Thyroid-stimulating hormone decreases the risk of osteoporosis by regulating osteoblast proliferation and differentiation

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Research article

Keywords: osteoporosis, TSH, BMD, osteoblast

DOI: https://doi.org/10.21203/rs.3.rs-47941/v1

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Abstract

Background

As the incidence of secretory osteoporosis increases, bone loss and osteoporosis and their relationships with thyroid-stimulating hormone (TSH) have received increased attention. In this study, the role of TSH in bone metabolism and the underlying possible mechanisms were investigated.

Methods

We analyzed serum triiodothyronine (FT3), tetraiodothyronine (FT4), TSH and bone mineral density (BMD) levels of 114 men with normal thyroid function. In addition, osteoblasts from rat calvarial samples were treated with different doses of TSH for different times at each time point. The related gene and protein expression levels were investigated.

Results

Comparing the BMD between the high-level and low-level serum TSH group showed that TSH serum concentrations were positively correlated with BMD. TSH at concentrations of 10 mU/mL and 100 mU/mL significantly increased the mRNA levels of ALP, COI1 and Runx2 compared with those of the control (P < 0.05, P < 0.01). BMP2 activity was enhanced both with increased TSH concentration and with increased time. The protein levels of Runx2 and osterix were increased in a dose-dependent manner.

Conclusions

The circulating concentrations of TSH and BMD were positively correlated with normal thyroid function in males. TSH promoted osteoblast proliferation and differentiation in rat primary osteoblasts.

Background

Osteoporosis, the seventh most common disease in the world, has become a serious public health problem due to the increased life expectancy and an aging society. This condition may lead to broken bones and substantially affects an individual’s physical and mental health and quality of life. In recent years, bone loss and osteoporosis and their relationships with thyroid-stimulating hormone (TSH) and thyroid-stimulating hormone receptor (TSHR) have received increasing attention due to the increasing incidence of secretory osteoporosis.

Homeostasis requires a balance between bone resorption and bone formation. Osteoblasts and osteoclasts continue to reshape the bone [1–3], unless the balance is disrupted, leading to osteoporosis and osteoporosis [4].
Hyperthyroidism is known to be associated with significant bone loss [5]. The expression of the TSHR mRNA and protein in osteoblasts and osteoclasts has been identified [6–9]. However, TSHRa and TSHRb were not detected in primary osteoblasts and osteoclasts [7], indicating that TSH does not exert paracrine effect on these cells [10]. Our initial histomorphological experiments using mice showed significant decreases in the trabecular bone volume, bone-like surface, bone-like thickness, and osteoblastic surface in Tshr/- and Tshr+/- mice compared with Tshr+/+ mice, while the osteoclast surface was significantly increased. Furthermore, TSH inhibited osteoclast differentiation in vitro, which was manifested as decreased numbers of tartrate-resistant acid phosphatase (TRAP) -positive cells, and the levels of differentiation markers, including tartaric acid-resistant acid phosphatase, matrix metalloproteinase-9 and proteinase K, were decreased in RAW264.7 cells [4]. Our results confirm that TSH increases bone volume, improves the bone microstructure, and improves bone strength, at least in part, by inhibiting osteoclast formation.

The effect of TSH on osteoblasts is controversial. The model system used by Baliram includes mouse embryonic stem cells (ES), which are induced to form mature mineralized osteoblasts. TSH promotes osteoblast differentiation mainly by activating protein kinase C and up-regulating the atypical Wnt signaling pathway intermediates Fri4 and Wnt5a [11]. As shown in the study by Sampath et al., TSH stimulates osteoblast differentiation and function [8]. However, Tsai et al. previously only observed low levels of TSHR expression, TSH binding and cAMP activation in human osteoblasts, and postulated that TSH was unlikely to play a physiological role in osteoblasts [12]. In contrast, TSH inhibits osteoblast differentiation and type I collagen expression in a Runx2- and Osterix-independent manner by reducing the activity of the Wnt and VEGF signaling pathways. TSH inhibits osteoblasts and reduces the expression of type I collagen, bone sialoprotein and osteocalcin. Inhibition of the expression of the LDL receptor-associated protein 5 (LRP5) mRNA suggests that these effects are mediated by the Wnt signaling pathway [6].

Therefore, although TSHR is expressed in osteoblasts, recent studies have produced contradictory results, suggesting that TSH may enhance, inhibit or have no effect on osteoblast differentiation and function [10].

In the present study, 114 Chinese men with normal thyroid function were investigated in a cross-sectional study, and their serum TSH, free triiodothyronine (FT3), and free thyroxine (FT4) levels and bone mineral density (BMD) were measured. In addition, the effects of TSH on the proliferation and differentiation of osteoblasts were analyzed during the protogenesis of rat osteoblasts.

**Methods**

**Subjects** The study population was collected by the Jinan health organization between October and November 2009. 731 men were recruited. The exclusion criteria included participants with chronic diseases, hepatic disease, renal disease, thyroid diseases or other endocrine diseases, taking medications, such as glucocorticoids, thyroid hormone, bisphophonate, calcitonin, calcium or active...
vitamin D analogs. In total, 114 men were enrolled in the present analysis. All consent forms were completed. The study was approved by the Ethics Committee of Shandong Provincial Hospital.

**Sample collection** A questionnaire was administered to each participant and included general information, such as name, ethnicity, date of birth, address, identification card number, educational status, profession, family income, telephone number, status of medical insurance, involvement in sports, dietary intake, history of smoking and drinking, and family history and personal history of diseases and their treatments. Height and body weight were measured to calculate body mass index (BMI) using the following formula: BMI = body weight (kg)/height (m$^2$). Fasting blood samples were drawn for serum TSH, FT3 and FT4 analyses.

**Analysis of blood samples** Serum TSH, FT3 and FT4 concentrations were determined with electrochemiluminescence immunoassays (CobasE601, Roche, Basel, Switzerland) in the Clinical Laboratory of Shandong Provincial Hospital affiliated with Shandong University. The normal reference range of TSH was 0.55–4.78 mU/L; however, the normal reference ranges of FT3 and FT4 were 3.5–6.5 pmol/L and 11.5–22.7 pmol/L, respectively.

**Bone mineral density measurement** BMD was measured at the low-median region of the left forearm, which is not dominant in most Chinese people, by a trained technologist using a dual-energy X-ray absorptiometry (DXA) fan-beam bone densitometer (Japan Osteosys Co., Ltd., EXA-3000). Based on the World Health Organization (WHO) diagnostic criteria for osteoporosis, osteoporosis was defined as a BMD T-score < 2.5, whereas a BMD T-score ≥-1 was considered normal. BMD T-scores between −2.5 and −1 were defined as osteopenia[13].

**Primary culture of osteoblasts** Osteoblastic cells were isolated from neonatal rat pup calvaria using sequential digestion with slight modifications. Briefly, calvaria were dissected from eight neonatal (1–3 d old) rat pups. After removal of sutures and adherent mesenchymal tissues, calvaria were subjected to 30 min digestions at 37 °C in a water bath at 200 rpm containing 0.25% trypsin. Sediments were collected for the second digestion in a tube containing 0.1% collagenase type II at 37 °C in a water bath at 200 rpm. Cells were extracted from the supernatant and washed with PBS twice, and they were resuspended in DMEM/F12 containing 10% FBS with 1% penicillin/streptomycin and 1% glutamine solution and transferred into culture dishes. The dishes were incubated at 37 °C with 5% CO$_2$ in an incubator.

**Osteoblast phenotype and function identification** The third generation of osteoblasts were cultured in 12-well culture plates at a density of 5*104/mL. We observed the typical morphological characteristics of osteoblasts with osteoblast HE staining. Alkaline phosphatase (ALP) staining was performed using an ALP kit (GENMED, China) according to the manufacturer's protocol. ALP-positive cells containing three or more nuclei were defined as osteoblasts. Calvaria-derived osteoblasts were seeded at a density of 5*104/mL and cultured for 16 d in DMEM/F12 supplemented with 10% FBS, 1% penicillin/streptomycin and 1% glutamine. Mineralization of osteoblasts was ascertained by Alizarin red (Solarbio, China)
staining. For Alizarin red staining, cells were similarly plated and fixed and stained with 2% Alizarin red for 5 min, washed with PBS several times, and finally allowed to air dry at room temperature. Finally, the cells were observed with a microscope.

**Osteoblast growth curve** Osteoblasts of the P3 generation with good growth were used to generate single cell suspensions, and the cell concentration was adjusted to 2.5*10^4/mL. Then, the cells were inoculated in 96-well plates at 200 µL per hole. After the inoculation, six cell samples were selected for the MTT colorimetric test every 24 h for 7 d. The specific procedure was as follows: 20 µL of 5 mg/mL MTT solution was added to each well and further incubated at 37 ºC. After 4 h, the medium was discarded, and 150 µL of dimethyl sulfoxide (DMSO) was added to solubilize the dark blue formazan crystals at 37 ºC for 10 min. Absorbance was recorded at 490 nm with an automatic enzyme standard instrument. The growth curve was generated with time as the X-axis and the OD value as the Y-axis.

**Cell proliferation** The proliferative effect of TSH was determined by the MTT assay. Calvarial osteoblasts were suspended in DMEM/F12 medium and plated at a density of 2.5*10^4 cells/well in a 96-well plate for incubation until the cells reached near confluence. Cells were serum-starved for 2 h, after which TSH was added to the appropriate wells at different concentrations (0, 1, 10, 100 mU/mL) in triplicate for different times (12, 24, 48 h). After the exposure period, 10 µL of 5 mg/mL MTT solution was added to each well and further incubated at 37 ºC. After 4 h, the medium was discarded, and 100 µL of DMSO was added to solubilize the dark blue formazan crystals at 37 ºC for 10 min. Absorbance was recorded at 490 nm with an automatic enzyme standard instrument (Labsystems, Finland).

**Quantitative real-time PCR** Total RNA was extracted using TRIzol (TaKaRa Biotechnology, China) according to the manufacturer's instructions. cDNA was synthesized from 500 ng of total RNA using ReverTraAce reverse transcriptase (TaKaRa Biotechnology, China) and oligo dT primers (TaKaRa Biotechnology, China). The PCR primers were as follows:

- **Alp**, Forward (F) 5’-CATCGCCTATCAGCTAATGCACA-3’,
- Reverse (R) 5’-ATGAGGTCCAGGCCATCCAG-3’,
- **BMP2**, F 5’-ACCGTGCTCAGCTTCCATCAC-3’,
- R 5’-CTATTTCCAAAGCTTCCATTTT-3’,
- **COL1**, F 5’-GACATGTTCAGCTTTGTGGACCTC-3’,
- R 5’-AGGGACCCTTAGGCCATTGTGTA-3’,
- **OSX**, F 5’-CACCCATTGCCAGTAATCTTCGT-3’,
- R 5’-GGACTGGAGCCATAGTGAGCTTCT-3’,
- **Runx2**, F 5’-CATGGCCGGGAATGATGAG-3’,
R 5’-TGTGAAGACCGTTATGGTCAAAGTG-3’,
BGP, F 5’-GGACCCTCTCTCTGCTCACTCT-3’,
R 5’-CTTACTGCCCTCCTGCTTGG-3’,
beta-actin, F 5’-ACCCAGATCATGTTTGAGAC-3’,
R 5’-GTCAGGATCTTACATGAGGTAGT-3’.

Real-time PCR was performed on a LightCycler 480 (Roche Diagnostics, Germany) with an initial denaturation step of 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 20 s at an annealing temperature of 60 °C. We used the comparative Ct method to calculate mRNA expression levels. We normalized the Ct values of the samples of interest against the Ct values of β-actin.

**Western blot analyses** The cellular protein was extracted in lysis buffer containing RIPA and 1 mM PMSF. The protein was measured using a BCA kit (Sheneng Bocai Biological Technology, China): 50 µg of protein was electrophoresed in 10% SDS-polyacrylamide gel under reducing conditions and electrotransferred to nitrocellulose membranes (Millipore). After the membranes were blocked in 5% BSA, they were incubated with anti-runx2 (1:600), anti-osterix (1:100) (Santa Cruz Biotechnology, USA), and anti-actin at a dilution of 1:3,000 and then probed with anti-rabbit or anti-mouse secondary antibodies (ZSGB-Bio, China). The images were detected with an imaging instrument.

**Statistical analysis**

All data are expressed as the mean ± SD. Differences between groups were assessed by one-way ANOVA, and the mean comparisons were performed by the SNK test using SPSS17.0 software. P < 0.05 was considered statistically significant.

**Results**

**Subject characteristics**

The 114 men were divided into two groups based on the mean of TSH. The low-level serum TSH group (TSH ≤ 1.65 mU/L) included 39 participants, and the high-level serum TSH group (TSH > 1.65 mU/L) included 75 participants. Table 1 presents the subject characteristics.
Table 1
Characteristics of the subjects in the two groups.

|                  | N  | Age(y)     | Height(cm) | Weight(kg) | BMI(kg/m²) |
|------------------|----|------------|------------|------------|------------|
| low-level serum TSH group | 39 | 44.46 ± 17.0 | 170.02 ± 6.45 | 68.48 ± 7.84 | 23.66 ± 2.11 |
| high-level serum TSH group | 75 | 40.81 ± 16.3 | 171.66 ± 6.50 | 70.19 ± 9.66 | 23.42 ± 2.91 |

The values are expressed as the mean ± SD.

TSH: thyroid-stimulating hormone, BMI: body mass index

**Interrelationships between serum TSH and BMD**

The serum FT3 and FT4 levels between the two groups did not show significant changes as the serum TSH concentration increased. Forearm BMD increased in the high-level serum TSH group compared with the low-level serum TSH group (P < 0.05) (Table 2).

Table 2
The forearm BMD, serum FT3 levels, and FT4 levels in the two groups.

|                  | N   | FT3(pmol/L)   | FT4(pmol/L)   | BMD(g/cm²) |
|------------------|-----|---------------|---------------|------------|
| low-level serum TSH group | 39  | 5.6 ± 0.47    | 17.26 ± 2.0   | 0.48 ± 0.08 |
| high-level serum TSH group | 75  | 5.53 ± 0.47   | 17.28 ± 2.0   | 0.51 ± 0.06 |

p value  0.632  0.818  0.018

The values are expressed as the mean ± SD.

FT3: free triiodothyronine, FT4: free tetraiodothyronine, BMD: bone mineral density

**Cell morphological identification of osteoblasts**

HE staining was observed using a microscope (Fig. 1). The cell body was small and appeared triangular, square or polygonal, and some cells showed fusion. The cell side showed various protrusion, though which they connected to the adjacent cells. The large nucleus was located in the central or side region of the cell. Consistent with the typical biochemical characteristics of osteoblasts, the cytoplasm appeared violet. After 16 d of Alizarin red staining, many round or elliptical opaque red nodules were observed under a microscope. These findings confirmed that the nodule was a mineralized nodule, further demonstrating that the cell showed mineralization.
Generation of the osteoblast growth curve

The growth trend of SD rat osteoblasts was an S-shaped curve (Fig. 2). The first 2 d after inoculation was the adjustment period, and at 3 d, cells entered the logarithmic phase. Proliferation peaked at approximately 5 d and plateaued beginning at 6 d. The growth trend of osteoblasts was consistent with that reported in most of the literature.

TSH promotes osteoblast proliferation

We first investigated whether cell proliferation could be regulated by TSH in primary osteoblasts. Osteoblasts were treated with TSH for different times and at each time point with different doses. MTT assays showed that at the 12 h and 24 h points, the OD value of the TSH 100 mU/mL group was higher than that of the other three groups (P < 0.05), and the mean differences in the OD values between the other three groups were not statistically significant (P > 0.05). After cultivation for 48 h, the mean OD value of the 10 mU/mL TSH group was higher than that of the control group (P < 0.05). The OD value of the TSH 100 mU/mL group was higher than that of the control group (P < 0.001), while there were no differences between the OD values of the 1 mU/mL experimental group and the control group (P > 0.05) (Fig. 3).

According to the statistical results, all of the above concentrations of TSH showed no inhibitory effects of osteoblast proliferation. The 100 mU/mL treatment showed the strongest promotion of cell proliferation. Furthermore, 10 mU/mL TSH promoted osteoblast proliferation, but the effect was weaker than that of the 100 mU/mL treatment. The 1 mU/mL treatment had no effect on osteoblast proliferation. In conclusion, TSH can promote osteoblast proliferation.

TSH promotes osteoblast differentiation

After starving for 2 h, primary osteoblasts were cultured in the absence or presence of TSH (1, 10 and 100 mU/mL) for 48 h. Osteoblast differentiation was analyzed by determining the RNA levels of the osteoblast marker proteins ALP, COL1, BMP2 and Runx2. As shown in Fig. 4, TSH at concentrations of 10 mU/mL and 100 mU/mL significantly increased the mRNA levels of ALP, COL1 and Runx2 compared with those of the control (P < 0.05, P < 0.01), and the differences between the 1 mU/mL TSH treatment group and the control group were not significant (P > 0.05). BMP2 mRNA levels in the groups treated with 10 and 100 mU/mL TSH were significantly increased compared with those in the control group (P < 0.01, P < 0.001), and there was no significant difference between the 1 mU/mL TSH treatment group and the control group (P > 0.05). All of the above results showed that TSH could increase the mRNA levels of osteoblast function-related genes, such as ALP, COL1, BMP2 and Runx2, in a dose-dependent manner. We also detected the mRNA levels of ALP and BMP2 at the 12, 24, and 48 h time points after treatment with 100 mU/mL TSH. ALP and BMP2 activity increased over time and peaked at 48 h (P < 0.05, P < 0.01). Moreover, the effects of TSH on Runx2 and osterix protein expression were analyzed by Western blot analysis. As shown in Fig. 4(G), Runx2 and Osterix protein expression was promoted by TSH (10 and 100 mU/mL).
Discussion

TSH is a glycoprotein hormone produced by thyrotroph cells in the anterior pituitary, which plays an important role in the regulation of thyroid development and function. In thyroid tissue, the effect of TSH is mediated by TSHR, a member of the seven-transmembrane helical G-protein-coupled receptor family. Osteoporosis is associated with thyroid dysfunction and has traditionally been considered a secondary result of changes in thyroid function [14]. However, in subsequent studies, the expression of the TSHR mRNA and protein in normal osteoblasts was observed, suggesting that TSH may exert a direct effect on bone formation [15].

In addition to these in vivo experiments, studies have reported associations between higher than normal levels of free T3 and free T4 with reduced bone density and an increased risk of non-vertebral fractures. High free T4 and free T3 levels are associated with low bone density in the hip joint, while high free T4 levels are also associated with potential bone loss at the hip joint [16]. However, thyroid hormone has been shown to stimulate cells to secrete insulin-like growth factor I that further stimulates osteoblast progenitor cells and finally promotes the differentiation and proliferation of osteoblasts. T3 binds to thyroid hormone receptors on osteoblasts, affecting cell proliferation, protein synthesis, and matrix formation, but high-dose T3 inhibits osteoblast proliferation. T3 binds to thyroid hormone receptors on osteoclasts and promotes the proliferation and activity of osteoclasts. Based on these results, thyroid hormone exerts a regulatory effect on bone growth, maturation and transformation [17].

Therefore, researchers have been unable to exclude a role for T3 in patients with hyperthyroidism and only study the role of TSH in osteoporosis. Subclinical thyroid disease refers to a normal serum thyroid concentration, and only the TSH concentration is higher or lower than the normal range. Therefore, this situation is considered an appropriate model for studying the direct effects of TSH on bone metabolism, while eliminating the direct effects of thyroid hormone on bone metabolism [18]. Of the 5,458 (mean age: 72 years, 49.1% female) individuals in the six prospective cohorts, 451 (8.3%) were diagnosed with subclinical hypothyroidism and 284 (5.2%) were diagnosed with subclinical hyperthyroidism. At 36,569 follow-up years, patients with subclinical hyperthyroidism had greater annual bone loss in the femoral neck than patients with hyperthyroidism. Subclinical hyperthyroidism is associated with increased bone loss at the femoral neck, which may increase the risk of fracture [19]. In older women without significant thyroid dysfunction [20], low TSH levels are independently associated with decreased bone density in the femoral neck. A meta-analysis of 1,371 studies and 70,298 participants also showed that subclinical thyroid dysfunction is an important risk factor for fractures [21].

Suppressed levels of TSH were correlated with low BMD in hyperthyroid[22], particularly in postmenopausal women [23]. Even TSH levels in the normal range show a similar relationship in the elderly and are associated with an increased risk of hip fracture in healthy women [24]. Thus, the duration of TSH inhibition is a predictor of major osteoporotic fractures. A cross-sectional study reported a high incidence of vertebral fractures during TSH inhibition, even in patients with a normal bone density. Vertebral fractures are very common in women with low TSH levels and a bone density in the
osteoporotic range [25]. Siderova et al. described the negative effect of hyperthyroidism on bone mineral density. In contrast, TSH exerts a positive effect on this parameter. TSH receptor antibodies are often present at a high titer in patients with Graves’ ophthalmopathy and may exert a protective effect on bone [26].

In the present study, after adjusting for age, body mass index, serum levels of FT4, higher serum TSH levels were observed in the group with a higher BMD than that in the low serum TSH group and normal thyroid function was observed in the male group (intraoperatively, 0.05), indicating that the BMD of males would also increase with an increase in serum TSH concentration. The results are consistent with previous studies.

Epigenetic mesenchymal osteoblasts derived from undifferentiated pluripotent stem cells synthesize and secrete osteoid components, such as collagen and glycoproteins, which participate in osteoid calcification and regulate osteoclast activity. These molecules play an important role in bone formation. Primary osteoblasts are often isolated as also the main cell source for in vitro experiments. Two main methods of osteoblast isolation have been described: enzymatic digestion and tissue blocking. The former is more widely used than the latter. Osteoblasts were successfully extracted from the rat skull by enzymatic digestion. The cells grew well in culture and showed activity. Since fibroblasts showed high adhesion and fast adhesion, we used the repeated adhesion method to remove fibroblasts and obtain high-purity osteoblasts. The cells were identified by HE staining and morphological observations of biological characteristics, and the extracted cells were confirmed to have typical features of osteoblasts.

MTT colorimetry showed that 10 mU/mL and 100 mU/mL TSH promoted proliferation of osteoblasts, and the dose of 100 mU/mL was the most effective. Using real-time PCR analysis, genes related to osteoblast function, such as COL1, BMP2, and Runx2, were further detected in cells treated with different concentrations of TSH. In this study, TSH increased the expression of genes related to osteoblast function, such as ALP, COL1, BMP2 and Runx2, compared with the control group. At concentrations of 10 mU/mL and 100 mU/mL, the changes in gene expression were statistically significant (P < 0.05, < 0.01, and P < 0.001). The effect of 100 mU/mL TSH on the mRNA levels of genes associated with osteoblast function, including ALP, COL1, BMP2 and Runx2, was stronger than the effect of 10 mU/mL TSH, suggesting that TSH exerted a dose-dependent effect on the mRNA levels of the osteoblast function-related genes ALP, COL1, BMP2 and Runx2. Then, we investigated the expression of ALP and BMP2 mRNAs at 12, 24, and 48 h. TSH increase the levels of the ALP and BMP2 mRNAs over time, confirming that TSH had directly promoted osteoblast differentiation and bone formation.

Bone morphogenetic proteins (BMPs) are generally considered the main factors inducing bone repair. According to the mechanistic analysis, BMPs bind to BMPR, promoting its association with Smad coactivators and p38-MAPK, thereby activating downstream transcription factor Runx2. This transcription factor directly targets the promoter region of ALP and osteocalcin to regulate osteogenesis and differentiation [27]. The Runx2 protein regulates osteoblast differentiation by directly affecting the transcriptional activity of ALP, BGP and the downstream COL1 and OPN molecules [28]. BMP2 is involved
in TSH-induced osteoblast proliferation and differentiation. Therefore, we speculated that the BMP-SmADs-Runx2-Osterix pathway might be involved in this process. Further studies are needed to explore the specific mechanisms.

TSHR mediates signaling through many different pathways, although the GS-cAMP pathway is considered the primary pathway. The development of functionally biased TSHR agonists, in which one signaling pathway may be superior to the other, may facilitate the development of drugs that selectively modulate treatment-related physiological functions, such as PTH1R signaling [29]. As shown in the study by Boutin, TSH-mediated up-regulation of IL-11, ALPL, and OPN occurs through different G-protein-coupled signaling pathways. Based on these findings, the development of TSHR agonists biased towards the ashutin-ashutin-1 and Gq/11-ERk1/2 pathways may contribute to the treatment of osteoporosis [30].

In the past few years, small molecules have received increasing attention as therapeutic options for regulating TSHR signaling [31]. Their chemical properties make them resistant to proteolytic enzymes, and thus these molecules are ideal therapeutic agents [32]. Molecular docking and experimental studies have shown that the TSH protein binds to TSHR and signals through TSHR [33, 34]. In addition to macrophages, the mouse pituitary is also a source of a new TSH splice variant (TSH-KVV), which may retain its biological effects [33–35]. A small molecule (MS-438) appears to increase osteoblast formation through the PKA signaling pathway [36]. Other studies have reported the biological effects of small molecules on osteoblasts overexpressing TSHR [37], and two small-molecule TSHR antagonists with a lower binding affinity than required for clinical use have been identified [38, 39].

We only conducted cell-based experiments, and our clinical examination was a retrospective study of a small number of patients. Since the single bone density measurement in the forearm does not adequately reflect bone metabolism, further studies, such as in vivo animal experiments and large-sample clinical studies, are needed to accurately assess signal transduction in osteoblasts. Studies aiming to elucidate the relationship between TSH and osteoporosis will provide a new basis for the treatment of thyroid disease and osteoporosis.

**Conclusion**

In the present study, we demonstrated that TSH serum concentrations were positively correlated with BMD in men with normal thyroid function. TSH can promote osteoblast proliferation. In addition, we confirmed that TSH increased the expression of key osteoblast differentiation genes, such as ALP, BMP2, COL1, and Runx2, in a dose-dependent manner. TSH directly or indirectly affects the osteoblast microenvironment and promotes bone differentiation and bone formation.

**Abbreviations**

TSH: thyroid-stimulating hormone; FT3:triiodothyronine; FT4:tetraiodothyronine; BMD: bone mineral density; TSHR: thyroid-stimulating hormone receptor; ES: embryonic stem; BMI: body mass index;
DXA: dual-energy X-ray absorptiometry; ALP: Alkaline phosphatase; DMSO: dimethyl sulfoxide; BMPs: Bone morphogenetic proteins

Declarations

-Ethics approval and consent to participate

Written informed consent was obtained from all participants. All procedures performed in studies involving human and animal participants were approved by the Ethics Committee of Shandong Provincial Hospital, Jinan, China.

- Consent for publish

This manuscript does not report personal data such as individual details, images or videos; therefore, consent for publication is not applicable.

-Availability of data and materials

The datasets used in the analyses described in this study are available from the corresponding author on reasonable request.

- Competing interests

The authors declare that they have no conflict of interest.

-Funding

This work was supported by the National Natural Science Foundation [grant number: 81370892]; the Key Research and Development Plan of Shandong Province [grant numbers: 2016GSF201025, 2016GGH3118 and 2017G006006]; and the Taishan Scholar Construction Project Special Funding [grant number: TS201712092]. The funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

-Authors’ contributions

T.D., W.Z., Y.Z., M.Z., and Z.H. performed all the experiments and data analysis; T.D. and W.Z. wrote the manuscript; C.Y. and X.Z. reviewed and edited the manuscript; Y.W. and J.X. developed the research ideas and experimental design, and reviewed and modified the manuscript.

-Acknowledgements

We thank all the men who participated in our study.

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Figures
Figure 1

HE staining of P3 primary osteoblasts was observed under a microscope. The cell side showed various protrusions, though which they connected to the adjacent cells. The large nucleus was located in the central or side region of the cell (A1*50, A2*100, A3*400). The cytoplasm appeared violet due to ALP staining (B*400). On d 16 of osteoblast culture, there were many round or elliptical opaque nodules that were stained red by Alizarin red (C1*100, C2*200).
Figure 2

The growth trend of P3 primary osteoblasts was an S-shaped curve. The first 2 d after inoculation were the adjustment period, and starting from d 3, cells entered into the logarithmic phase, showed peak proliferation at approximately 5 d, and began to plateau on d 6. The growth trend of osteoblasts was consistent with that in most of the literature reported.
Cell proliferation was regulated by TSH in primary osteoblasts. Osteoblasts cocultured with different concentrations of TSH (0, 1, 10, and 100 mU/mL) were detected at 12, 24, and 48 h by MTT assays. Data are expressed as the mean±SD. *P<0.05 versus the control group (TSH = 0 mU/mL) at the corresponding time period. ***P<0.001 versus the control group (TSH = 0 mU/mL) at 48 h.
Figure 4

Effect of TSH on mRNA expression of ALP, COI1, BMP2 and Runx2. The mRNA expression of ALP (A), COI1 (B), BMP2 (C) and Runx2 (D) was determined by real-time RT-PCR. ALP (E) and BMP2 (F) mRNA levels were detected at 0, 12, 24 and 48 h of 100 mU/mL TSH treatment. Data are expressed as the mean±SD. *P<0.05, **P<0.01, ***P<0.001 versus the control group. As shown by Western blot analysis, Runx2 and osterix protein expression was promoted by TSH (10 and 100 mU/mL) (G).