion

Preliminary studies in our laboratory have revealed that the core circadian gene *Bmal1* is possibly involved in regulation of MSC senescence with age, but its mechanism of action had not been investigated (23,25). *Bmal1* may act in MSC osteo-differentiation in two ways. First, it may affect MSC proliferation by affecting numbers of osteoblasts originating from MSCs (5,28). Second, *Bmal1* may act directly on one or several stages of osteogenesis. Both mechanisms can cause bone loss due to MSC senescence. Our preliminary results supported the first mechanism involving *Bmal1* promotion of MSC proliferation through Wnt signalling (25). On the other hand, reduced osteogenic ability of MSCs is positively related to reduction in *Bmal1* expression during ageing (23). However, no evidence regarding interaction between *Bmal1* and Wnt signalling in MSC osteo-differentiation had been previously reported.

Considering the circadian rhythm, many studies have shown that osteogenic markers ALP, Ocn and type I collagen fluctuate based on day and night, demonstrating effects of clock genes in regulation of the balance between bone formation and cell proliferation (29,30). Fu *et al.* verified that both period (Per) and cryptochrome (Cry), the negative regulating factors of *Bmal1*, had an effect on bone metabolism. When Per or cryptochrome was knocked out, their mice appeared to experience excessive bone formation and loss of osteogenic response caused by leptin activity (31). Calvaria research studies conducted by Zvonic *et al.* have shown that 26% TCFs in osteogenesis exhibit volatility between day and night. Such factors may be independent of central control factors or further control factors such as adipose tissue, and acted by multiple signals to regulate bone metabolism (32).

More evidence directly linking *Bmal1*, the core gene of circadian rhythm, and bone metabolism, have been discussed. Miyamoto *et al.* found reduced expression of bone sialoprotein and dentin matrix protein I after removing retinoic acid-related orphan receptor α (RORα), a positive regulating factor of *Bmal1*, in human MSCs, by RNA interference. This is downstream of Wnt signalling in osteogenesis, and participates in expression of bone sialoprotein and dentin matrix protein I during osteo-differentiation and bone formation (33). Thus, RORα seemed to directly combine *Bmal1* and Wnt signalling in osteo-differentiation. Hinoi *et al.* studied the cartilage cell line ATDC5, and their results demonstrated that the Bmal1/Clock dimer is linked to E-box, coded by *type II collagen* gene and plays the role of a reporting gene. Meanwhile, linkage is inhibited by transcription of *Per* gene, another important member of the clock gene family (34).Moreover, a recent study by Janich and colleagues has reported lower expression of Wnt-related genes including *Lef1* and *Wnt10a* in *Bmal1* KO mice (27). These findings further suggest the importance of clock genes and *Bmal1* in osteogenesis.

In our study, we found that after 7 days osteo-induction with Wnt signalling blocked by Dkk1, Bmal1 mRNA and protein levels fell significantly. However, after 14 days osteo-induction, no significant difference was found compared to the control group. These result showed that *Bmal1* participated at this particular stage of osteo-induction; however, the mechanism remained unclear. Considering variation of Wnt signalling, specially *β*-catenin, in these processes, some evidence may be considered. A recent study by ChIP analysis on the interaction between *Bmal1* and Wnt signalling in adipogenesis, demonstrated that *β-catenin* is the target gene of *Bmal1* (35). Thus, reduction in *β-catenin*, as the target gene of *Bmal1*, may have a negative feedback effect such as RORα (positive regulating factor of *Bmal1*), which has a slight promotional effect on *Bmal1* expression at lower protein levels. Moreover, with low expression of *Bmal1*, target genes, including *β-catenin*, had lower mRNA and protein levels. The intrinsic mechanism may be attributed to initial downregulation of β-catenin protein level by Dkk1, which eventually changes β-catenin mRNA level. Hence, overexpression of *Bmal1* in the Dkk1 + *Bmal1* group, and expression of β-catenin, both at mRNA and protein levels, increased as expected. These results indicate that *Bmal1* most likely had a synergistic effect on Wnt signalling during MSC osteogenesis, specially after 7 days osteo-induction, critical time of *Bmal1* activity in MSC proliferation and differentiation. Considering that ALP expression with circadian rhythm activity had the same changing trend with *Bmal1*, *ALP* could be the target gene of *Bmal1* just as the case with *β-catenin*. Further studies need to be performed to test this hypothesis.

Results here have shown comprehensive inhibition of Dkk1 on Wnt signalling from ligand Wnt3a to nuclear transcription factor TCF1. The mechanism of Dkk1-mediated suppression of Wnt signalling by blocking LRP5/6 binding Wnt ligands has been verified (36). As the downstream target gene of Wnt signalling, it is easy to understand reduced expression of TCF1 mRNA in the Dkk1 group. That *Wnt3a* is the target gene of Wnt signalling has been suggested, but needs to be tested (37).The result of Wnt3a mRNA being lower in the Dkk1 group may provide evidence of this.

The ability for MSC to undergo osteogenesis falls with increasing age (23). Given that the first stage of osteoblastic differentiation *in vitro* is proliferation, we initially tested effects of a physiological dose of Dkk1

on proliferation and senescence. No difference was found during early passages. This finding may be caused by reduction in concentration of Dkk1 in the culture medium due to its short half life. Subsequently, this may allow Wnt signalling to recover to its normal state and cause normal levels of cell population growth and proliferation. Meanwhile, studies on proliferation of hMSCs *in vitro* have demonstrated that at early passages, as cells begin to proliferate rapidly, high levels of Dkk1 are secreted (19). This finding indicates that Dkk1 is a promoter of proliferation at early passages. In this respect, effects of Dkk1 on MSC proliferation in our experiment *in vitro* was probably reasonable.

No significant enhancement of MSC osteogenesis has been shown with *Bmal1* overexpression. This might due to the good osteogenic capability of passage 2 cells and poorer transfection effect of MSCs, compared to other cells and cell lines (38). In contrast, effects of Dkk1 protein on osteogenesis *in vitro* were clear, MSCs did not form calcium nodules, and expression of osteo-related factors fell greatly. Meanwhile, enhanced expression of *Bmal1* rescued decreasing trend of osteogenesis in the Dkk1 group to some extent, and activity of Wnt signalling (expression of Wnt 3a, β-catenin and TCF1) in Dkk1 + *Bmal1* group slightly increased, indicating that *Bmal1* might be positively regulated in osteogenesis, by Wnt signalling. Considering that under normal circumstances ageing progresses along with reducing osteogenetic ability, these results demonstrate the positive role of *Bmal1* in preventing ageing during MSC differentiation. However, inhibition of Wnt signalling by Dkk1 was not reversed completely by *Bmal1* transfection. This result reveals that effects of osteogenic inhibition with a physiological dose of Dkk1 was more powerful. For mechanisms of bone ageing, efforts to explore interactions between clock genes and signalling of development may yield more positive results. Moreover, molecular regulation of osteogenesis in MSCs may provide us with better understanding of bone ageing and may guide development of novel treatments for osteoporosis.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:
Fig. S1. Overexpression of *Bmal1* in MSCs.
Fig. S2. Effect of MSC osteogenesis after *Bmal1* transfection.