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Overexpression of Wnt7b antagonizes the inhibitory effect of dexamethasone on osteoblastogenesis of ST2 cells

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Abstract

Introduction: It is well established that glucocorticoid-induced osteoporosis is highly associated with preosteoblast differentiation and function. This study is based on the premise that Wnt7b can promote bone formation through Wnt signalling pathway because it can stimulate preosteoblast differentiation and increase its activity. However, it is unknown whether Wnt7b can rescue the inhibited osteoblast differentiation and function caused by exogenous glucocorticoid.

Material and methods: In this study we used Wnt7b overexpression ST2 cells to explore whether Wnt7bcan rescue the inhibited osteoblast differentiation and function, which can provide strong proof to investigate a new drug for curing the glucocorticoid induced osteoporosis.

Results/Conclusion: We found that Wnt7b can rescue the suppressed osteoblast differentiation and function without cell viability caused by dexamethasone. **(Endokrynol Pol 2023; 74 (1): 83–88)**

Key words: *Wnt7b; osteoblast; dexamethasone*

Introduction

Endogenous glucocorticoids have a pleiotropic effect in various physiological processes. Glucocorticoids act as life-saving treatment when insufficient endogenous glucocorticoids occur, such as in Addison's disease. However, excess glucocorticoids cause an opposing manifestation (Cushing's syndrome) compared to Addison's disease. Both Addison's disease and Cushing's syndrome show an increased risk of fracture [1]. Because glucocorticoids are widly used in the therapy of autoimmune and chronic inflammatory diseases, its side effects cannot be ignored. Glucocorticoid-induced osteoporosis (GIOP) is one of the most deleterious side effects during glucocorticoid therapy. Among the patients receiving glucocorticoid treatment, over 30% suffer an osteoporotic fracture, and over 10% develop osteonecrosis [2]. In the pathophysiology of GIOP, glucocorticoids effect both haematopoietic- and mesenchymal-derived bone cells. In the early stage of GIOP, glucocorticoids result in a transient increase of osteoclast activation and osteoclast function [3]. Meanwhile, glucocorticoids continuously reduce osteogenesis and activity of osteoblast and promote the adipogenic differentiation from mesenchymal stem cells [4]. Hence, long-term use of glucocorticoids decreases both bone mass and bone strength, which makes the bones fragile. The risk of fracture, especially hip fracture and vertebral fracture, increases with time in glucocorticoid treatment. Patients receiving glucocorticoids therapy are recommend to evaluate the fracture risk, using for example Fracture Risk Assessment (FRAX), and those with high fracture risk are encouraged to take a pharmacological anti-osteoporotic treatment [5]. However, the FRAX instrument estimates the glucocorticoid use without dose and duration. A new modified FRAX tool has been developed for patients received treated with glucocorticoids [6]. At present, oral bisphosphonates are the most commonly used because they are cost-effective and hence are recommended as first-line therapy for GIOP. The main mechanism of bisphosphonates is their function as antiresorptive drugs. Because reduction in bone formation plays a major role in long-term glucocorticoids therapy, bone anabolic agents are a preferred option. Teriparatide shows a better prognosis compared with bisphosphonates. Teriparatide has demonstrated efficacy in increasing bone mineral density at the spine and hip and reducing new vertebral fractures in patients with GIOP [7]. Due to its high cost, teriparatide can be considered for patients at very high risk of fracture. New bone anabolic agents are needed for GIOP.

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Wnt signalling is important for trabecular and cortical bone mass [8]. During osteoblast differentiation Wnt signalling promotes preosteoblasts to express osterix and therefore induce them to differentiate into mature osteoblast via both canonical and noncanonical pathways [8]. Also, Wnt signalling acts on mesenchymal progenitor cells to determine its osteogenic versus adipogenic lineage fate [9]. There are a series of extracellular proteins that can regulate Wnt signalling, including Dickkopf-related protein 1 (DKK1), secreted frizzled-related protein (SFRP), and sclerostin (and its homologue Wise) [10]. The DKK1 and sclerostin families antagonise Wnt signalling by binding to Wnt receptor LRP5/6 [12, 13]. SFRP proteins are Wnt-interfering molecules that bind to Wnt proteins directly [12, 13].

Evidence has shown that Wnt signalling is restrained in the pathogenesis of GIOP [14]. The Dkk-1 enhanced by glucocorticoids inhibits the Wnt signal in human osteoblasts, and the silenced Dkk-1 recuses the suppressed activity of human osteoblast imposed by glucocorticoids [15, 16]. In vivo, osteocyte-specific Dkk-1 deletion protects against glucocorticoid-induced bone loss [17]. Female mice lacking SOST/sclerostin have shown a high bone mass and increased bone formation with an unchanged bone resorption when exposed to glucocorticoids [18]. Therefore, Wnt signalling is a potential therapeutic target for GIOP. A previous study showed that antibody against sclerostin prevented glucocorticoid-induced bone loss in mice [19]. Nowadays, an antisclerostin antibody, romosozumab, is applied for the treatment of patients with severe postmenopausal osteoporosis [20]. However, romosozumab caused a numerical increase in serious cardiovascular adverse events in a clinical trial [20]. Therefore, new bone anabolic agents must be sought [21].

Wnt7b has been identified as an important Wnt ligand in osteoblast differentiation [22]. In vitro, Wnt7b overexpression induced the formation of bone nodules, activity of osteoblast, and osteoblast differentiation. During mice embryonic bone development, Wnt7b was expressed in perichondrial cells, and ablation of Wnt7b induced a lower ossification [23]. When exposed to a high concentration, Wnt7b was downregulated in osteoblast [24, 25]. Here, our study explores whether Wnt7b can rescue the phenotype in osteoblast induced by dexamethasone.

Material and methods

Reagents and cell culture

ST2 cells were maintained in growth medium (α-minimum essential medium [α-MEM] with 10% foetal bovine serum [FBS] and 1% penicillin and streptomycin [P/S]). To examine the effects of dexamethasone on osteoblast differentiation, ST2 cells were seeded in 12-well plates at 2 x 10^5 cells/well and cultured with growth medium. After leaving overnight, ST2 cells were treated with osteogenic medium (α-MEM containing 10% FBS, 1% P/S, 100 ng/mL Bmp2, 50 μg/ml L-ascorbic acid, and 10 mM β-glycerophosphate) in the presence of varying concentrations of dexamethasone (0, 100 nM, 1 uM, 10 uM, or 100 uM). The medium was changed every 2 days. The cells subsequently underwent alkaline phosphatase (ALP) staining at day 7 and Von Kossa staining at day 14, respectively. All cells were cultured at 37°C in a 5% CO_2 incubator with culture medium changed every 2–3 days. The α-MEM medium, P/S, and FBS were obtained from Gibco-BRL (Gaithersburg, MD, USA). We dissolved dexamethasone (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) and restored it in the dark.

Generation of Wnt7b-expressing stable ST2 cells

Before transfection, we diluted Wnt7b- or GFP- (control) virus in appropriate growth medium to achieve > 90% infection according to GFP detection. ST2 cells were infected with Wnt7b- or GFP-expressing lentivirus for 12 hours. Then the viruses were removed. After 48 hours of infection, ST2 cells were selected with puromycin, and stable ST2 cells were expanded for further use. We verify the infection by fluorescent microscopic imaging and by quantitative polymerase chain reaction (qPCR) assay of Wnt7b expression.

Cell viability assay

To evaluate the cytotoxic effects of dexamethasone, ST2 cells were seeded in 96-well plates overnight with a density of 1000 cells/well. Subsequently, the cells were treated with varying concentrations of dexamethasone (0,10 nM, 100 nM, 1 uM, 10 uM, or 100 uM) for 24, 48, and 72 hours. The cell viability was detected using the CCK-8 method (Dojindo Laboratories, Japan) as per the manufacturer's instruction. Absorbance optical density (OD) values at 450 nm were acquired by microplate reader.

ALP staining

Activity of alkaline phosphatase, an early marker of osteoblast differentiation, was evaluated using an ALP staining kit (#OB02C, BZ Biotechnology, Suzhou, Jiangsu, China) as described previously [25]. Briefly, ST2 cells were rinsed with PBS, fixed with 4% PFA, and then stained with staining mixture containing naphthol AS-MX phosphate, N, N-dimethylformamide, and fast blue BB salt for 20 minutes in dark conditions at room temperature (RT).

Von Kossa staining

Von Kossa staining was carried out to determine the mineralization of the extracellular matrix as described previously [26]. ST2 cells were fixed with cold (4°C) methanol for 20 minutes, rinsed with double-distilled water (ddH2O), and then stained with 5% silver nitrate solution under bright light for 30 minutes. Afterwards, cells were washed with ddH_2O to remove unstained solution. The mineralised areas were stained brown/black.

Results

Dexamethasone suppressed the viability of ST2 cells

We first carried out CCK-8 assays to investigate the impact of glucocorticoids on osteoblasts. We treated ST2 cells with increasing concentrations (0, 10 nM, 100 nM, 1 uM, 10 uM, or 100 uM) of dexamethasone in growth medium, and performed CCK-8 assays at 24, 48, and 72 hours after treatment. We repeated every concentration 6 times. Compared with the control group, dexamethasone showed a cytotoxic effect on ST2 cells

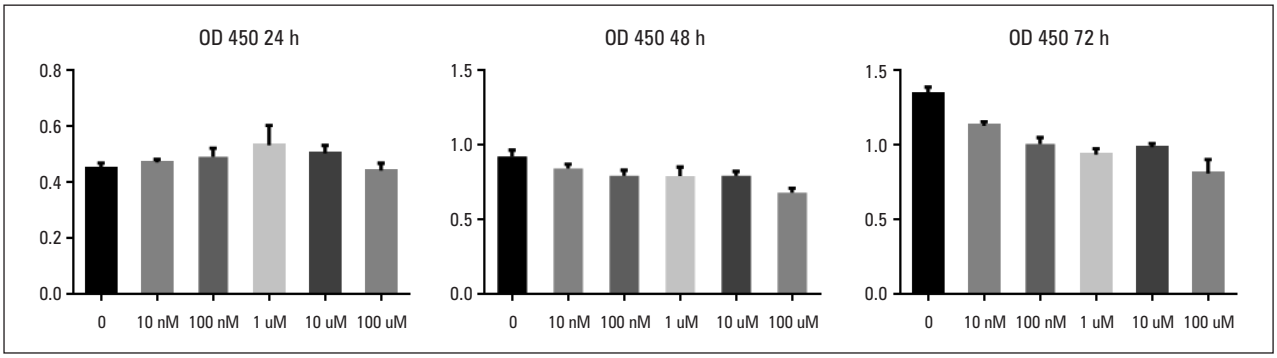

Figure 1. *Dexamethasone-suppressed cell viability of ST2 cells*

correlating with dosage and time (Fig. 1). The preliminary results suggested that dexamethasone restrains ST2 cell proliferation.

Dexamethasone suppressed osteoblast differentiation of ST2 cells

Next, we determined whether dexamethasone inhibited osteoblast differentiation of ST2 cells. To this end, ST2 cells were treated with osteogenic medium (OM) to induce osteoblast differentiation in the presence of various concentrations of dexamethasone for 7 days and 14 days. We first performed ALP staining to assess osteoblast differentiation phenotype. ST2 cells in OM without dexamethasone showed a high ALP activity, while dexamethasone suppressed ALP activity (Fig. 2A). We then assessed the capability of ST2 cells to form a mineralized extracellular matrix in conditions of dexamethasone via Von Kossa staining. As expected, OM treatment induced formation of mineralized bone nodules after osteogenic induction for 14 days. In contrast, as little as 100 nM of dexamethasone was sufficient to block matrix mineralization in ST2 cells (Fig. 2B) Thus, dexamethasone impaired cell viability, ALP activity, and mineralization of ST2 cells. To achieve the maximal effect of dexamethasone on cell viability and osteoblast differentiation, we used 100 um dexamethasone in the subsequent studies.

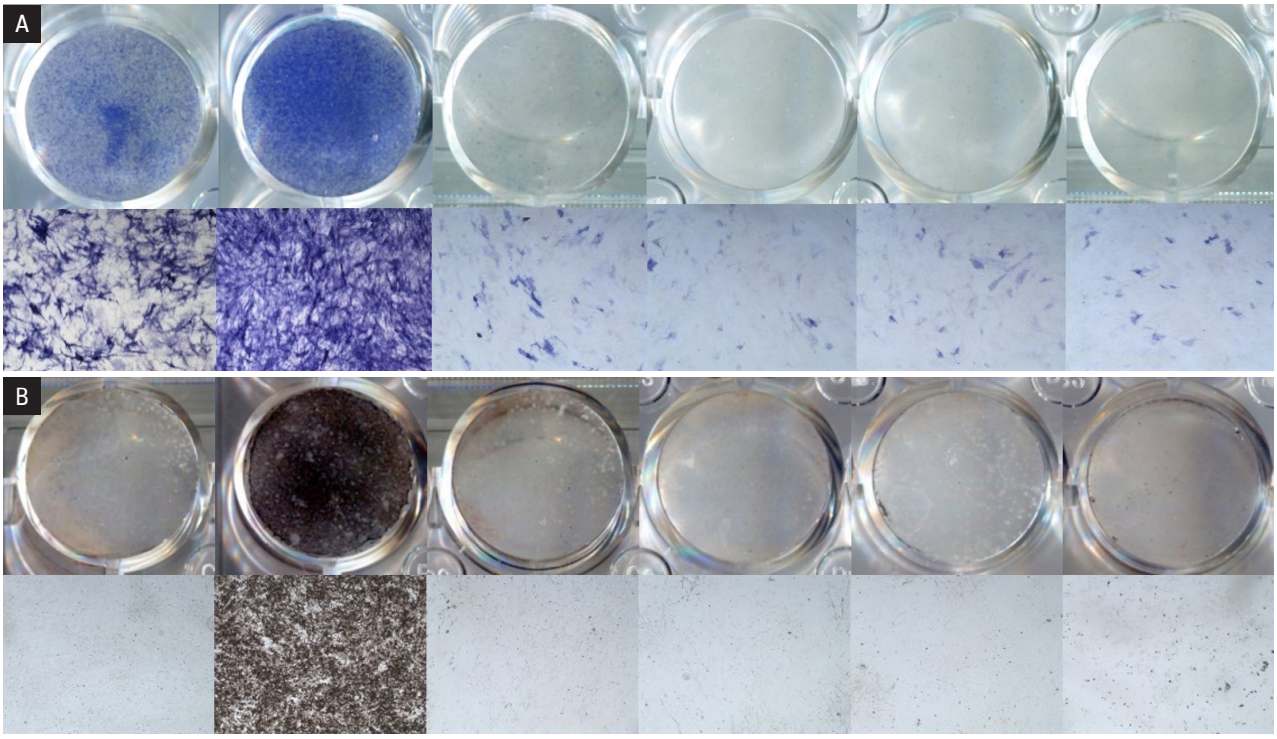

Figure 2. *Dexamethasone (Dex)-suppressed osteogenesis of ST2 cells.* **A**. *Dex-suppressed ALP activity in ST2 cells; Dex-blocked matrix mineralization in ST2 cells*

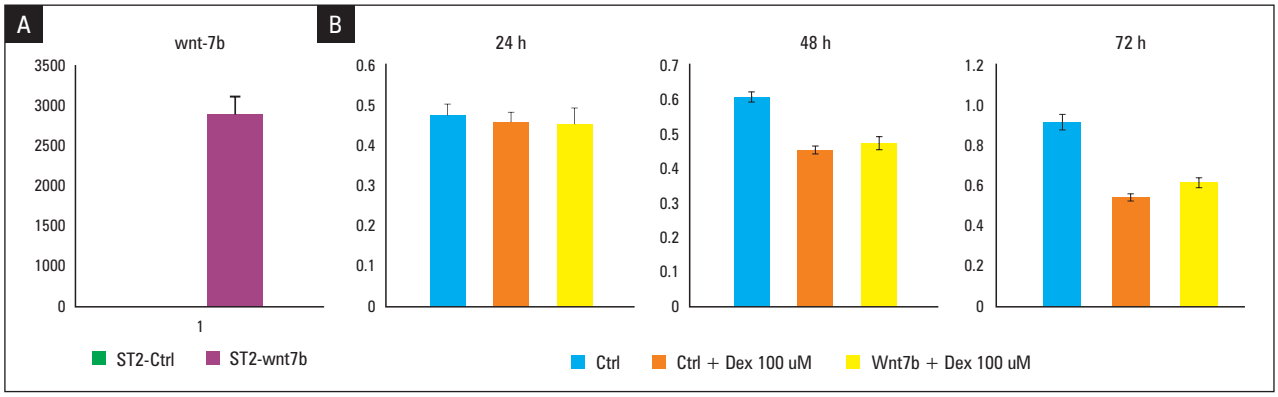

Figure 3. *Overexpressing Wnt7b did not rescue the inhibitory effect of dexamethasone (Dex) on ST2 cell.* **A.** *ST2 expressing Wnt7b verified by quantitative polymerase chain reaction (qPCR);* **B.** *Overexpressing Wnt7b did not alter the inhibitory effect of Dex on ST2 cell viability*

Overexpressing Wnt7b did not affect the impact of dexamethasone on ST2 viability

To investigate whether Wnt7b rescues detrimental effects caused by dexamethasone, we established ST2 cells that stably overexpressed Wnt7b (Fig. 3A). qPCR analysis confirmed that expression of Wnt7b was remarkably increased in Wnt7b-expressing ST2 cells, compared with control cells. After confirming the overexpression of Wnt7b in ST2 cells, we then selected 100 um dexamethasone as the control concentration. CCK-8 assays in different times suggested that upregulated Wnt7b did not save the decreased cell viability (Fig. 3B).

Overexpressing Wnt7b attenuates the inhibitory effect of dexamethasone on osteoblast differentiation

To test the effect of Wnt7b overexpression on dexamethasone-induced inhibition of osteoblast differentiation, we repeated ALP staining and Von Kossa staining at the same time point. With the condition of 100 um dexamethasone, mineralized bone nodules and ALP activity in Wnt7b-overexpressed ST2 cells were dramatically increased compared with ST2 cells (Fig. 4). Meanwhile, Wnt7b-overexpressed ST2 cells in OM with dexamethasone showed high ALP activity and excellent mineralization, which was similar to ST2 cells in OM (Fig. 4). Hence, Wnt7b can rescue the suppressed osteoblast differentiation and bone matrix mineralization without cell viability in ST2cells.

Discussion

In this study, we have demonstrated that dexamethasone impaired the cell viability and osteogenic ability in ST2 cells. Dexamethasone restrained the cell viability in a time- and dose-dependent manner. Overexpression of Wnt7b in the ST2 cells reversed suppression of osteogenesis caused by dexamethasone without affecting cell viability.

During osteogenesis, Wnt7b expression levels were elevated at the early stage of osteogenesis and degraded at the late stage of osteogenesis [26]. Sclerostin and DKK-2 were expressed at late stages of osteoblast differentiation, consistent with the expression of osteocalcin and following the expression of Wnt7b [27, 28]. At the cellular level, Wnt7b enhances proliferation and osteoblast differentiation, and maintains the number of mesenchymal stem cells [29]. A study showed that Wnt7b was inversely correlated with age in human bone marrow stromal cells [30]. Wnt7b was upregulated, induced by mechanical loading in young adult mice; it was not responsive in old mice [31]. In aged mice, inducible expression of Wnt7b facilitates bone formation and leads to a significantly increase in bone mass [32]. These data suggested that target of Wnt7b to aged osteoporosis may be explored as a potential therapy. Although Wnt signalling pathway participates in pathogenesis of GIOP, studies about the function of Wnt7b in GIOP are scarce.

Wnt7b has been identified as an anabolic regulator in bone formation. In vitro, Wnt7b promotes osteoblast differentiation and increases bone nodules in mineralization medium [32]. In vivo, Wnt7b significantly enhances both the number and function of osteoblasts and improves bone mass [33]. Among the 19 Wnt proteins, Wnt7b functions in Wnt non-canonical signalling pathway. In a previous study, Wnt7b was shown to stimulate bone formation by activating mTORC1 through PI3K-AKT signalling [33]. Because Wnt7b also activates PKCδ via phosphoinositide signalling, PKCδ activation may facilitate in Wnt7b-induced osteoblastogenesis [23, 33]. Wnt7b not only stimulates osteoblast differentiation but also maintains the progenitor cell pool by activating the Ca^{2+}-Nfatc1 pathway

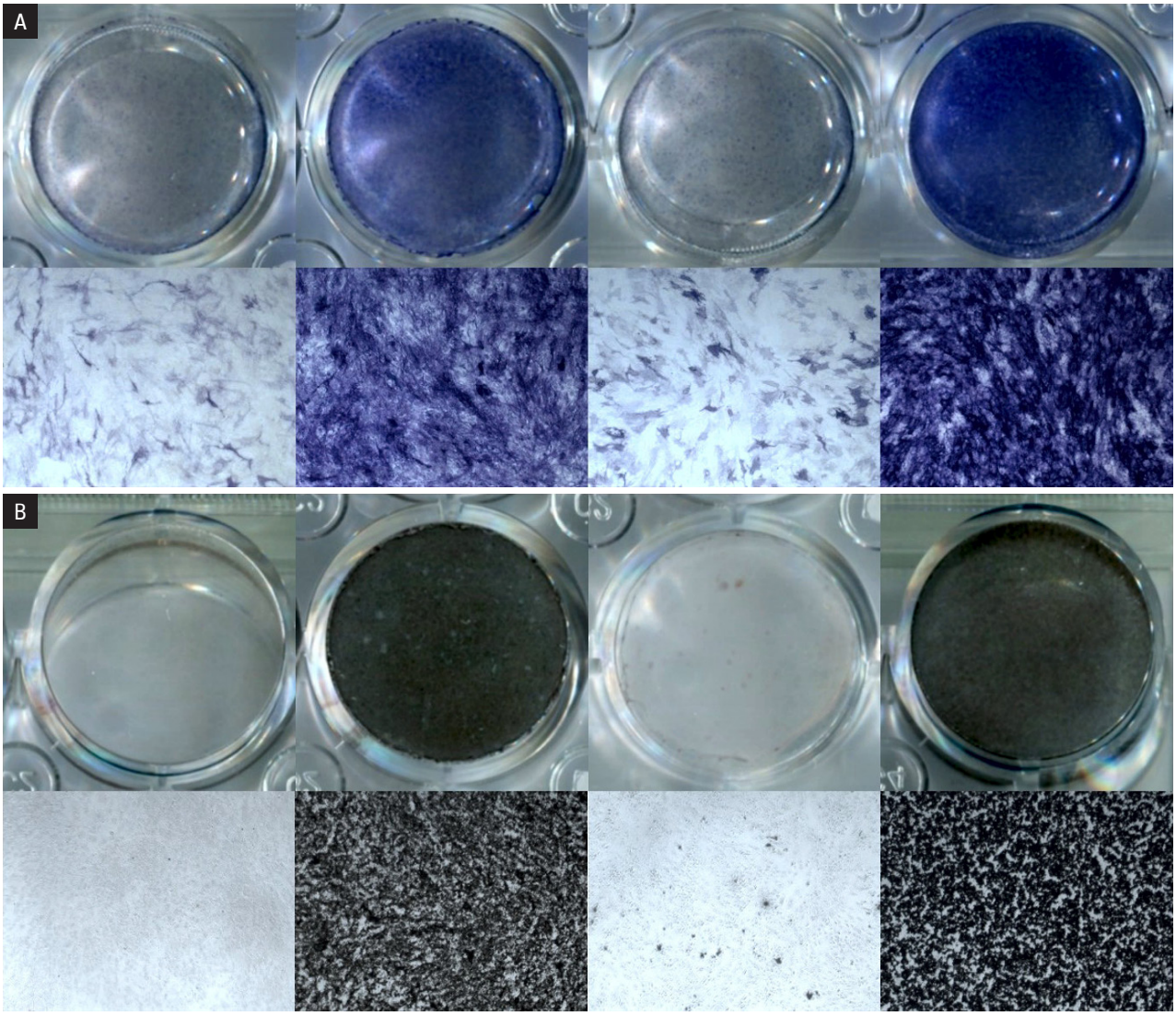

Figure 4. *Overexpressing Wnt7b rescues the suppression of dexamethasone.* **A.** *Overexpressing Wnt7b rescued the inhibitory effect of alkaline phosphatase (ALP) activity in ST2 cells;* **B.** *Overexpressing Wnt7b rescued the inhibitory effect of matrix mineralization in ST2 cells*

[29]. Although it may not add to the high-bone-mass phenotype, Wnt7b appeared to suppress osteoclast numbers in in vivo experiences [23]. Moreover, glucose metabolism has been shown to be essential in supporting Wnt7b-induced bone formation [34]. Based on this evidence, Wnt7b acts by multiple non-canonical signalling pathway to promote bone formation. Further explorations are needed to demonstrate the function mechanism of Wnt7b in GIOP.

A recent study has shown that inducible Wnt7b overexpression in the osteoblast lineage reverses bone loss after chronic glucocorticoid treatment in mice [35]. Wnt7b rescues both osteoblast number and activity in the study. However, in our study, we used a CCK-8 kit to test cell viability after 24, 48, and 96 hours. Wnt7b overexpression did not alter the cell viability. In previous study, Wnt7b was induced in vivo for 2 weeks. We cannot figure out that Wnt7b did not function in a short time, it may have a slow effect in vitro. And then, we carried out ALP staining and Von Kossa staining at days 7 and 14. We found that Wnt7b rescue the ALP activity and mineralized nodule, which was accompany with the result reported in previous study.

Conclusions

We can conclude that Wnt7b reverses the osteogenesis both in vivo and in vitro. Therefore, Wnt7b shows a potent bone anabolic effect in glucocorticoid-induced osteogenesis inhibition and may be used as a therapy for GIOP.

Funding

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