Cent. Eur. J. Biol. • 6(6) • 2011 • 963-972
DOI: 10.2478/s11535-011-0079-8

VERSITA

Central European Journal of **Biology**

Osteogenic potency of stem cell-based genetic engineering targeting Wnt3a and Wnt9a

Research Article

Weerachai Singhatanadgit*, Manatsanan Varodomrujiranon

Faculty of Dentistry,
Thammasat University,
12121 Pathum-Thani, Thailand

Received 04 May 2011; Accepted 30 August 2011

Abstract: Bone engineering is a promising therapeutic approach to correct skeletal defects, and genetically-modified stem cells have been implicated in engineering new bone. However, the use of genetically-modified human mesenchymal stem cells targeting an osteogenic growth factor Wnt is not yet investigated. In the present study, a proliferation assay and the alkaline phosphatase (ALP) activity and expression of runt-related transcription factor 2 (Runx2) and osteocalcin (OC) transcripts were investigated to examine the effect of Wnt2 overexpression, Wnt3a overexpression, and Wnt9a knockdown on cell proliferation and osteoblast differentiation of bone marrow-derived mesenchymal stem cells (BMSCs). The results showed that the expression of Wnt2 and Wnt3a was up-regulated throughout the osteoblast differentiation period of BMSCs, whereas that of Wnt9a was down-regulated. Overexpression of Wnt3a stimulated cell proliferation while knockdown of Wnt9a increased the ALP activity and the expression of Runx2 and OC. Double transfection producing Wnt3a overexpression and Wnt9a knockdown simultaneously resulted in up-regulation of osteoblast differentiation markers, *i.e.*, the ALP activity and the Runx2 expression. In conclusion, simultaneous genetic modification of Wnt3a overexpression and Wnt9a knockdown enhances osteoblast differentiation of BMSCs, suggesting its osteogenic potency to regenerate new bone *in vivo*.

Keywords: *Bone engineering • Genetic modification • Osteogenic potency • Stem cells • Wnt3a • Wnt9a*

1. Introduction

Bone engineering, currently considered as a promising therapeutic approach to correct skeletal defects, includes the use of three key components including stem cells, scaffolds, and signaling molecules such as growth factors [1,2]. Recently, genetic engineering technology has been implicated in stem cell-based bone engineering. During this process, genetically-modified stem cells are used to produce a sustained increase in osteogenic cytokines and growth factors [3], thus subsequently stimulating osteoblast differentiation of the implanted stem cells and the recipient stem cells [3,4]. For example, overexpression of bone morphogenetic protein-2 (BMP-2) in mesenchymal stem cells has been reported to increase new bone formation in bone defects [4]. In addition, genetic engineering of a number of other growth factor genes, such as BMP-4 and -7 [3], and of transcription factors, such as runt-related transcription factor 2 (Runx2) and osterix (Osx) [5-8], have also been shown to induce osteoblast differentiation of progenitor cells.

Wnts (mammalian homologue of *Drosophila* wingless) family of growth factors have been shown to play an important role in growth and development of various cell types [9,10], including those of mesenchymal stem cells [11]. It has also been reported that Wnts regulate the differentiation of stem cells in to muscle cells [12], nerve cells [13,14] and chondocytes [15,16], further suggesting that Wnts are involved in multilineage differentiation of stem cells. Wnt3a, Wnt5a and Wnt10b have been shown to stimulate osteoblast differentiation of stem cells by suppression of the adipogenic differentiation [17-19]. Moreover, it has been demonstrated that Wnt3a induces the ALP activity, a key marker of early osteoblast differentiation [17,20]. Recently, Wnt4 has been shown to enhance osteoblast differentiation of mesenchymal stem cells both *in vitro* and *in vivo* [21]. However, given that Wnt4 was expressed at the late stage of osteoblast differentiation

* *E-mail: s-wrch@tu.ac.th*

of mesenchymal stem cells, it would suggest that the osteogenic role occurs during the late stage of this process [21]. It has also been demonstrated that the expression of Wnt9a mRNA transcripts was down-regulated during the osteoblast differentiation of stem cells [22].

Although several lines of evidence suggest the osteogenic activity of Wnts, little is known about the role of genetically-modified human mesenchymal stem cells targeting Wnt genes in osteogenesis. The present study was therefore carried out to examine the effect of Wnt2 overexpression, Wnt3a overexpression and Wnt9a knockdown on cell proliferation and osteoblast differentiation of human bone marrow-derived mesenchymal stem cells (BMSCs). We have shown, for the first time, that double genetically-modified bone engineering using simultaneous Wnt3a overexpression and Wnt9a knockdown significantly facilitates osteoblast differentiation of BMSCs.

2. Experimental Procedures

2.1 Cell culture

Human bone marrow-derived mesenchymal stem cells (BMSCs; Lonza Biologics plc, Cambridge, UK) were cultured in α-Modified Eagle's Medium (α-MEM) (Gibco Life Technologies Ltd, Paisley, UK) containing 15% fetal calf serum (FCS) (PAA Laboratories, Yeovil, UK) (15% FCS-MEM) supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine (all from Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. BMSCs between passages 3 and 5 were used in the present study.

2.2 Osteoblast differentiation of BMSCs

For osteogenic differentiation, cells were plated ($2x10^4$ cells/cm^2) in 6-well plates and incubated for 48 h, at which time osteogenic media (OM) was added (10% FCS-MEM and osteogenic supplements containing dexamethasone, ascorbate-phosphate, and β-glycerolphosphate) and the cultures re-incubated for up to 14 days.

2.3 Knockdown of Wnt9a using small interfering RNA (siRNA)

BMSCs were transfected with either plasmid DNA or siRNA using a reverse transfection method with some modifications, as previously reported [23]. Briefly, on the day of transfection, exponentially-growing bone cells were harvested by trypsinization with trypsin-EDTA for 5 min at 37°C and resuspended in MEM without FCS and antibiotic supplements at $5x10^5$ cells/ml. For each reaction, transfection complexes were prepared in a final volume of 100 ml of FCS/antibiotics-free α-MEM by mixing 4 ml of metafectene (2 mg/ml; Invitrogen) with 10 ml of a 'scramble' siRNA, which has limited sequence similarity to known genes, or with Wnt9a siRNA (10 mM; Insight Biotechnology, Wembley, UK). After incubating at room temperature for 30 min, 100 ml of the transfection complexes were gently mixed with 900 ml of cell suspension, prepared as described above, and then placed into 6-well plates. The cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in air. One milliliter of α-MEM containing 20% FCS and 2x antibiotic supplements were then added to each well and the cells incubated for another 24 h prior to assay. Cells transfected with scramble siRNA were used as a control. In preparation for cell transfection and subsequent ALP activity assay of the transfected cells, a 96-well plate was used, and the number of cells and the amount of reagents adjusted accordingly, in accordance with the manufacturer's recommendations.

2.4 Overexpression of Wnt2 and Wnt3a

Plasmid DNA for Wnt2 and Wnt3a were obtained using the method described previously [24]. Briefly, total RNA was isolated from BMSCs and cDNA was synthesized, as described above. The Wnt2 and Wnt3a were amplified using specific primers, as follows:

Wnt2 forward 5'
ACAGGATCCATGGCCCCACTCGGATA 3'
reverse 5'
CTGCTCGAGCTTGCAGGTGTGCAGGTC 3'
Wnt3a forward 5'
ACAGGATCCATGAACGCCCCTCTCGGT 3'
reverse 5'
CTGCTCGAGTGTAGCGGTTGTCCAGTC 3'

PCR products of Wnt2 and Wnt3a were cut and ligated into pcDNA3.1 mammalian expression vector, and then transformed into JM 109 and DH5α competent cells. The plasmids were purified and sequenced, and the Wnt2 plasmid, Wnt3a plasmid, and control plasmid transfected into BMSCs using reverse transfection as described previously [23,24]. Cells transfected with control plasmid were used as a control. In preparation for cell transfection and subsequently performing the ALP activity assay of the transfected cells, a 96-well plate was used, and the number of cells and the amount of reagents adjusted accordingly, in accordance with the manufacturer's recommendations.

2.5 Western blotting (WB)

Samples were first washed once with PBS and lysed in ice cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl,

1 mM EDTA, 1 mM phenylmethyl sulphonyl fluoride (PMSF), and 1 mg/ml of each of aprotinin, leupeptin, pepstatin (all from Sigma). Protein concentration was measured using the Biorad DC protein assay kit (Biorad, Hemel Hempstead, UK). The samples (50-150 mg) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12-20% acrylamide gels for 2 h at 100 V and 30 mA, then transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfermembrane, Sigma) and run for 45 min at 100 V and 200 mA. The membranes were placed in 100% methanol for 10 s to remove water and then blocked with 5% bovine serum albumin (BSA) in PBS (blocking buffer) for 1 h at room temperature. Immunoblotting was carried out by incubating the membranes overnight at 4°C with the following primary antibodies diluted 1:500 in blocking buffer: anti-Wnt2, anti-Wnt3a, anti-Wnt9a, and anti-a-tubulin (all from Insight Biotechnology, Wembley, UK). The membranes were washed three times with 1% Tween-20 in PBS, and the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako), diluted 1:2500 in blocking buffer, were added for 2 h. After three washes with 1% Tween-20 in PBS, the immunoreactive bands were visualized using the enhanced chemiluminescence ECL Plus system (Amersham Biosciences, Little Chalfont, UK), with Lumigen PS-3 Acridan as substrate.

2.6 Quantitative real-time PCR (Q-PCR)

Total RNA was isolated and first strand cDNA synthesized from 1 mg RNA. The first strand cDNA were used for amplifications performed with specific primers for Wnt2, Wnt3a, Wnt9a, Runx2, and osteocalcin (OC) (Applied Biosystems, Cheshire, UK). Primer sequences were designed with the Primer Express® program from Applied Biosystems. For Q-PCR analysis in an iQ5 iCycler (Bio-RAd, Bradford, UK), the TaqMan® Gene Expression Assays with the Assays-on-Demand™ Gene Expression products were used. TaqMan PCR reaction mixtures were set up in accordance with the manufacturer's recommendations. Briefly, a 5 ml aliquot of cDNA was used in a final volume of 25 ml reaction mixture containing 12.5 ml of 2x TaqMan® Universal PCR Master Mix (P/N 4304437), 1.25 ml of 20x Assays-on-Demand™ Gene Expression Assay mix, and 6.25 ml of nuclease-free water (all from Applied Biosystems). Thermal cycler conditions consisted of AmpErase® UNG activation at 50°C for 2 min, AmpliTaq Gold® DNA polymerase activation at 95°C for 10 min and 40 PCR cycles, each of which was 95°C for 15 s and 60°C for 60 s. All PCR reactions were performed in triplicate and each of the signals was normalized to the GAPDH signal in the same reaction. The data were analyzed using the iQ™5 optical system Software (Bio-Rad). The data are expressed as the mean ± SD obtained from the triplicate experiments at each indicated time point (defined as 100% in the control culture).

2.7 Proliferation assay

Transfected cells were cultured at a density of $5x10^3$ cells/well in 96 well culture plates for 0, 1, and 3 days. Cell proliferation was determined by the MTT assay by incubation of cells with 0.2% MTT solution at 37°C for 4 h. The reaction was then stopped by dimethylsulfoxide (DMSO) and glycine buffer. The end product color was then analyzed by measuring absorbance at 490 nm (A_{490}) which corresponds to the viability of cells. The data are expressed as the percent cell viability compared with that in the control sample at each indicated time point, defined as 100%.

2.8 Alkaline phosphatase (ALP) activity assay

Transfected cells were cultured in OM in a 96 well culture plate for up to 3 days, and the ALP activity assay was performed as previously described [23]. Briefly, the ALP activity of the cell layer was measured in triplicate wells by rinsing twice with PBS and then incubating the cells with 200 ml of 5 mM *p*-nitrophenyl phosphate (p-NPP) in 50 mM glycine, 1 mM $MgCl_2$, and 150 mM 2-amino-2-methlyl-1-propanol buffer with pH 10.5 at 37°C for 1 h. A 50 ml aliquot of 3 M NaOH was added to stop the enzymatic reaction, and the amount of *p*-nitrophenol (p-NP) produced, corresponding to the ALP activity, was measured at A_{405}. The data are expressed as the percent ALP activity compared with that in the control sample at each indicated time point, defined as 100%.

2.9 Statistical analysis

The data are presented as the mean percent ± SD of measurements from three independent experiments, with the experiments being performed in triplicate at least. Statistical differences between the mean of the test groups and the control group were analyzed by single sample *t*-test, with $P<0.05$ considered significant. The *t*-test program in the SPSS 11.0 software (SPSS, Chicago, IL) was used for the analyses.

3. Results

3.1 Expression of Wnt2, WNt3a and Wnt9a mRNA transcripts during osteoblast differentiation of BMSCs

The quantitative real-time PCR results in Figure 1 demonstrate that throughout the course of osteoblast

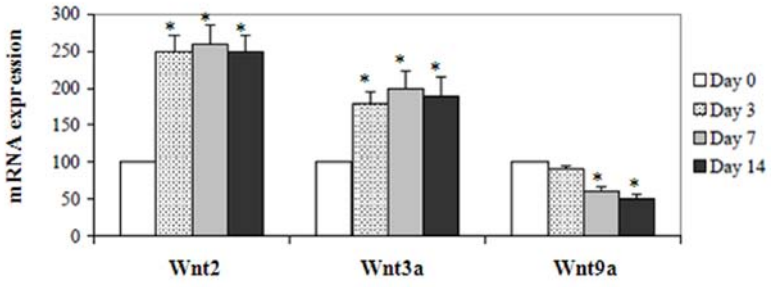

Figure 1. Expression of Wnt2, 3a and 9a transcripts during osteoblast differentiation of BMSCs. Cells were cultured in osteogenic media for 0, 3, 7 and 14 days. Total RNA was subsequently extracted and the expression of Wnt2, 3a and 9a mRNA was determined by quantitative real-time PCR, as described in the Experimental Procedures. The results are presented as the mean ± SD of the expression level in the sample at day 0 (control culture), defined as 100% (N=6). Similar results were observed in three independent experiments. * $P<0.05$ *vs.* control culture.

differentiation of BMSCs in the present study, the expression of Wnt2 and Wnt3a transcripts was increasingly up-regulated, approximately 2.5- and 2-fold, respectively, from day 3 to day 14 when compared with that on day 0. The results also showed that although no significant change in the expression of Wnt9a gene was observed on day 3, the level of Wnt9a expression on day 7 and day 14 was markedly down-regulated to approximately 50% of that observed on day 0.

3.2 Expression of Wnt2, Wnt3a and Wnt9a in genetically-modified BMSCs

The expression patterns of these factors during osteoblast differentiation (as shown in Figure 1) suggested that this process could be enhanced by Wnt2 and Wnt3a, and be inhibited by Wnt9a. To test this possibility, we first performed gain-of-function and loss-of-function experiments by using plasmid-based overexpression of Wnt2 and Wnt3a, and siRNA-mediated knockdown of Wnt9a, respectively. The transfection efficiencies of siRNAs and plasmids assessed by flow cytometry were found to be approximately 80% and 60%, respectively (data not shown). BMSCs were transfected with Wnt2 plasmid, Wnt3a plasmid, and Wnt9a siRNA, and the expression of these antigens was determined using WB as described in the Experimental Procedures. The results showed that 3 days post-transfection, the expression of Wnt2 and Wnt3a proteins increased more than 2-fold compared with that in the control group, whereas the protein level of Wnt9a was approximately 25% of that observed in the control group (Figure 2)

3.3 Effect of Wnt2 overexpression, Wnt3a overexpression and Wnt9a knockdown on BMSC proliferation

The results in Figure 3 show that transfection of BMSCs with Wnt3a plasmid significantly enhanced proliferation of BMSCs at 1 and 3 days post-transfection (by

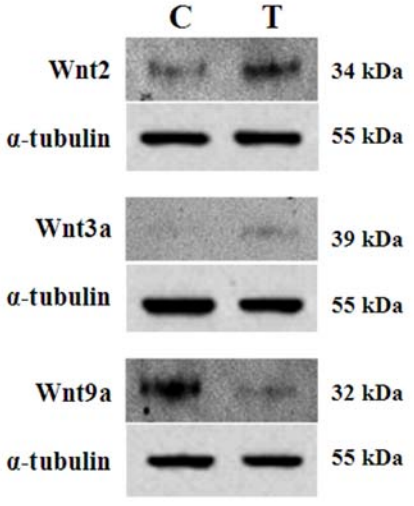

Figure 2. Expression of Wnt2, 3a and 9a proteins in transfected BMSCs. Cells were transfected with control plasmid/siRNA (C) or Wnt2 plasmid Wnt3a plasmid and Wnt9a siRNA (T) for 3 days. Total protein was subsequently extracted and the expression of Wnt2, 3a and 9a was determined by western blot, as described in the Experimental Procedures. α-Tubulin was used as an internal control. Similar results were observed in two independent experiments.

approximately 2-2.5 fold) when compared with that of the control. In contrast, transfection with either Wnt2 plasmid or Wnt9a siRNA had little, if any, effect on BMSC proliferation (Figure 3). The results suggest the stimulatory effect of Wnt3a on cell proliferation of BMSCs.

3.4 Effect of Wnt2 overexpression, Wnt3a overexpression and Wnt9a knockdown on osteoblast differentiation of BMSCs

In the present study, osteoblast differentiation of BMSCs was determined by measuring the ALP activity and the expression of key osteoblastic markers Runx2 and OC. The results showed that 3 days post-transfection Wnt9a

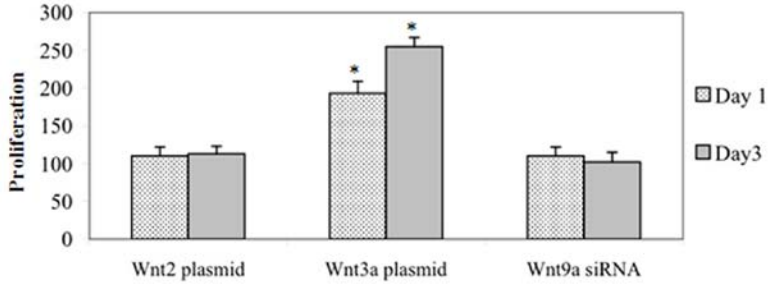

Figure 3. Effect of Wnt2 overexpression, Wnt3a overexpression and Wnt9a knockdown on proliferation of BMSCs. Cells were transfected with Wnt2 plasmid Wnt3a plasmid and Wnt9a siRNA for 3 days, and the cell proliferation was measured as described in the Experimental Procedures. The results are presented as the mean percentage ± SD of that obtained from the triplicate experiment at each indicated time point (defined as 100% in the control culture). Similar results were observed in three independent experiments. * $P<0.05$ *vs.* control culture.

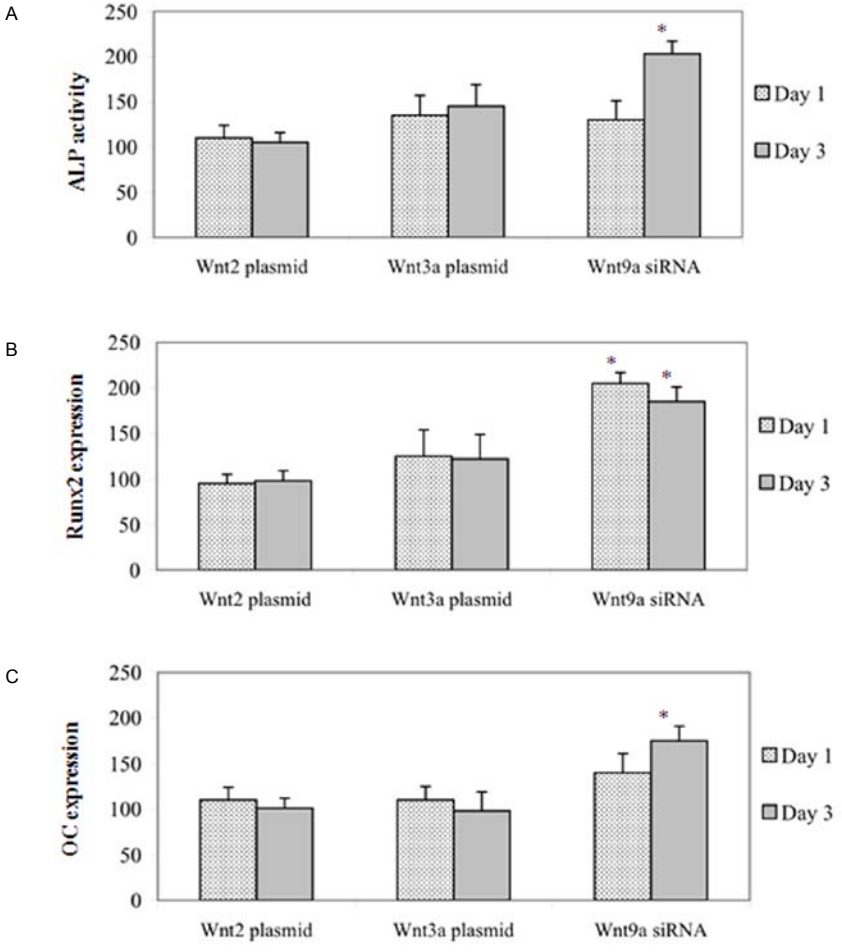

Figure 4. Effect of Wnt2 overexpression, Wnt3a overexpression and Wnt9a knockdown on osteoblast differentiation of BMSCs. Cells were transfected with Wnt2 plasmid Wnt3a plasmid and Wnt9a siRNA for 0, 1 and 3 days, and the ALP activity (A) and the expression of Runx2 mRNA (B) and OC mRNA (C) was examined as described in the Experimental Procedures. The results are presented as the mean ± SD of that obtained from the triplicate experiment at each indicated time point (defined as 100% in the control culture). Similar results were observed in three independent experiments. * $P<0.05$ *vs.* control culture.

siRNA, but not Wnt2 plasmid, significantly stimulated the ALP activity (Figure 4A) and the expression of Runx2 and OC transcripts (Figure 4B and 4C, respectively) compared with the corresponding controls. Although the stimulatory effect of Wnt3a overexpression on osteoblast differentiation was not statistically significant, it seemed

to up-regulate these osteoblast-associated markers. The results thus suggested that Wnt3a overexpression and Wnt9a knockdown may cooperatively enhance osteoblast differentiation of BMSCs.

3.5 Effect of double target genetic modification by Wnt3a overexpression and Wnt9a knockdown on osteoblast differentiation of BMSCs

To examine the combined role of simultaneous Wnt3a overexpression and Wnt9a knockdown in osteoblast differentiation of BMSCs, cells were transfected with Wnt3a plasmid and Wnt9a siRNA simultaneously, and osteoblast differentiation was assessed as mentioned previously. The results showed that the present genetic engineering targeting Wnt3a and Wnt9a significantly increased the ALP activity and the expression of Runx2 in BMSCs by more than 250% of that in the control cells, although it had little effect on the OC expression (Figure 5).

4. Discussion

Genetic engineering of a number of osteogenic transcription factors and growth factors has been used to stimulate cell proliferation and osteoblast differentiation of mesenchymal stem cells [1,2]. Genetically-engineered pluripotent mesenchymal cells expressing transcription factor genes such as Runx2 and Osx [5-8], and growth factor genes such as BMP-2, -4 and -7 [3,4], showed an increase in their osteogenic activity, thus facilitating both *in vitro* and *in vivo* new bone formation. In addition to these osteogenic factors, Wnt signaling has recently been shown to induce *in vitro* mesenchymal cell proliferation and differentiation [25,26], and to accelerate *in vivo* bone regeneration [27-29]. However, the use of stem cell-based genetic engineering by targeting Wnts in bone engineering is not yet well established. The present study examined the osteogenic activity of Wnts using genetic modification of Wnt genes in BMSCs. We showed that during osteoblast differentiation of BMSCs, the expression of Wnt2 and Wnt3a was significantly up-regulated, whereas that of Wnt9a was down-regulated, suggesting a potential osteogenesis-associated role of these growth factors. This prompted us to examine the osteogenic role of stem cell-based genetic engineering targeting these growth factors by forced overexpression of Wnt2 and Wnt3a, and by knockdown of Wnt9a in BMSCs. The results have shown that simultaneously combined Wnt3a overexpression and Wnt9a knockdown significantly stimulated early osteoblast differentiation of BMSCs, presumably through up-regulation of the Runx2 expression and the ALP activity.

In the present study, the expression of Wnt2 was up-regulated while BMSCs differentiated along with the osteoblastic lineage, suggesting its potential role in this process. In addition, it has previously been reported that Wnt2 plays an important role in the differentiation of embryonic stem cells [30]. However, the present results revealed little, if any, effect of Wnt2 on the cell proliferation and the level of the ALP activity and Runx2 and OC expression, all of which are key markers of osteoblast differentiation. This suggests that Wnt2 may not be necessary in the differentiation of BMSCs along with the osteoblastic lineage. It is also possible that in contrast to embryonic stem cells, BMSCs were not responsive to Wnt2 stimulation, possibly due to the lack of sufficient and appropriate receptors and intracellular signaling molecules specific to Wnt2 in BMSCs. Further studies are required to examine these hypotheses.

Unlike Wnt2 overexpression, the results in the present study demonstrated that the increased Wnt3a level significantly stimulated proliferation of BMSCs, as has previously been reported [17,20,31,32]. Moreover,

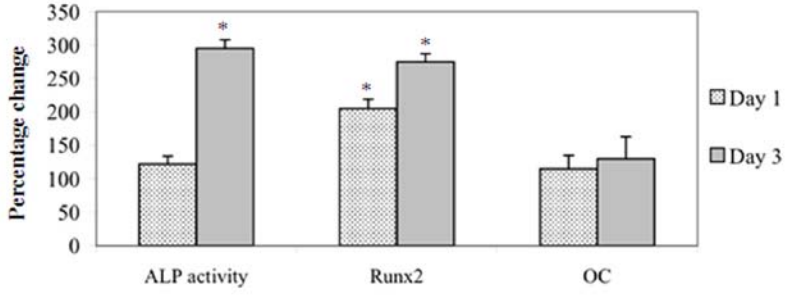

Figure 5. Effect of combined Wnt3a overexpression and Wnt9a knockdown on the ALP activity and on the expression of Runx2 and OC mRNA in BMSCs. Cells were simultaneously transfected with Wnt3a plasmid and Wnt9a siRNA for 0, 1 and 3 days, and the ALP activity and the expression of Runx2 and OC mRNA were measured, as described in the Experimental Procedures. The results are presented as the mean ± SD of that obtained from the triplicate experiment at each indicated time point (defined as 100% in the control culture). Similar results were observed in three independent experiments. * $P<0.05$ *vs.* control culture.

Wnt3a overexpression appeared to increase osteoblast differentiation, although its effect was not statistically significant in the present study. It is noteworthy that the effect of this growth factor on osteoblast differentiation remains controversial. Although most of previous studies have reported its inhibitory effect [22,33], there are a few reports describing its stimulatory effect [17,20], as also observed in the present study. The conflicting results could possibly be due to differences in the state of differentiation of the cells used, species of the cells, methods used to activate Wnt3a signaling and Wnt3a concentration, as previously reported [34]. It is thus important to further examine the detailed mechanism of the Wnt3a signaling pathway. Our unpublished data suggest that recombinant human Wnt3a (rhWnt3a) significantly stimulates BMSC proliferation, and hence up-regulation of recombinant Wnt3a signaling during the early stage of osteoblast development (day 0-7 in osteogenic culture), resulting in enhanced osteogenesis in BMSCs used in the present study (data not shown). This is consistent with the findings of Baksh and Tuan [25] which showed the stimulatory effect of Wnt3a on cell proliferation and thus increasing the pool of cells capable of differentiating along with osteoblastic lineage. It is therefore likely that in the experimental settings used in the present study, Wnt3a overexpression and rhWnt3a showed similar but not identical biological activity, at least in terms of cell proliferation and early osteoblast differentiation of BMSCs.

The present results also demonstrated that suppression of Wnt9a expression had little effect on BMSC proliferation and that Wnt9a siRNA-transfected BMSCs had a significantly greater level of the ALP activity and expression of Runx2 and OC mRNA (key early and late osteoblastic differentiation markers, respectively) compared with that of the corresponding control. Although a previous report has shown that Wnt9a knockdown stimulates cell proliferation of human breast cancer cells [35], the results of the present study showed little, if any, of this effect on BMSC. One possibility causing such conflicting findings is that the BMSCs used in the present study and the cancer cells could respond to Wnt9a differently, possibly due to their differences in receptors and intracellular signaling molecules specific for Wnt9a.

Double transfection of Wnt3a plasmid and Wnt9a siRNA resulted in a significant increase in the ALP activity and the expression of Runx2, suggesting its stimulatory role in early osteoblast differentiation. These findings suggest that Wnt3a and Wnt9a could act as potential novel therapeutic targets for re-building bone, as has recently been reported for the synergism between Wnt3a and other osteogenic factors, such as BMP and heparin [36,37]. Moreover, a previous study showed that canonical Wnt signaling stimulated the expression of Runx2 and OC in a SFRP1-null mouse (secreted frizzled-related protein-1), which exhibited activated Wnt signaling and a high bone mass phenotype [38]. It is possible that a number of signals, not only the Wnt signal, were positively and negatively affected in the SFRP1-null mouse, and thus synergistically stimulated the expression of the osteogenic genes. This supports the results of the present study, which demonstrated that Runx2 expression was up-regulated in combined Wnt3a overexpression and Wnt9a knockdown.

In the present study, although Wnt9a knockdown significantly up-regulated the expression of the key marker of late osteoblast differentiation OC, simultaneous Wnt3a overexpression and Wnt9a knockdown had only little, if any, effect on this marker. It has been shown that while Wnt3a plays a stimulatory role in early osteoblast differentiation, it may suppress the late stage of osteoblast differentiation of BMSCs [17,22,39]. Such effects of Wnt3a could possibly attenuate the Wnt9a knockdown-mediated OC up-regulation in BMSCs, suggesting that in order to achieve a maximal stimulation of osteoblast differentiation, Wnt3a overexpression may be required only in the early stage of the differentiation process, while a sustained Wnt9a knockdown is needed throughout the osteoblast differentiation of BMSCs used in stem cell-based bone engineering. It is noteworthy that due to the limitation of transient transfection carried out in the present study, long term effects of these genetically modified cultures targeting Wnt3a and Wnt9a could not be assessed. Further studies on genetic modification using a stable transfection approach are required to monitor long term effects of these growth factors on osteoblast differentiation of BMSCs.

The results of the present study demonstrated that Wnt3a and Wnt9a significantly affect osteoblast differentiation of BMSCs, although signal transduction initiated by these growth factors has also been shown to influence chondrogenic differentiation [40-42]. It is possible that a growth factor may activate a number of different signaling proteins and transcription factors required in both osteogenic and chondrogenic pathways, such as β-catenin, mitogen-activated protein kinases (MAPKs), Smads and Runx2 [21,43,44], and thus contribute to transcriptional events associated with osteoblastic and chondrogenic differentiation.

Treatment of several pathologic bone conditions such as fractures, cancer and congenital disorders has been problematic since the defects are difficult to heal [3]. This requires effective regeneration of new bone. A stem cell-based genetic engineering approach has previously been suggested to be promising for

inducing rapid and sustained osteogenesis [3,45]. In the present study, we have genetically manipulated BMSCs to undergo osteoblast differentiation, especially at the early stage, by targeting Wnt3a and Wnt9a simultaneously, as shown by stimulatory effects on the expression of two key markers of early osteoblast differentiation, Runx2 and ALP activity. Consequently, this likely led to an initiation and a further increase in the extent of mineralized nodule formation *in vitro*, thus promoting mineralized matrix formation. The results in the present study suggest the osteogenic potential of this stem cell-based genetic engineering targeting the Wnt genes on bone healing *in vivo*. This strategy may overcome the disadvantages associated with the use of the gold standard autologous bone graft method such as donor-site morbidity, post-operative bleeding, infection, and chronic pain [46,47].

In conclusion, the present study has introduced the use of double gene modification by overexpressing Wnt3a and knocking down Wnt9a simultaneously for enhancing osteoblast differentiation of BMSCs. For therapeutic applications, this promising approach may be possible to regenerate new bone in defective skeletal bones caused by congenital defects, trauma and tumors.

Acknowledgements

Financial support for the present study was received from Thammasat University, Thailand.

References

[1] Langer R., Vacanti J.P., Tissue engineering, Science, 1993, 260, 920-926
[2] Bianco P., Robey P.G., Stem cells in tissue engineering, Nature, 2001, 414, 118-121
[3] Kimelman N., Pelled G., Helm G.A., Huard J., Schwarz E.M., Gazit D., Review: gene- and stem cell-based therapeutics for bone regeneration and repair, Tissue Eng, 2007, 13, 1135-1150
[4] Gazit D., Turgeman G., Kelley P., Wang E., Jalenak M., Zilberman Y., et al., Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy, J Gene Med, 1999, 1, 121-133
[5] Tai G., Polak J.M., Bishop A.E., Christodoulou I., Buttery L.D., Differentiation of osteoblasts from murine embryonic stem cells by overexpression of the transcriptional factor osterix, Tissue Eng, 2004, 10, 1456-1466
[6] Tu Q., Valverde P., Li S., Zhang J., Yang P., Chen J., Osterix overexpression in mesenchymal stem cells stimulates healing of critical-sized defects in murine calvarial bone, Tissue Eng, 2007, 13, 2431-2440
[7] Wu L., Wu Y., Lin Y., Jing W., Nie X., Qiao J., et al., Osteogenic differentiation of adipose derived stem cells promoted by overexpression of osterix, Mol Cell Biochem, 2007, 301, 83-92
[8] Zhang X., Yang M., Lin L., Chen P., Ma K.T., Zhou C.Y., et al., Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose--derived stem cells in vitro and in vivo, Calcif Tissue Int, 2006, 79, 169-178
[9] Gordon M.D., Nusse R., Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors, J Biol Chem, 2006, 281, 22429-22433
[10] Wodarz A., Nusse R., Mechanisms of Wnt signaling in development, Annu Rev Cell Dev Biol, 1998, 14, 59-88
[11] Etheridge S.L., Spencer G.J., Heath D.J., Genever P.G., Expression profiling and functional analysis of wnt signaling mechanisms in mesenchymal stem cells, Stem Cells, 2004, 22, 849-860
[12] Belema B.F., Technau A., Ebelt H., Schulze M., Braun T., Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells, Mol Cell Biol, 2005, 25, 9509-9519
[13] Lange C., Mix E., Rateitschak K., Rolfs A., Wnt signal pathways and neural stem cell differentiation, Neurodegener Dis, 2006, 3, 76-86
[14] Peters S., Mix E., Bauer P., Weinelt S., Schubert B., Knoblich R., et al., Wnt-5a expression in the rat neuronal progenitor cell line ST14A, Exp Brain Res, 2004, 158, 189-195
[15] Andrade A.C., Nilsson O., Barnes K.M., Baron J., Wnt gene expression in the post-natal growth plate: regulation with chondrocyte differentiation, Bone, 2007. 40, 1361-1369
[16] Nishioka K., Dennis J.E., Gao J., Goldberg V.M., Caplan A.I., Sustained Wnt protein expression in chondral constructs from mesenchymal stem cells, J Cell Physiol, 2005, 203, 6-14
[17] de Boer J., Wang H.J., Van Blitterswijk C., Effects of Wnt signaling on proliferation and differentiation of human mesenchymal stem cells, Tissue Eng, 2004, 10, 393-401
[18] Kang S., Bajnok L., Longo K.A., Petersen R.K., Hansen J.B., Kristiansen K., et al., Effects of Wnt signaling on brown adipocyte differentiation and metabolism mediated by PGC-1alpha, Mol Cell Biol, 2005, 25, 1272-1282

[19] Takada I., Suzawa M., Matsumoto K., Kato S., Suppression of PPAR transactivation switches cell fate of bone marrow stem cells from adipocytes into osteoblasts, Ann N Y Acad Sci, 2007, 1116, 182-195

[20] Caverzasio J., Manen D., Essential role of Wnt3a-mediated activation of MAP kinase p38 for the stimulation of alkaline phosphatase activity and matrix mineralization in C3H10T1/2 mesenchymal cells, Endocrinology, 2007, 148, 5323-5330

[21] Chang J., Sonoyama W., Wang Z., Jin Q., Zhang C., Krebsbach P.H., et al., Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK, J Biol Chem, 2007, 282, 30938-30948

[22] Boland G.M., Perkins G., Hall D.J., Tuan R.S., Wnt 3a promotes proliferation and suppresses osteogenic differentiation of adult human mesenchymal stem cells, J Cell Biochem, 2004, 93, 1210-1230

[23] Singhatanadgit W., Salih V., Olsen I., Up-regulation of bone morphogenetic protein receptor IB by growth factors enhances BMP-2-induced human bone cell functions, J Cell Physiol, 2006, 209, 912-922

[24] Parisuthiman D., Mochida Y., Duarte W.R., Yamauchi M., Biglycan modulates osteoblast differentiation and matrix mineralization, J Bone Miner Res, 2005, 20, 1878-1886

[25] Baksh D., Tuan R.S., Canonical and non-canonical Wnts differentially affect the development potential of primary isolate of human bone marrow mesenchymal stem cells, J Cell Physiol, 2007, 212, 817-826

[26] Si W., Kang Q., Luu H.H., Park J.K., Luo Q., Song W.X., et al., CCN1/Cyr61 is regulated by the canonical Wnt signal and plays an important role in Wnt3A-induced osteoblast differentiation of mesenchymal stem cells, Mol Cell Biol, 2006, 26, 2955-2964

[27] Qiu W., Andersen T.E., Bollerslev J., Mandrup S., Abdallah B.M., Kassem M., Patients with high bone mass phenotype exhibit enhanced osteoblast differentiation and inhibition of adipogenesis of human mesenchymal stem cells, J Bone Miner Res, 2007, 22, 1720-1731

[28] Minear S., Leucht P., Jiang J., Liu B., Zeng A., Fuerer C., et al., Wnt proteins promote bone regeneration, Sci Transl Med, 2010, 2, 29ra30

[29] Popelut A., Rousval B., Fromentin O., Feghali M., Mora F., Bouchard P., Tooth extraction decision model in periodontitis patients, Clin Oral Implants Res, 2010, 21, 80-89

[30] Wang H., Gilner J.B., Bautch V.L., Wang D.Z., Wainwright B.J., Kirby S.L., et al., Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies, J Biol Chem, 2007, 282, 782-791

[31] Nemoto E., Koshikawa Y., Kanaya S., Tsuchiya M., Tamura M., Somerman M.J., et al., Wnt signaling inhibits cementoblast differentiation and promotes proliferation, Bone, 2009, 44, 805-812

[32] Otto A., Schmidt C., Luke G., Allen S., Valasek P., Muntoni F., et al., Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration, J Cell Sci, 2008, 121, 2939-2950

[33] de Boer J., Siddappa R., Gaspar C., van Apeldoorn A., Fodde R., Van B.C., Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells, Bone, 2004, 34, 818-826

[34] Quarto N., Behr B., Longaker M.T., Opposite spectrum of activity of canonical Wnt signaling in the osteogenic context of undifferentiated and differentiated mesenchymal cells: implications for tissue engineering, Tissue Eng Part A, 2010, 16, 3185-3197

[35] Xiang Y., Lin G., Zhang Q.J., Tan Y.Q., Lu G.X., Knocking down Wnt9a mRNA levels increases cellular proliferation, Mol Biol Rep, 2008, 35, 73-79

[36] Fukuda T., Kokabu S., Ohte S., Sasanuma H., Kanomata K., Yoneyama K., et al., Canonical Wnts and BMPs cooperatively induce osteoblastic differentiation through a GSK3beta-dependent and beta-catenin-independent mechanism, Differentiation, 2010, 80, 46-52

[37] Ling L., Dombrowski C., Foong K.M., Haupt L.M., Stein G.S., Nurcombe V., et al., Synergism between Wnt3a and heparin enhances osteogenesis via a phosphoinositide 3-kinase/Akt/RUNX2 pathway, J Biol Chem, 2010, 285, 26233-26244

[38] Gaur T., Lengner C.J., Hovhannisyan H., Bhat R.A., Bodine P.V., Komm B.S., et al., Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression, J Biol Chem, 2005, 280, 33132-33140

[39] Liu G., Vijayakumar S., Grumolato L., Arroyave R., Qiao H., Akiri G., et al., Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells, J Cell Biol, 2009, 185, 67-75

[40] Reinhold M.I., Kapadia R.M., Liao Z., Naski M.C., The Wnt-inducible transcription factor Twist1 inhibits chondrogenesis, J Biol Chem, 2006, 281, 1381-1388

[41] Spater D., Hill T.P., O'sullivan R.J., Gruber M., Conner D.A., Hartmann C., Wnt9a signaling is required for joint integrity and regulation of Ihh during chondrogenesis, Development, 2006, 133, 3039-3049

[42] Surmann-Schmitt C., Widmann N., Dietz U., Saeger B., Eitzinger N., Nakamura Y., et al., Wif-1 is expressed at cartilage-mesenchyme interfaces and impedes Wnt3a-mediated inhibition of chondrogenesis, J Cell Sci, 2009, 122, 3627-3637

[43] Milat F., Ng K.W., Is Wnt signalling the final common pathway leading to bone formation?, Mol Cell Endocrinol, 2009, 310, 52-62

[44] Komori T., Signaling networks in RUNX2-dependent bone development, J Cell Biochem, 2011, 112, 750-755

[45] Gamradt S.C., Lieberman J.R., Genetic modification of stem cells to enhance bone repair, Ann Biomed Eng, 2004, 32, 136-147

[46] Silber J.S., Anderson D.G., Daffner S.D., Brislin B.T., Leland J.M., Hilibrand A.S., et al. Donor site morbidity after anterior iliac crest bone harvest for single-level anterior cervical discectomy and fusion, Spine, 2003, 28, 134-139

[47] Kneser U., Schaefer D.J., Polykandriotis E., Horch R.E., Tissue engineering of bone: the reconstructive surgeon's point of view, J Cell Mol Med, 2006, 10, 7-19