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Different Effects of Oxidized Parathyroid Hormone (ox-PTH) and Non-oxidized Parathyroid Hormone (n-oxPTH) on Sclerostin (SOST): based on in vitro studies

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percent
µl	Microliter
µg	Microgram
25(OH)D	25-hydroxyvitamin D
1,25(OH)2D	1,25-dihydroxyvitamin D
BMP	Bone Morphogenetic Protein
BMD	Bone Mineral Density
cAMP	Cyclic Adenosine Monophosphate
CKD	Chronic Kidney Disease
CVD	Cardiovascular Disease
CHX	Cycloheximide
Ct	Cycle Threshold
CI	Confidence Interval
DAN	Differential screening selected gene Aberrant in Neuroblastoma
DMEM	Dulbecco's Modified Eagle Medium
dL	Deciliter
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-linked Immunosorbent Assay
ESRD	End-stage Renal Disease
FGF23	Fibroblast Growth Factor 23
g	Gram
HLA	Human Leukocyte Antigen
hPTH1-34	Human Parathyroid Hormone 1-34
H_2O_2	Hydrogen Peroxide
iPTH	Intact PTH
IQR	Interquartile Range
KTRs	Kidney Transplant Recipients
KO	Knockout
kg	Kilogram

LIST OF ABBREVIATIONS

LDL	Low Density Lipoprotein
LRP	LDL-receptor-related Protein
L/l	Liter
Met	Methionine
Met18 (ox)-PTH	PTH Oxidized at Met 18
Met8 (ox)-PTH	PTH Oxidized at Met 8
Met8,18 (di-ox)-PTH	PTH Oxidized at Met 8 and 18
MEF2	Myocyte Enhancer Factor 2
ml	Milliliter
N-oxPTH	Non-oxidized PTH
NFH_2O	Nuclease-free H_2O
nm	Nanometer
nmol	Namomole
ng	Nanogram
Ox-PTH	Oxidized PTH
PTH	Parathyroid Hormone
PTH1R	Parathyroid Hormone 1 Receptor
PHPT	Primary Hyperparathyroidism
pH	Potential of Hydrogen
PKA	Protein Kinase A
PTHrP	PTH-related Protein
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
rPTH	Rat PTH
Runx2	Runt-related Transcription Factor 2
RT	Reverse Transcription
SOST	Sclerostin
T2DM	Type 2 Diabetes Mellitus
T1DM	Type 1 Diabetes Mellitus
VC	Vascular Calcification

1 INTRODUCTION

1.1 Parathyroid hormone

Parathyroid hormone (PTH) is produced by parathyroid glands located at the posterior of the thyroid gland [1]. In vivo, PTH can initiate functions in bones, intestines, and kidneys through binding to the PTH1 receptor (PTH1R) expressed by osteoblasts. In the bone, PTH stimulates the release of calcium and phosphate [2]. In the intestines, PTH acts on the absorption of calcium and phosphorus. In the kidney, PTH activates vitamin D, meanwhile, promotes the transformation of 25-hydroxyvitamin D (25(OH)D) to 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), also called calcitriol, by activating the enzyme 1-α-hydroxylase, which, in turn, increases calcium and phosphate reabsorption in the intestines [2]. In addition, PTH stimulates calcium reabsorption but impedes phosphate reabsorption in the distal tubules of kidneys [2] (Figure 1). PTH is a key hormone for calcium homeostasis through many ways of regulation, resulting in the increase of calcium levels. In turn, circulating calcium levels also play a pivotal role in regulating PTH levels. Once increased level of extracellular calcium is detected, intracellular calcium concentrations increase through the signal transduction across the calcium-sensing receptor of cell surface and intracellular pathways, consequently suppressing PTH exocytosis. Meanwhile, hypercalcemia can stimulate the degradation and release of PTH fragments [3]. In addition, calcium also regulates the synthesis of PTH. In addition to extracellular calcium, the main regulator of the processes as above mentioned, extracellular $1,25(OH)_2D$, phosphate, and fibroblast growth factor 23 (FGF23) also influence PTH synthesis and secretion [2].

Previous investigation has identified the role of increased blood PTH levels in clinical practice. Primary and secondary hyperparathyroidism are characterized with excessive PTH secretion, which can result in bone diseases and vascular calcification [4]. The disorders of excess levels of PTH have been also investigated in general population [5,6]. It is reported that increased PTH concentrations are related to high risks of cardiovascular disease (CVD) and death rate [5,6]. Similar results were reported in patients with chronic kidney disease (CKD) [7]. However, a meta-analysis found there was no clear association between serum levels of PTH and all-cause mortality in CKD patients [8]. A possible explanation is that the systemic review did not exclude the studies in CKD patients on dialysis [8]. Since studies are controversial in patients

undergoing dialysis. Some studies indicated that increased circulating PTH levels were associated with higher risk of mortality in dialysis patients [9-12], whereas, other studies either found no association [13], or even in an inverse association [14-16]. Thus, Cozzolino et al. suggested PTH should be adequately controlled without over-suppression, especially in patients with CKD receiving dialysis [17]. Similarly, in kidney transplant recipients (KTRs), circulating PTH levels were significantly increased [18,19]. Moreover, increased serum PTH concentrations were associated with the estimated glomerular filtration rate (eGFR) [18], and were a potential predictor for renal allograft loss in KTRs [19].

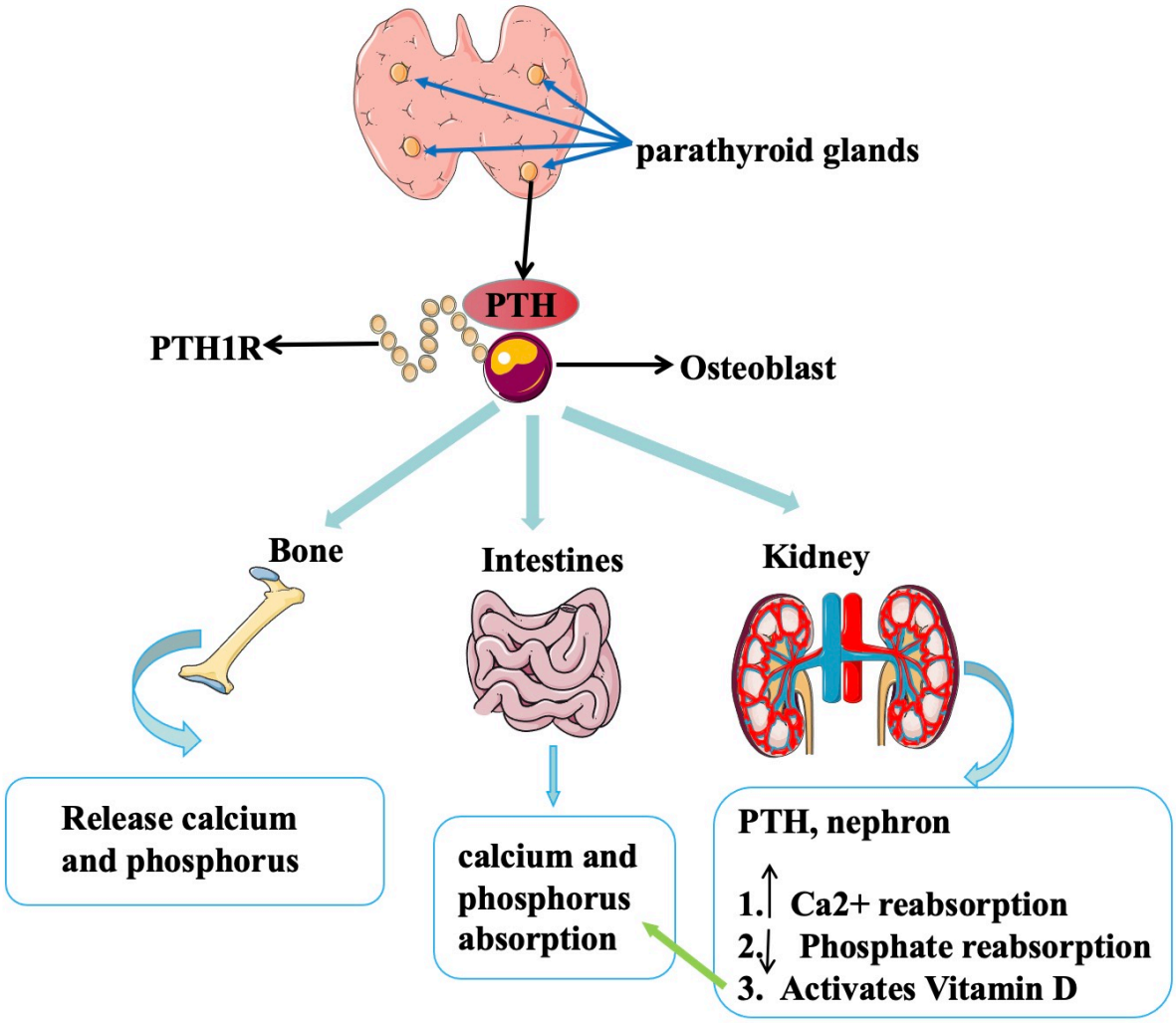


Figure 1. Effects of PTH on bone, intestines, and kidneys. Parathyroid glands secrete PTH in vivo, which binds to PTH1R expressed by osteoblasts. The affinity between PTH and PTH1R can differently impact bones, intestines, and kidneys. In the bone, PTH promotes the release of calcium and phosphorus, while in the intestines, PTH promotes the absorption of calcium and phosphorus. Moreover, PTH facilitates calcium reabsorption and vitamin D activation but suppresses phosphate reabsorption in the kidneys. The activated vitamin D in the kidneys can in turn promote the absorption of calcium and phosphorus in the intestines. This figure is on my own elaboration.

PTH is a single-chain polypeptide hormone consisting of 84 amino acid residues [3]. Two methionine (Met) residues at positions 8 and 18 within PTH, which are inclined to be oxidized in vivo, which may lead to conformational changes. Consequently, the biological activity of oxidized form of PTH is impaired due to the conformational changes of PTH. As oxidized PTH does not stimulate the PTH1R to generate cyclic adenosine monophosphate (cAMP), it is most likely biologically inactive [20]. It has been reported that the non-oxidized PTH (n-oxPTH) and the oxidized PTH (oxPTH) have different bioactivities. However, the previous PTH assays emphasize measuring full-length PTH but fail to differentiate n-oxPTH from oxPTH, giving rise to several-folder higher readout than the actual bioactive PTH in population study [20]. Moreover, the Met residue 18 of PTH was susceptible to becoming oxidized due to its location, whereas the PTH at Met residue 8 is difficult to be oxidized because of a hydrophobic pocket [21]. In addition, it is reported that PTH oxidized at Met18 (Met18 (ox)-PTH) maintains partial biological activity compared to PTH oxidized at Met8 (Met8 (ox)-PTH) [2,22]. The different biological activities of the various ox-PTH forms contribute to the different conformational changes. A previous study showed that Met8 (ox)-PTH had a substantial change of secondary structure, whereas Met18 (ox)-PTH caused a relatively small conformation change [23]. Meanwhile, the PTH oxidized at Met8 and Met18 (Met8,18 (di-ox)-PTH) had the biggest impact on conformation changes when compared with individual oxidations [23]. Accordingly, the bioactive sequences of different ox-PTH are as follows: Met18 (ox) PTH > Met8(ox) PTH > Met8, Met18 (di-ox) PTH [23].

Over the past few decades, PTH measurements have been evolved from the first-generation assays to the currently available second-generation and third-generation assays which are based on a sandwich immunoassay. For the first-generation assays, single antibody is used which can simultaneously detect several inactive hormone fragments [24]. However, the second-generation PTH assay applies two antibodies, by which one antibody was designed against the C-terminal amino acid region 34-84, whereas another can recognize the N-terminal residues of 23-24 or 26-32 [25]. Therefore, this second-generation PTH assay measures the full-length PTH and C-terminal fragments, mainly the PTH fragments 7-84. The N-terminal antibody of the third-generation assay is raised against a more proximal part (amino acid region 1-4). Although the third-generation assay solely measures full-length PTH, there is still an

ongoing debate on whether using second or third-generation PTH assays is preferable [25]. PTH results are acquired from second-generation PTH assays in most nephrology centers. Notably, the second and third-generation assays cannot distinguish oxPTH from n-oxPTH. First described in 2012, the "4th generation" assay was recently developed to measure n-oxPTH alone[20]. In this assay, oxPTH is first excluded by specific monoclonal antibodies in an affinity, while the remained n-oxPTH can be actuarily measured through automated PTH assays [26].

As early as 1934, PTH was described as being oxidized [27]. That year, Tweedy et al. investigated the effects of various chemical agents on the PTH's biological activity. Dogs were injected with extracts of PTH treated with different concentrations of hydrogen peroxide (H_2O_2). As H_2O_2 concentration increases, oxidation causes the significant decrease in calcium concentration, indirectly showing that PTH can be inactivated by oxidation [27]. Subsequently, many other in vitro studies observed the changed biological activities by oxidation of PTH. Several animal studies demonstrated that infused ox-PTH induced by H_2O_2 failed to act on kidney and bone in vivo experiments, resulting in a decline in calcium and 1,25$(OH)_2$D levels[28,29]. Moreover, some studies investigated that PTH can stimulate adenyl cyclase of renal and bone fragments to produce the second messenger - cAMP [30]. These findings demonstrated that ox-PTH possessed a reduced signaling capacity and failed to activate the cellular adenylate cyclase [30]. Furthermore, some researchers also explored the altered effects of ox-PTH on activating alkaline phosphatase [31], relaxing guinea-pig trachea [32], constricting blood vessels [33], activating mitochondrial adenosine triphosphate synthase [34], stimulating cardiac action [35], and influencing FGF23 synthesis [36] in vitro as well.

Although evidence is compelling for ox-PTH in rat models, little is known as to PTH oxidation in humans [2]. In the beginning, it was generally assumed that oxidation of PTH did not take place in living organisms [37]. As a consequence, less interest had been directed toward this field in clinical practice until Hocher et al. launched a creative assay, enabling it to measure n-oxPTH in an easy way [20]. Several studies found that circulating n-oxPTH concentrations, namely completely bioactive PTH, were much lower than that of measured intact PTH (iPTH), even though their exact percentages were unclear [38,39]. Previous data on associations of iPTH with mortality were consistent

with the U-shaped association previously built by others, suggesting increased risk with too high or too low iPTH [40,41]. In comparison, the association between n-oxPTH and mortality in kidney disease was controversial. Subsequently, accumulating clinical studies analyzed the relationship between n-oxPTH and clinical outcomes. In 2013, Tepel et al. first assessed the clinical implications of n-oxPTH measured by three-generation assays in 340 hemodialysis patients [42]. Survival analysis showed that patients with high tertile of circulating n-oxPTH had higher survival rates than those with low tertile of n-oxPTH concentrations [42]. Moreover, multivariate analysis demonstrated that the odds of death increased with age, whereas higher n-oxPTH concentrations decreased the odds of death after adjusting confounding factors [42]. In all patients, iPTH had a strong correlation with n-oxPTH, whereas subgroup analysis in patients with high iPTH (> 70ng/L) showed iPTH was strongly associated with oxPTH levels but not n-oxPTH [42]. Next year, Hocher et al. published an abstract that analyzed 2867 patients of the EVOLVE trial (ClinicalTrials.gov: NCT00345839) [43]. They found that iPTH was strongly associated with oxPTH but weakly associated with n-oxPTH. Their findings showed that n-oxPTH, but not iPTH or oxPTH, was associated with cardiovascular and all-cause mortality [43]. In KTRs, only n-oxPTH was reported to be significantly correlated with graft loss after adjustment for known confounding factors, such as sex, age at transplantation, age at study entry, serum phosphorus, calcium excretion, hemoglobin, urinary protein, eGFR, pH, urinary albumin creatine ratio, and 25(OH)D [44]. Furthermore, a strong association between iPTH and oxPTH was observed in this cohort [44]. These studies supported the measurement of n-oxPTH in clinical settings. Meanwhile, n-oxPTH may be superior to iPTH in evaluating cardiovascular mortality and renal endpoints in kidney diseases.

On the other hand, the clinical implications of n-oxPTH have been disputed by other researchers. A prior study showed that n-oxPTH was not associated with any of the examined clinical outcomes, such as CKD progression, acute heart failure, all-cause mortality, and atherosclerotic events in CKD from stages 2 to 4 [45]. In contrast, iPTH continued to be associated with all-cause mortality after adjusting cardiovascular and kidney risk factors in multivariate regression analyses [45]. N-oxPTH measurements were not superior to second-generation PTH measurements in predicting all-cause mortality in participants without dialysis [45]. Recently, one of our studies showed that ox-PTH and iPTH concentrations markedly increased, whereas circulating n-oxPTH

moderately increased with the decrease in eGFR in two cohorts, including CKD children and KTRs [36]. Another study explored the implications of n-oxPTH for assessing bone turnover in CKD [26]. The researchers found that the abilities of n-oxPTH and iPTH to discriminate low/non-low and high/non-high bone turnover were significant and comparable, suggesting no added values of measuring n-oxPTH for assessing bone turnover in CKD patients [26].

Circulating iPTH was strongly associated with oxPTH in CKD patients with or without dialysis. Given the involvement of oxidative stress in patients with CKD, there was a high proportion of oxidized PTH in CKD. Previously, it has been reported that Met18(ox)-PTH is more likely to be oxidized than Met8(ox)-PTH did in the organisms. Therefore, we assume that Met18(ox)-PTH concentrations are higher than that of Met8(ox)-PTH in vivo. This hypothesis has been supported by prior studies [2,26]. Moreover, it has been suggested that n-oxPTH may be prone to reflect the PTH bioactivity, whereas iPTH may be better described as a consequence of oxidative stress. Currently, despite the partial loss of biological activity of oxPTH being demonstrated, the roles of n-oxPTH and oxPTH with residual biological activity, such as Met18(ox)-PTH, are associated with some pertinent questions and concerns about its clinical implications for clinical diagnostics and decision-making remains unclear.

1.2 Sclerostin

Sclerostin (SOST) is a 190-amino-acid secreted glycoprotein with catabolic effects on bone, encoded by the SOST gene. It has been reported that the SOST gene is mainly expressed in osteocytes, but also in many other organs or tissues such as osteoblasts, cartilage, bone marrow, kidney, liver, and heart; however, it mainly expresses in osteocytes [46]. The amino acid sequence of sclerostin is closely similar to that of the DAN family of glycoproteins, and DAN (differential screening selected gene aberrant in neuroblastoma) family proteins that act as antagonists of bone morphogenetic protein (BMP) signaling[46]. It was initially reported that SOST exerts its regulatory function through modulating BMPs. However, the argument is inconsistent regarding the interaction between sclerostin and BMP signaling. It was showed that sclerostin can directly antagonize BMP signaling pathways [47,48], whereas others showed that

SOST is not a classical BMP antagonist [49,50]. Furthermore, Krause et al. demonstrated that the antagonist action of SOST on BMP signaling is mediated through specific targeting on BMP7 in osteocytes [47]. At cellular level, SOST can directly inhibit of the Wnt/β-catenin signaling pathway to promote osteogenic pathways for elevation of bone mass [31, 33, 35, 36], despite the incomplete elucidation of SOST actions in modulating bone formation (Figure 2). Wnt ligands bind to LDL-receptor-related protein (LRP) 5 or 6, activating the canonical Wnt/β-catenin signaling. Alternatively, SOST may bind to the LRP5/ LRP6 complexes and prevent them from interacting with the Wnt ligands [1] (Figure 2). But the notion was challenged in a recent study which questioned the importance of the interaction between SOST and the Wnt co-receptor LRP5 in inhibiting bone formation in osteoblasts [37, 38]. Although the molecular basis of interaction between SOST and LRP5 in inhibiting bone formation remained unclear, it is clear that SOST is a negative regulator of bone growth [1].

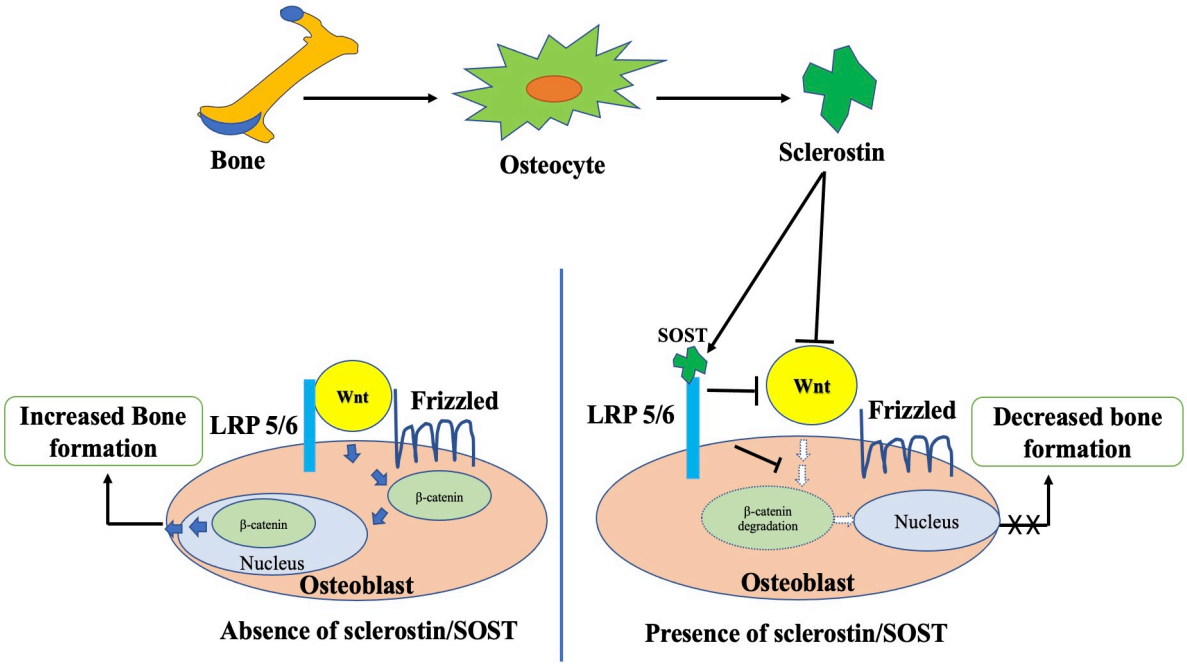


Figure 2. Potential mechanisms of Sclerostin/SOST action in the osteoblast. Sclerostin (SOST) is primarily produced by osteocytes in the bone. In the absence of sclerostin/SOST, the Wnt signaling pathway is activated by the binding of Wnt ligands to receptors on the cell surface, which leads to the recruitment and activation of several downstream proteins, including β-catenin. β-catenin is a transcription factor that translocates to the nucleus and promotes the expression of genes involved in osteoblast differentiation and activity, seen in the left panel. SOST is known to act as a negative regulator of bone formation. SOST exerts its function by inhibiting the Wnt signaling pathway, which is essential for the differentiation and activation of osteoblasts. Specifically, sclerostin binds to LRP5/6 co-receptors, which are required for Wnt signaling activation, and prevents them from interacting with Wnt ligands, seen in the right panel. This figure is on my own elaboration.

A series of basic studies explored the SOST actions in various animal models. In SOST knockout (KO) mice, bone formation, bone volume, and bone strength were significantly increased, demonstrating its negative role in regulating bone formation [51]. Furthermore, Li et al. observed impaired healing in SOST KO mice, showing the critical role in wound healing. Their findings showed that callus mass, bone formation, and bone strength were greater in SOST KO mice [52]. In addition, Yee et al. explored the specific cell type contributing to high bone mass phenotype in SOST KO mice by building a mice model characterized by conditional deletions of SOST function. They found that some cell types from the Prx1-osteoprogenitor-derived lineages predominantly contribute to the amount of SOST protein in bone [53]. In animal models of osteoporosis, a systematic review concluded that treatment of antibodies to SOST improved bone structure, mass, and strength [54]. In models of mature ovariectomized rats, SOST antibody administration significantly increased mRNA gene expression of several osteocytes in the tibia and vertebra compared to the control group [55]. Even in aged male rats, the inhibition of SOST by the treatment of SOST antibody increased bone mass, formation, and strength [56].

Previous research into the biology and mechanism of SOST actions revealed that SOST plays a key role in many skeletal disorders. In clinical practice, disorders of the SOST in vivo, such as decreased SOST levels due to the inactivation of the SOST gene, lead to certain clinical phenotypes, including sclerosteosis and van Buchem's disease [57,58]. These diseases are characterized by high bone mass. Both diseases' mechanisms probably contribute to the inactivation of the SOST gene, stimulating the osteoanabolic pathway it regulates. Furthermore, the role of SOST was also explored in other types of diseases. In patients with diabetes mellitus, increased SOST concentrations were observed in type 2 diabetes mellitus (T2DM) [59,60] and associated with a high risk of vertebral fractures independent of bone turnover and bone mineral density (BMD) [59]. Upregulated SOST levels might delay the healing of T2DM - associated bone fracture [61]. Moreover, SOST levels in T2DM were higher than those in type 1 diabetes mellitus (T1DM) or controls [62]. In T1DM, no association was observed between SOST and the biomarker of bone metabolism [63,64]. In rheumatoid arthritis, serum SOST levels were an independent risk factor for abdominal aortic calcification [65].

In recent years, the role of SOST has been discussed in the field of renal diseases. Accumulating studies have explored SOST-associated mortality risk in non-transplanted patients with CKD. In patients with CKD without dialysis, serum SOST levels were higher than controls and increased with the stages of CKD. Moreover, high SOST concentrations correlated with an increased risk of cardiovascular events and all-cause mortality even after multiple adjustments [66]. However, several studies reported that the association of SOST with cardiovascular and all-cause mortality is conflicting in dialysis cohorts. Some studies showed that SOST is related to all-cause mortality in dialysis patients [67-71], whereas other studies did not find this association [72-75]. Among them, three studies found that SOST is associated with cardiovascular mortality [67,69,72], but one study did not identify this association [73]. Furthermore, Pelletier et al. reported that serum SOST increased with declining GFR [76-78], and SOST was independently associated with GFR in CKD after multiple regression analyses [76]. In a study conducted the following year, Cejka et al. showed that urinary excretion of SOST increased with decreasing eGFR; however, this finding was not associated with serum SOST levels in the CKD stages from 1 to 5 [79].

Circulating SOST levels significantly increased in CKD and end-stage renal disease (ESRD), where serum SOST was independently associated with vascular calcification (VC) [80,81] and atherosclerotic disease [77,82]. Although previous findings about the association of SOST with cardiovascular events, all-cause mortality, and VC are controversial, it is clear that SOST is an important biomarker of disease progression in CKD and may be a novel therapeutic target. In terms of KRTs, Bonani et al. conducted a prospective study comparing serum SOST levels before and after one year of renal transplantation while exploring the association of SOST with BMD [83]. They found that pre-transplant SOST concentrations were increased in all patients while significantly decreasing 15 days after kidney transplantation, parallel to renal function improvements [83]. There was no association between SOST and BMD in KRTs [83]. Considering VC is prevalent and progressive in KRTs, Evenepoel et al. studied the correlation between SOST and the progression of VC [84]. The finding showed that up to 84% of participants presented VC at baseline, and more than half of KRTs underwent progression of VC [84]. Furthermore, serum SOST levels at baseline were negatively associated with VC progression after adjusting traditional factors such as age, gender, and PTH levels [84].

Moreover, the serum SOST levels in KRTs with the high arterial stiffness group were higher than those in the low arterial stiffness group [85]. Further multivariate logistic regression analysis showed that SOST was an independent predictor of peripheral arterial stiffness, suggesting SOST may be involved in the pathogenesis of peripheral arterial stiffness in KRTs [85]. One of our prior studies demonstrated that circulating SOST was independently associated with all-cause mortality in multiple proportional hazards regression analyses after adjustment for known confounding factors [86]. Cardiovascular disease (CVD) is the leading cause of death in KTRs [87]. It is reported that more than half of the populations with KRTs directly died from CVD [88,89]. In addition, the Wnt signaling pathway is believed to be associated with vascular calcification [90] and CVD [91]. The progression of VC and atherosclerosis are risk factors for the increased incidence of CVD. This probably explained that increased SOST concentrations - a known inhibitor of Wnt signaling - contributed to the cardiovascular death rate.

Estrogen, mechanical loading, and PTH have been reported as regulators of sclerostin expression [46]. Several studies have shown that estrogen treatment markedly decreases circulating SOST levels in postmenopausal women [92-94], and increased SOST is associated with age [94]. Furthermore, circulating sclerostin is higher in male CKD patients than female CKD patients [76,78,95]. These findings are consistent with the fact that estrogen mediates the reduction in SOST. Moreover, researchers conducted studies on the role played by mechanical loading in the expression of SOST. They found that the SOST-positive staining intensity and number in osteocytes were significantly reduced after mechanical loading [96]. In contrast, the SOST was upregulated after mechanical unloading in the female mice model. Currently, many pieces of research have proven the mutual regulation between PTH and SOST.

1.3 Interaction of sclerostin and parathyroid hormone

1.3.1 Preclinical perspectives

Cell culture and animal studies initially investigated the association between SOST and PTH. In this context, Keller et al. showed that SOST mRNA expression in PTH-treated calvariae four hours after the last human PTH 1-34 (hPTH (1-34)) administration was decreased by 40% in a mouse model of calvaria local bone formation when compared with the vehicle-treated group [97]. This study treats mice with subcutaneous injections of 100 nmol/l of hPTH (1-34) twice daily for five days or a vehicle. Moreover, a significant reduction of SOST expression in the femur was also observed in a single subcutaneous injection at a dose of 80 ug/kg of rat PTH 1–34 (rPTH (1-34)) six hours after rPTH (1-34) injection and in estrogen-deprived six-month-old rats that were treated with a moderate dose of rPTH (1-34) (5 ug/kg) in vivo [97]. Similar trends were also observed in in vitro experiments [97]. Their findings showed that PTH statistically inhibited SOST gene expressions in calvaria cultures and UMR-106 osteosarcoma cells, presenting a similar time course. Meanwhile, there was a dose-response trend of hPTH (1-34) in UMR-106 cells [97]. In the same year, Teresita et al. reported that SOST mRNA expression dramatically decreased in vertebral bone by 80 - 90% after a continuous infusion in mice using PTH for four days in vivo [98]. Meanwhile, in vitro experiments showed that PTH directly inhibited SOST expression in cell cultures, such as primary neonatal murine calvaria and osteocytic MLO-A5 cells [98]. In subsequent studies, it was consistently shown that PTH inhibited both in vitro and in vivo SOST expression [99,100]. In our preliminary report, we found both n-oxPTH and Met18(ox)-PTH peptides with concentrations of 3 nmol/l and 10 nmol/l profoundly downregulated the SOST mRNA expressions in UMR-106 cells [36]. Moreover, PTH also impacts SOST protein expressions. In 2005, Teresita et al. showed that immunoblot analysis and immunostaining significantly reduced SOST protein in osteocytes [98]. PTH administration decreased SOST staining density in mice mandibular condylar cartilage and subchondral bone [101]. Similarly, Yam et al. demonstrated that, compared to the controls, PTH (1-34) treatment decreased SOST expression in the cartilage by immunohistochemical staining [102]. Furthermore, increased serum PTH levels in primary hyperparathyroidism (PHPT) mice decreased circulation SOST levels and SOST expression in calvaria [103].

On the other hand, SOST probably also mediates the role of PTH in stimulating bone formation [104]. Rhee et al. showed that the stimulatory impact of the PTH receptor signaling pathway on periosteal bone formation requires SOST downregulation and consequent Wnt activation, but the modulation of PTH by SOST is relatively weak [105]. In the mice model, a lack of PTH signaling was associated with an increased expression of SOST [106]. Moreover, Saini et al. showed that the regulation of sclerostin expression plays a pivotal role in PTH/PTH-related peptide type 1 receptor signaling in osteocytes, impacting the catabolism and anabolism of PTH [107]. In the mice model with SOST overexpressing or SOST deficiency, PTH-induced bone formation was blunt due to disorders of SOST secretion, suggesting SOST, a bone formation inhibitor, mediates PTH bone anabolism [108].

1.3.2 Clinical perspectives

Based on previous basic studies, a significant negative association between SOST and PTH is clear. Except for preclinical studies, the question of whether PTH plays a regulatory role in SOST levels has also been investigated in humans. We will introduce relevant researches in two aspects, including non-renal and renal disease. In human subjects with PHPT, circulating sclerostin levels were inversely associated with blood PTH levels [109,110] and were lower than those in euparathyroid or hypoparathyroid controls [110]. In healthy men, serum SOST levels decreased throughout the PTH infusion [111]. However, blood SOST levels in patients who had undergone parathyroidectomy were much higher compared with subjects with active PHPT, and the levels returned to normal by postoperative day 10 [112]. Moreover, serum SOST levels in postmenopausal women negatively correlated with circulating PTH levels compared with premenopausal women [92]. In addition, intermittent PTH therapy dramatically declined peripheral serum SOST and marrow plasma SOST in postmenopausal women [113]. These findings indicate that PTH downregulates SOST production in bone. Moreover, the findings from a study conducted by Drake et al. supported a hypothesis that the anabolic effects of PTH on bone were at least partly regulated by decreased SOST production [113]. A previous study demonstrated that SOST was inversely associated with PTH in non-dialysis patients, whereas no significant association was identified between SOST and PTH in both T1DM and T2DM

patients. This suggested that the repressive transcriptional impact of PTH on sclerostin production may be diminished [62]. Cejka et al. demonstrated that the level of SOST is negatively correlated with the level of iPTH in CKD patients with stage 5. Meanwhile, in predicting bone turnover and osteoblast activity, SOST outperformed iPTH [114]. In maintenance hemodialysis patients, a significant negative correlation was observed between serum sclerostin levels and log iPTH [115,116]. Similarly, serum iPTH is associated with serum sclerostin in patients undergoing peritoneal dialysis [117].

1.3.3 The importance of interactions between sclerostin and parathyroid hormone

Based on previous preclinical and clinical studies, it is widely reported that SOST production is negatively regulated by PTH, which appears to be complex on bone metabolism in whole organisms. It has been found that continuous PTH treatment stimulates catabolic bone modeling, whereas intermittent PTH treatment stimulates anabolic bone modeling [118]. Taken together with all previous findings, in animal models and humans, we assume that the anabolic properties of PTH may be mediated at least in part by inhibiting SOST production. These findings improve our understanding of mechanisms of the actions of PTH on bone. In addition, studies in dialysis patients are controversial on the roles of PTH and SOST in predicting mortality [4,86]. These conflicting outcomes may be due to the lack of studying the role of their relative levels. Thus, except further focusing on the separate effects of PTH and SOST, exploring their negative correlation and relative levels in dialysis patients were warranted.

On the other hand, pharmacologic therapy of PTH and its analogues, as well as inhibitors of SOST have been utilized in postmenopausal osteoporosis. Administration of PTH and its analogs teriparatide and abaloparatide, is often accompanied by side effects, including headache, nausea, dizziness, and leg cramps [119]. Additionally. Animal studies showed PTH analogues were associated with osteosarcoma [119]. Moreover, romosozumab, a monoclonal antibody to SOST, was reported to increase cardiovascular risk in osteoporosis patients [120]. The relevant adverse effects of these anabolic agents often give rise to interruptions in the treatment of osteoporosis, and reduce the patients' life quality. Considering the negative association between PTH

and SOST, we assume that PTH and its analogues or anti-SOST antibodies can advantageously be reduced in dose without affecting the osteoporosis treatment effect to decrease the toxicity of these anabolic drugs. Therefore, measuring the dynamic variation of PTH and SOST may be beneficial to guide the osteoporosis therapy.

1.3.4 Potential mechanisms of interactions between sclerostin and parathyroid hormone

Currently, the precise mechanisms underlying how PTH downregulates SOST gene expression are not fully understood. PTH may directly bind to the SOST promoter region to inhibit its transcription, resulting in reduced SOST gene expression. Besides, there are other possible underlying mechanisms that have been proposed: Firstly, PTH may inhibit SOST gene expression by inducing proteasomal degradation of Runt-related transcription factor 2 (Runx2), which is a transcription factor that regulates the expression of several bone-related genes, including SOST [121]. Specifically, Runx2 has been shown to bind to the SOST promoter region and upregulate its transcription, leading to increased production of SOST, which is a key regulator of bone remodeling [122]. By inducing proteasomal degradation of Runx2, PTH may therefore indirectly downregulate SOST gene expression and reduce SOST production. This mechanism may be particularly relevant in conditions such as osteoporosis, where increased sclerostin production by osteocytes is associated with impaired bone formation and increased risk of fractures. Secondly, PTH downregulates osteocyte-specific SOST expression by inhibiting the activity and/or expression of myocyte enhancer factor 2 (MEF2) in osteocytes [104,123]. MEF2 is a transcription factor that plays a crucial role in the transcriptional activation
of the SOST bone enhancer, which is important for the expression of SOST in osteocytes [123-125]. Studies have shown that the expression of MEF2 and SOST is co-localized in osteoblasts, and the expression of three types of MEF2 genes (MEF2A, MEF2C, and MEF2D) is necessary for endogenous SOST expression in UMR-106 cells, which are a model of osteoblastic cells [123]. By inhibiting MEF2 activity or expression, PTH may therefore indirectly downregulate SOST gene expression in osteocytes and reduce sclerostin production. This mechanism may be associated with

primary hyperparathyroidism, in which elevated levels of PTH are related to increased bone resorption and decreased bone density. Lastly, there have been several in vitro

studies demonstrating PTH inhibits SOST expression and protein levels primarily by activating cAMP/PKA downstream of the PTH1R [97,98,104]. Activation of the PTH1R by PTH leads to increased production of cAMP, which in turn activates PKA [126]. PKA can then phosphorylate various downstream targets, including transcription factors and co-regulators that regulate gene expression, and also promote proteasomal degradation of SOST protein. One study found that PTH-induced downregulation of SOST expression in osteocytes was dependent on PKA activation, and that inhibition of PKA activity or cAMP production attenuated the effects of PTH on SOST expression [97]. Another study also demonstrated that PTH-induced inhibition of SOST expression was dependent on cAMP/PKA activation, and that the effects of PTH could be mimicked by treatment with a cAMP analog [127] (Figure 3).

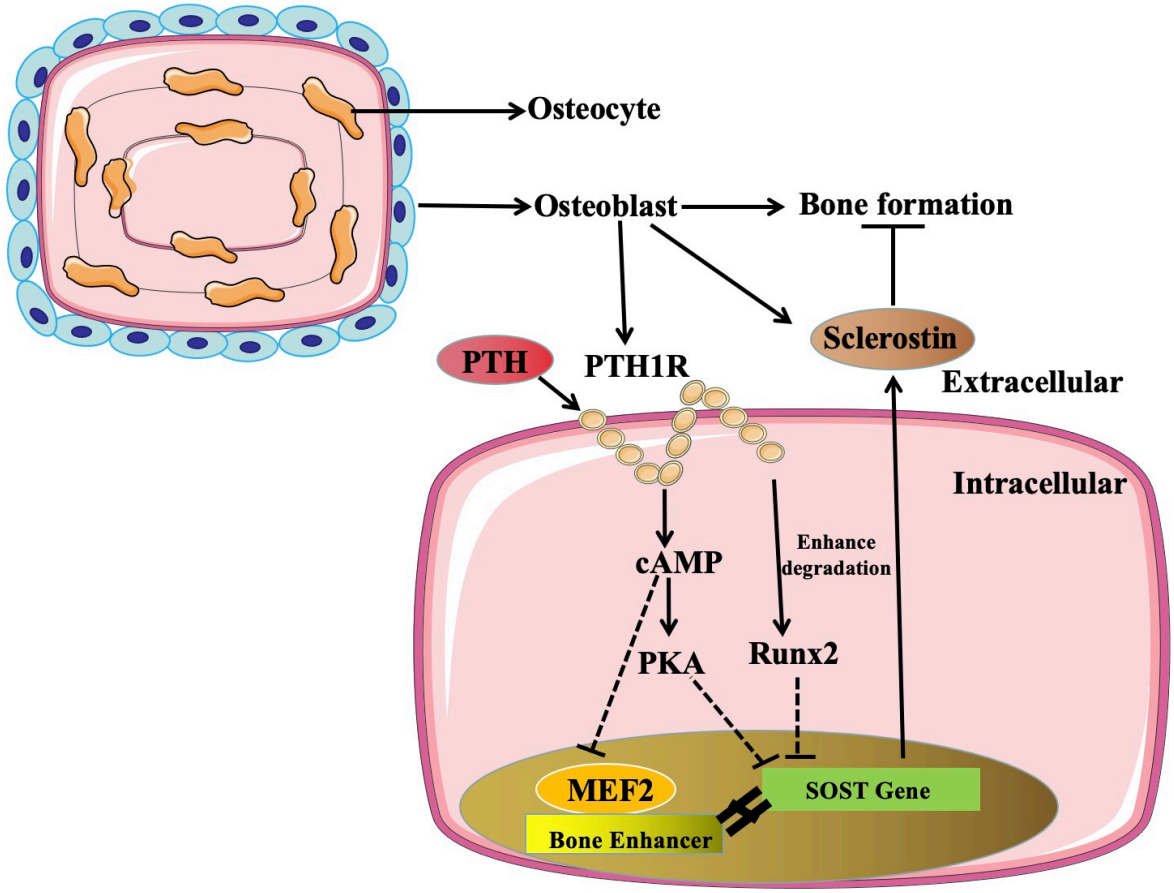


Figure 3. Mechanisms of interaction between PTH and SOST. Osteocytes can simultaneously express PTH1R and SOST. The binding of PTH and PTH1R activates the cAMP/PKA signaling pathway, inhibiting SOST gene expression. Furthermore, the binding of PTH and PTH1R can impede the actions and/or expression of MEF2 in osteocytes, which, in turn, can impact the transcriptional activation of the SOST bone enhancer, inhibiting SOST expression. Additionally, the interactions of PTH and PTH1R can promote the degradation of Runx2, facilitating SOST production. As a result, increased Runx2 degradation decreases SOST formation. This figure is on my own elaboration.

1.4 Aim of the study

One of our previous publications showed that that only bioactive PTH peptides, including n-oxPTH and Met18 (ox)-PTH, can stimulate FGF23 production in UMR106 cells in vitro experiments. Based on the findings of the studies mentioned all above, it is clear that PTH was negatively associated with SOST in animal and human studies. In addition, it is widely reported that n-oxPTH and oxPTH have different bioactivities. Furthermore, prior studies conducted both in vitro and in vivo strongly suggested that it would be advantageous to investigate inverse relationships between PTH and SOST in the future. As a part of this study, we explored the direct effects of various PTH peptides, such as n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH on SOST mRNA expression in cell cultures. Moreover, considering that four forms of PTH are present in organisms, including n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH, it is unclear how n-oxPTH interacts with oxPTH, or how various forms of oxPTH interact with one another. Further investigations were conducted in vitro to explore the effects of different combinations of four PTH peptides on the expression of the SOST gene.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1. Cell lines and cell culture material

The UMR106 rat osteoblast-like cells (ATCC, CRL-1661) were characterized by the response to PTH, bone-resorbing steroids, and prostaglandins. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose with 4,5 g/L glucose (L-glutamine) and supplemented with 5% fetal calf serum and penicillin (100 ug/ml) / streptomycin (100 ug/ml).

The UMR106 cells were separately activated with four forms of PTH peptides including n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH peptides at doses of 1, 3, 20, 30 nmol/l. As well, we tested two types of combinations of various PTH peptides in vitro, including n-oxPTH + Met8, Met18 (di-ox)-PTH peptides and Met18 (ox)-PTH + Met8, Met18 (di-ox)-PTH peptides to stimulate the UMR106 cells using the same conditions as activation using separate PTH peptides. Four forms of PTH peptides were purchased from JPT Peptide Technologies GmbH, Berlin, Germany, same as used by Zeng et al. recently [36].

2.1.2 Chemicals and reagents

The chemicals used in this study are shown in Table 1. The related reagents and kits, as well as their order number and manufacturer, are described in Table 2. Moreover, the related TaqMan assays are presented in Table 3.

Table 1. Chemicals used in this Study

Name	Manufacturer
CASY-ton	Roche (Germany)
Chloroform	Carl Roth (Germany)
DMEM, high glucose	Invitrogen (USA)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (Germany)
dNTP-Mix (dATP, dTTP, dCTP, dGTP)	Carl Roth (Germany)
Ethanol	Sigma-Aldrich (Germany)
FBS	Life Technologies (Germany)
Isopropanol	Merck (Germany)
Met8 (ox)-PTH	JPT Peptide Technologies (Germany)
Met18 (ox)-PTH	JPT Peptide Technologies (Germany)
Met8, Met18 (di-ox)-PTH	JPT Peptide Technologies (Germany)
n-oxPTH	JPT Peptide Technologies (Germany)
PBS 10×	Invitrogen (Germany)
Penicillin/streptomycin	Sigma-Aldrich (Germany)
RNase Inhibitor	Applied Biosystems (USA)
Trizol Regent	Life Technologies (Germany)
TrypLETM Express 10×	Invitrogen (Germany)
TaqMan Fast Advanced Master Mix system	Applied Biosystems (USA)

Table 2. Reagents and Kits used in this Study

Name	Catalog Number	Manufacturer
DNA-freeTM Kit (DNase Treatment and Removal Reagents)	AM1906	Invitrogen (Germany)
High-capacity cDNA reverse Transcription Kit	4368814	Applied Biosystems (USA)

Table 3. TaqMan Assays

Gene name	Species	Assay ID	Manufacturer
SOST	Rat	Rn00577971_m1; 4453320	Applied Biosystems (USA)
β-actin	Rat	4351370	Applied Biosystems (USA)

2.1.3 Devices and equipment

The name and manufacturers of devices and equipment used in this study are listed in Table 4.

Table 4. Devices and Equipment used in this Study

Name	Manufacturer
ABI StepOne plus Real-Time PCR System	Applied Biosystems (USA)
ABI 2720 Thermal Cycler	Applied Biosystems (USA)
Autoclave Typ ELVC 5075	SysTec GmbH (Germany)
CASY cell counter	Schärfe Systems (Germany)
Centrifuge Biofuge Pico/ Fresco/ Primo R	Heraeus (Germany)
GraphPad Prism 9.0	GraphPad Software (USA)
Laminar flow hood	Heraeus GmbH (Germany)
Light microscope DMIL	Leica (Germany)
Incubator for Cell culture HERAcell 150i	Heraeus GmbH (Germany)
Microplate-Reader infinite M200	Tecan (Germany)
Pipette Research	Eppendorf (Germany)
Shaker Vortex REAX 2000	Heidolph (Germany)
Thermoblock TechneDri-Block® DB-2D	Biostep GmbH (Germany)

2.2 Methods

2.2.1 Cell culture

The cryogenic vials of the UMR106 rat osteoblast-like cell lines were taken from the -80°C freezer and quickly melted by gentle agitation in a water bath at 37°C for approximately two minutes. The O-ring and cap were always kept out of direct contact with the water to reduce the possibility of contamination. The vial was removed from the water bath as soon as the contents were thawed and decontaminated by dipping in or spraying with 70% ethanol. Subsequently, the vial contents were rapidly transferred into a 75 cm^2 tissue culture flask with a pre-heated complete culture medium. The cell lines were then incubated in a suitable incubator at 37°C in a 5% CO2 solution in an air atmosphere and could grow adherently at the bottom of the flask. Medium renewals were performed two to three times per week.

The cells were passaged until 70 - 80% of confluence was achieved. In light of the subculturing procedure, the medium was removed, and the cells were rinsed with 1 × PBS once. Subsequently, the solution was removed, and 3 ml of 1% triple solution was added. The flask was incubated at 37°C until the cells detached. Thereafter, the cells were passaged with a sub-cultivation ratio of 1:2 or 1:6 and transferred into new culture flasks with fresh culture medium.

The number of cells was evaluated through the cell counter. Cells were seeded in six-well plates with 0.6×10^6 per well. The UMR106 osteoblast-like cells were grown for 24 hours with 2 ml per well and then removed from the previous medium before adding an equal volume of fresh culture medium into each well. The cells were treated with or without the different types of PTH 1-34 derivatives, such as n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH peptides with doses of 1 nmol/l, 3 nmol/l, 20 nmol/l, 30 nmol/l, for additional 24 hours. Similarly, we used various combinations of PTH peptides to stimulate the UMR106 cells including n-oxPTH (concentrations: 3 nmol/l, 10 nmol/l, 100 nmol/l) + Met8, Met18 (di-ox)-PTH (concentrations: 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, 100 nmol/l) peptides and Met18 (ox)-PTH (concentrations: 10 nmol/l, 100 nmol/l) + Met8, Met18(di-ox)-PTH (concentrations: 0 nmol/l, 3 nmol/l,

10 nmol/l, 30 nmol/l, 100 nmol/l) peptides under the same conditions. Thereafter, the supernatant and cells were harvested for the subsequent experiments.

2.2.2 RNA isolation

The collected cell samples were washed with 1 × PBS three times, and then 0.5 ml Trizol reagent was added per well. Finally, the cells were homogenized by pipetting up and down. The homogenized solutions were incubated for 5-10 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Subsequently, we added 0.1 ml of chloroform and vigorously shook the samples for 15 seconds. After incubating at room temperature for two to three minutes, we centrifuged the samples for 15 minutes at 12,000 × g at 4°C. Subsequently, we transferred the samples to the aqueous phase, including the RNA, to fresh tubes and added 1ul of glycogen into each EP tube before adding 0.25 ml of isopropanol to the aqueous phase and incubating them for 10 minutes at room temperature. Thereafter, the aqueous phase was centrifuged for 10 minutes at 12,000 × g at 4°C. Subsequently, total RNA precipitated and a white gel-like pellet formed on the side of the tube. Next, the supernatant was discarded, and we washed the pellet with 1 ml of 75% ethanol and centrifuged the mixture at 7500 × g for 5 minutes at 4°C. Again, the supernatant was removed until no liquid was visible, and we airdried the pellets for 5-10 minutes. Finally, we redissolved the RNA pellets in 20-30 µl nuclease-free (NF) H_2O (NFH_2O) according to the size of the pellets and stored them at -80°C for the following trials.

2.2.3 RNA purification

The procedure for RNA purification is as follows: RNA samples were added as DNase digestion reagents, including 10 x DNase I Buffer and rDNase I, and then mixed gently and incubated at 280 rpm for 20-30 minutes at 37°C. Subsequently, the RNA samples were resuspended by adding a DNase inactivation reagent. After that, we mixed them well before incubating the RNA samples at room temperature for 2 minutes. Then, the samples were centrifuged for 1.5 minutes at 10000 × g. Finally, we transferred the RNA

samples into fresh tubes. Then, concentrations of total RNA were assessed using UV spectroscopy by measuring the absorbance of a diluted RNA sample at 260nm. The equation is A260 × dilution factor × 40 ug/ml = μg RNA/ml. The volume details are described in Table 5.

Table 5. Components of DNA Free Kit

Reagents	Volume
10 x DNase I Buffer	0.1 volume based on the volume of Nuclease free water
rDNase I	1μl
DNase Inactivation Reagent	0.1 volume based on the volume of Nuclease free water

2.2.4 cDNA synthesis

In the cDNA reverse transcription (RT) system, 1 μg total RNA was added per 20 μl reaction using a high-capacity cDNA reverse transcription kit. We prepared the 10μl mixture containing 1 μg RNA sample and NFH_2O. Then we prepared an RT master mix using high-capacity cDNA reverse Transcription Kit, including RT Buffer, dNTP Mix, RT Random Primers, Reverse Transcriptase, RNase Inhibitor, and Nuclease free. Subsequently, we added 10 μl cDNA synthesis master mix into each 10 μl RNA mixture. Thereafter, the tubes with the 20 μl reaction were mixed completely and then briefly centrifuged to spin down all contents to the bottom of the tubes. Finally, the prepared mixtures were immediately placed in the thermocycler for cDNA synthesis.

Details about the RT master mix are shown in Table 6. The cDNA synthesis conditions are described in Table 7. All procedures were performed on ice. After cDNA synthesis was complete, cDNA products were stored at -20 °C until the next experiments.

Table 6. cDNA Synthesis Materials

Component	Volume/Reaction (µl)
10 x RT Buffer	2.0
25 x dNTP Mix (100mM)	0.8
10 x RT Random Primers	2.0
MultiScrie ^{TM} Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Nuclease-free H_2O	3.2

Table 7. cDNA Synthesis Conditions

Thermal Conditions				
Temperature (°C)	25	37	85	4
Time (minutes)	10	120	5	∞

2.2.5 Real-time quantitative polymerase chain reaction (RT-qPCR)

To begin with, 9 µl cDNA was pipetted in each well of the 96-well plate with 0.5 µl synthesized cDNA and 8.5 µl Nuclease free water per well. Then we mixed TaqMan fast advanced master mix with SOST or actin assays with a ratio of 10:1 to make assay mixtures and mixed them completely by carefully pipetting up and down. Next, we added 11 µl assay mixtures into each well and sealed the 96-well reaction plate with a foil cover using the brown-colored applicator. Finally, the plate was centrifuged briefly to spin down the samples to the bottom of the wells and discharge the air bubbles. Then we loaded the plate in the ABI StepOneTM plus RT-PCR machine. Every sample was in three-fold replicates along with endogenous controls. We made a no-template control for each experiment containing only the assay mixtures and NFH_2O. All steps were performed on ice. The details in the TaqMan master mix system and the setting parameters of the StepOne plus machine are presented in Tables 8 and 9. The measured Cycle threshold (Ct) values were normalized using beta-2-microglobulin and expressed as fold-over control samples.

Table 8. TaqMan Fast Advanced Master Mix System

PCR Reaction Mix Component	Volume per 20 µl Reaction for Three Replicates (µl)
cDNA	0.5
TaqMan master mix	10
SOST assay/Actin assay	1
NFH_2O	8.5

Table 9 Plate Experiment Parameters for TaqMan® Gene Expression Assays

Thermal Cycling Conditions		
Stage	**Temperature (°C)**	**Time (minutes : seconds)**
Hold	50	02:00
Hold	95	02:00
Cycle	95	00:01
(40 Cycles)	60	00:20

2.2.6 Statistical analysis

One-way ANOVA test was used to compare SOST gene expression in cultured UMR106 osteoblast-like cells induced by n-oxPTH and different forms of oxPTH-peptides, as well as different combinations of four forms of PTH peptides, followed by Dunnett's multiple comparisons test. The significance was defined if a p-value was less than 0.05. All data were analyzed using SPSS software for Windows (version 26; IBM, Chicago, IL, USA) and GraphPad Prism 9.0 (GraphPad Software, Inc. La Jolla. California, USA).

3 RESULTS

3.1 Separate effect of PTH peptides on SOST expression in cultured cells

To investigate how oxidized and non-oxidized PTH influence the SOST gene expression, we performed the cell culture experiment using UMR106 osteoblast-like cells. In this study, we stimulated UMR106 cells using n-oxPTH, Met18 (ox)-PTH, Met8, Met18 (di-ox)-PTH, and Met8 (ox)-PTH peptides for 24 hours in the incubator with the various concentrations: 1nmol/l, 3nmol/l, 20nmol/l, and 30 nmol/l, respectively. The levels of SOST expression were measured by qRT-PCR (Figure 4 a–d).

Our findings show that the n-oxPTH peptide strongly inhibited the SOST production in UMR106 cells, followed by Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH peptides. The Met8 (ox)-PTH had the weakest inhibitory impact on SOST gene expression in cell culture. As for all forms of PTH peptides, the levels of SOST inhibition were dependent on doses of PTH peptides. When compared with the control group, we can see that all dosages of n-oxPTH peptides significantly decreased the SOST mRNA levels in UMR106 cells ($P < 0.001$) (Figure. 4a). However, there was no difference between various concentrations of n-oxPTH peptides ($P > 0.05$). Similarly, Met18 (ox)-PTH markedly inhibited the gene expression of SOST at all dosages (1, 3, 20, 30 nmol/l) ($P < 0.001$) (Figure. 4b). Moreover, Met8, Met18 (di-ox)-PTH peptides led to a relatively weak inhibition of SOST gene expression at all dosages (1, 3, 20, 30 nmol/l) ($P < 0.05$) (Figure. 4c). However, Met8 (ox)-PTH peptides slightly impeded the SOST mRNA levels only at the concentrations of 20 nmol/l and 30 nmol/l. Notably, the intergroup difference of only occurred in Met8 (ox)-PTH peptides between concentrations of 1 nmol/l and 30 nmol/l ($P < 0.05$) in this study (Figure. 4d).

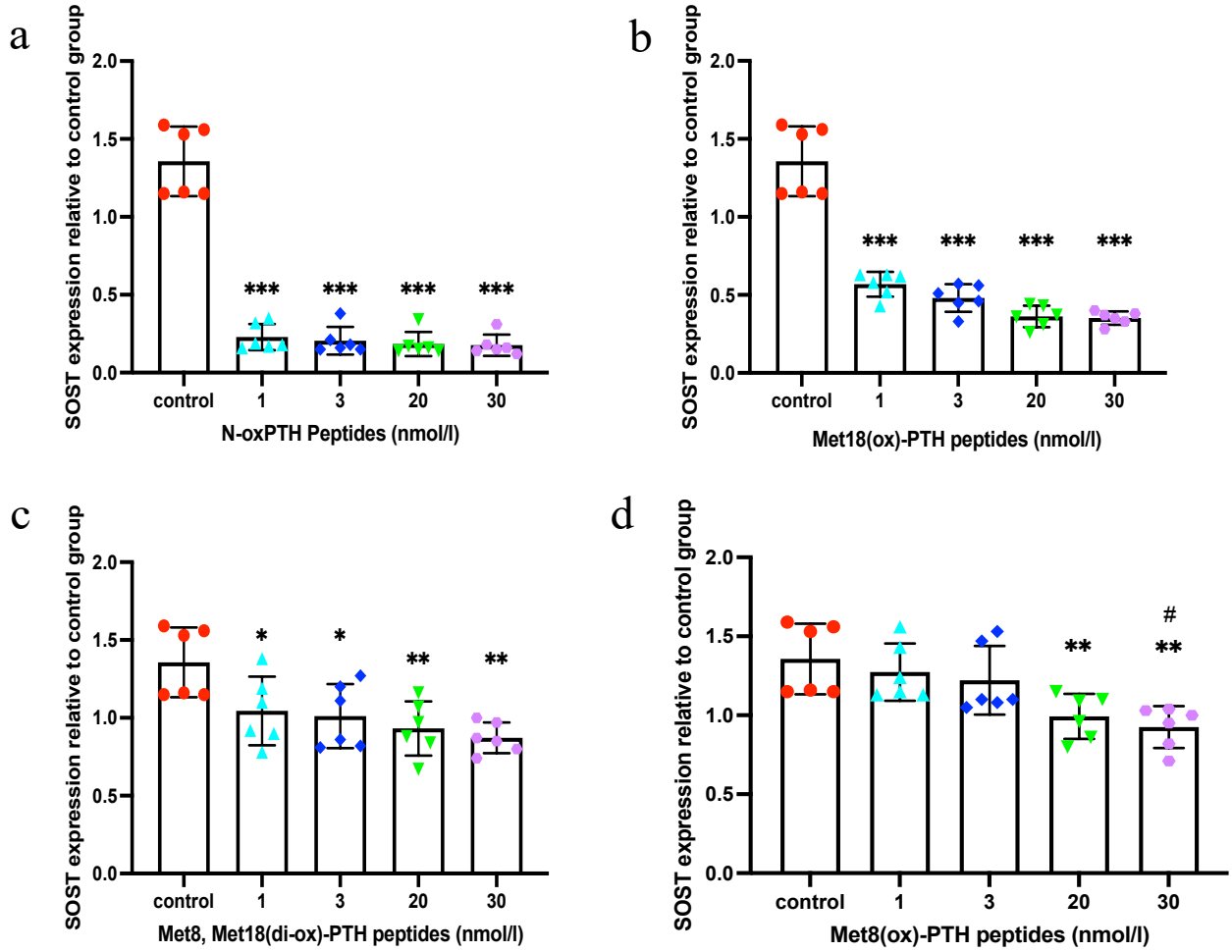


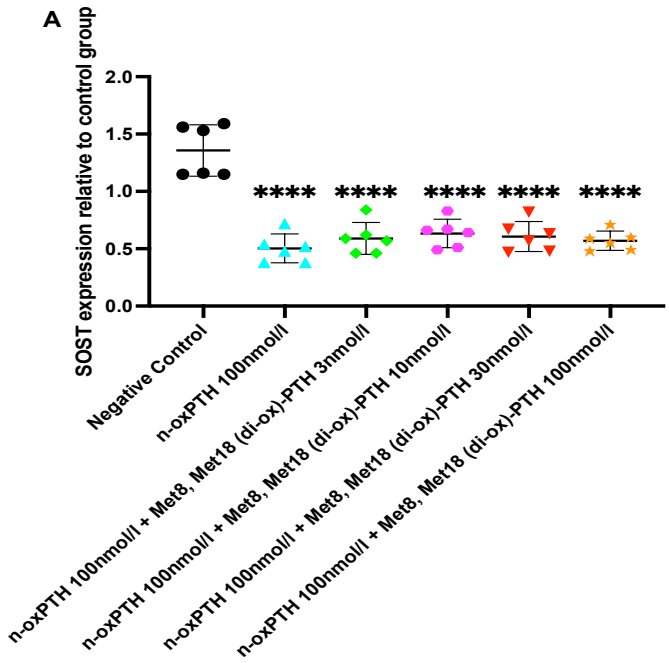
Figure 4. Effects of n-oxPTH and various forms of oxPTH on SOST gene expression in UMR106 rat osteoblast-like cells (a-d). We cultured the cells for the first 24 hours and then treated them with or without various PTH 1-34 derivatives for another 24 hours at 1, 3, 20, and 30 nmol/l, respectively. The experiment was repeated at least six times, and the levels of SOST mRNA expression were measured by qRT-PCR. **a and b** In terms of n-oxPTH and Met18 (ox)-PTH peptides, all dosages caused a significant decrease in SOST gene expression when compared with negative controls ($P < 0.001$). **c** A similar trend was also observed in the Met8, Met18 (di-ox)-PTH peptides, despite a relatively slight inhibition in contrast to negative controls ($P < 0.05$). **d** However, the Met8 (ox)-PTH peptides inhibited the SOST mRNA synthesis only at dosages of 20 and 30 nmol/l, instead of low dosages of 1 and 3 nmol/l when compared with negative control ($P < 0.05$). Moreover, a significant intergroup difference was observed between 1 nmol/l and 30 nmol/l of Met8 (ox)-PTH. The x-axis means the different concentrations. The y-axis represents the relative SOST expression. All data were calculated using one-way ANOVA test, after that, Dunnett's multiple comparisons test was performed. * $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. negative control group. $^{\#}P<0.05$, indicates 1 nmol/l vs. 3

nmol/l Met8(ox)-PTH peptides. I bars indicate standard deviation. Note: Partial results of this work were accepted on: Pflügers Archiv - European Journal of Physiology, Feb 2024.

3.2 Effect of combination of non-oxidized PTH and oxidized PTH peptides on sclerostin in cultured cells

To observe the direct interaction of n-oxPTH with oxPTH, and how this interaction affects the SOST gene expression, we further activated UMR106 cells using combinations of n-oxPTH and Met8, Met18 (di-ox)-PTH peptides in vitro. In this study, the applied concentrations of n-oxPTH peptides included 3 nmol/l, 10 nmol/l, and 100 nmol/l, respectively.

As for figure 5 (A-C), we found that all combinations of n-oxPTH with Met8, Met18 (di-ox)-PTH peptides significantly inhibited the expression of the SOST gene with regardless of various concentrations of n-oxPTH peptides when compared with the negative control ($P < 0.001$). In addition, the inhibition of SOST by the addition of different concentrations of 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, 100 nmol/l Met8, Met18 (di-ox)-PTH peptides to n-oxPTH was no significant difference.



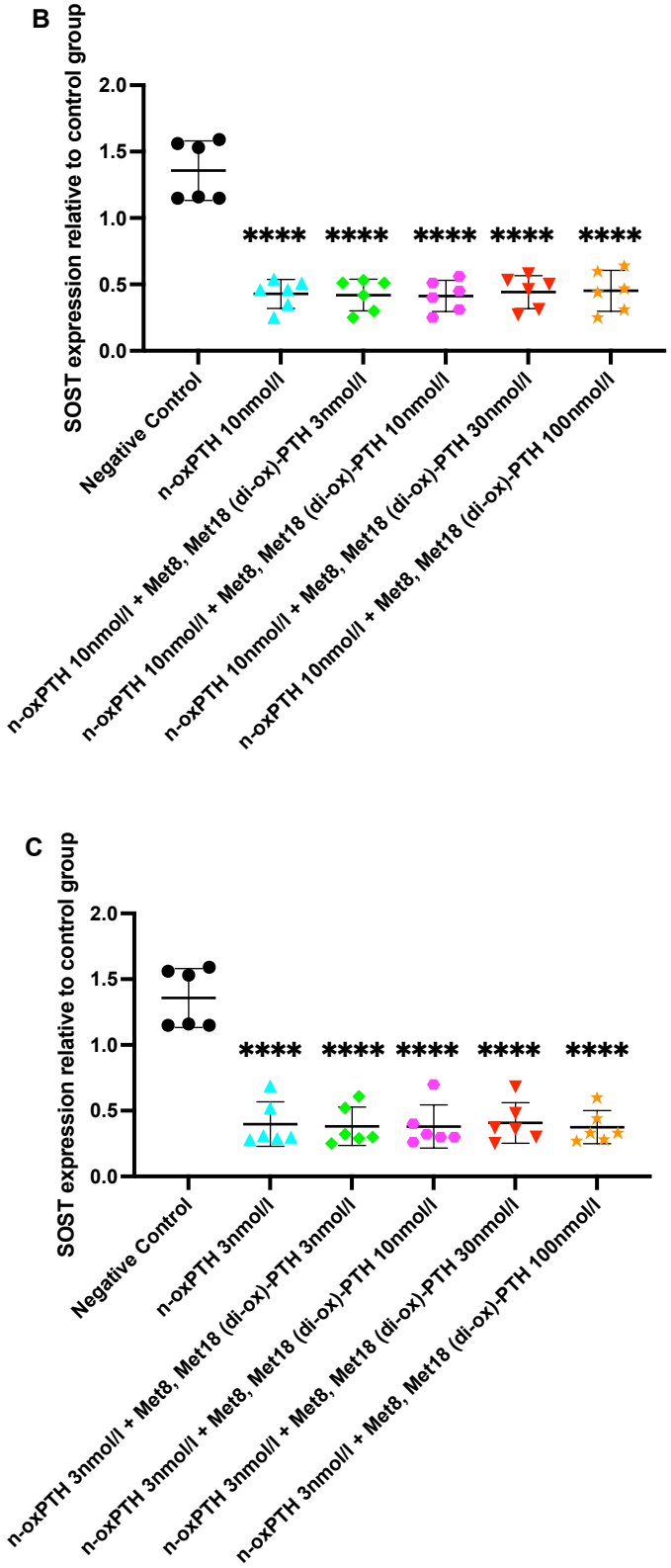


Figure 5. Effectiveness of co-stimulation of n-oxPTH and oxPTH on SOST gene expression (A-C). The UMR106 were incubated under the same conditions as the figure 4

described. The difference was used the different stimulations to treat UMR106 cell lines. In this part, we combined three concentrations of n-oxPTH peptides with five concentrations of Met8, Met18 (di-ox)-PTH peptides separately. The experiment was repeated at least six times, and the levels of SOST mRNA expression were measured by qRT-PCR. **A** The concentration of n-oxPTH was 100 nmol/l for each 6-well except for negative control, and combined with 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, and 100 nmol/l of Met8, Met18 (di-ox)-PTH in each 2 μl medium, respectively. **B and C** The concentrations of n-oxPTH were changed to 10 nmol/l and 3 nmol/l, respectively. Other conditions were the same as A. The x-axis means the different concentrations of combination of n-oxPTH and Met8, Met18 (di-ox)-PTH peptides. The y-axis represents the relative SOST expression. The statistical analysis method was the same as the figure 4. **** P<0.0001 vs. negative control group. I bars indicate standard deviation.

3.3 Effect of combination of various oxidized PTH peptides on sclerostin in cultured cells

To investigate the interplay between different forms of PTH peptides, and the effects of their coexistence on SOST expression, we activated the UMR106 cell lines using Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides at different doses (Figure 6 i-ii). In this study, we combined Met18 (ox)-PTH peptides at doses of 10 nmol/l and 100 nmol/l with various concentrations of Met8, Met18 (di-ox)-PTH peptides including 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, 100 nmol/l, respectively.

The findings showed that all combinations of Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides markedly decrease the generation of SOST with regardless of concentrations of Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides in contrast to the negative control ($P < 0.01$). Moreover, there was no statistical difference found on the inhibition of SOST gene expression among other groups. The addition of Met8, Met18 (di-ox)-PTH to Met18 (ox)-PTH in vitro did not affect the effect of Met18 (ox)-PTH on SOST production.

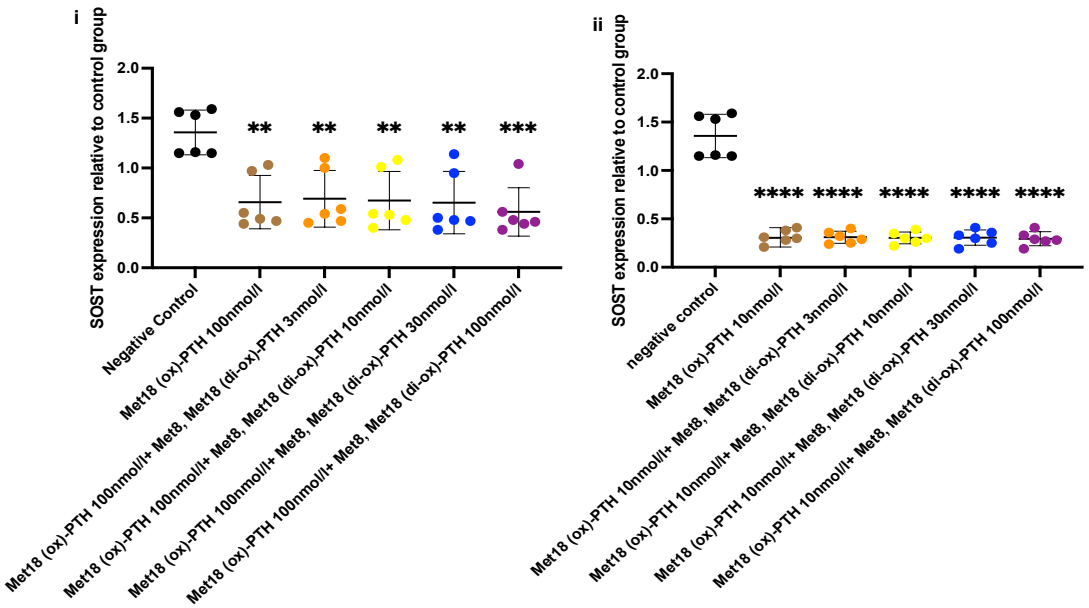


Figure 6. Inhibitory effect of co-stimulation of Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH on SOST gene expression (i, ii). The cells were activated by Met18 (ox)-PTH with doses of 10 nmol/l and 100 nmol/l, and separately combined with Met8, Met18 (di-ox)-PTH with concentrations of 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, and 100 nmol/l in each 2 µl medium. Other conditions were same as the figure 4 and the figure 5. The experiment was repeated at least six times, and the levels of SOST mRNA expression were measured by qRT-PCR. **i** The concentration of Met18 (ox)-PTH was 100 nmol/l for each 6-well except for negative control, and combined with 0 nmol/l, 3 nmol/l, 10, nmol/l 30 nmol/l, and 100 nmol/l of Met8, Met18 (di-ox)-PTH, respectively. **ii** Met18 (ox)-PTH concentration was decreased to 10 nmol/l. The x-axis means the different concentrations of combination of Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides. The y-axis represents the relative SOST expression. The statistical description was the same as the figure 4. $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$ vs. negative control group. I bars indicate standard deviation.

4 DISCUSSION

Our findings found that effects of n-oxPTH and Met18 (ox)-PTH peptides on SOST gene expression are more pronounced than effects of Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH in cultured UMR106 osteoblast-like cells. Additionally, both co-stimulation of n-oxPTH and Met8, Met18 (di-ox)-PTH peptides, as well as Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides significantly inhibit SOST production in vitro. Our primary findings showed that Met8, Met18 (di-ox)-PTH did not interact with either n-oxPTH or Met18 (ox)-PTH in ex vivo.

Cell culture and animal studies were initially used to investigate the association between SOST and PTH. As Keller and Kneissel demonstrated, in a mouse model of local bone formation, SOST mRNA expression in PTH-treated calvariae after the last hPTH 1-34 administration was significantly decreased when compared to vehicle-treated calvariae [97]. Moreover, it has been shown that rPTH (1-34) treatment at a dose of 80 ug/kg six hours after the single injection of rPTH (1-34) significantly decreased the expression of SOST in the femur of estrogen-deprived six-month-old rats treated with rPTH (1-34) (5 ug/kg) in vivo [97]. Experiments conducted in vitro also demonstrated similar trends. There was a dose-response trend of hPTH (1-34) in UMR-106 cells, also PTH inhibited the expression of SOST genes in both calvaria cultures and osteosarcoma cells [97]. In the same year, Bellido et al. reported that after continuous infusion of PTH using a microcosmic pump for four days in mice, SOST mRNA expression in vertebral bone decreased by 80 - 90%, which was accompanied by a significant reduction of SOST protein in osteocytes by quantitative immunoblots analysis and immunostaining [98]. Meanwhile, according to in vitro experiments, PTH inhibits SOST expression in cells cultured from primary neonatal murine calvaria cells and osteocytic MLO-A5, suggesting that PTH affects this type of cell directly [98]. Moreover, after a single injection of PTH, the SOST mRNA was transiently reduced by 50% within two hours [98]. In contrast to continuous infusion of PTH, the intermittent administration of PTH by daily injections for four days did not affect levels of SOST mRNA and protein in the bones of animals [98]. Their findings found the effects of continuous administration and intermittent administration of PTH on SOST production are controversial [98] - a finding that is also inconsistent with the results from the study conducted by Keller et al [97].

To further explore the effects of PTH administration on SOST, subsequently, Silvestrini et al. conducted a study about the actions of intermittent administration of PTH on SOST expression in three distinct bone segments of the rat tibia - metaphyseal, epiphyseal, and cortical diaphyseal bones [100]. After intermittent PTH administration, they showed that SOST mRNA and protein levels decreased in all three areas [100]. Moreover, Prediaux et al. showed that PTH significantly suppressed SOST mRNA levels in mesenchymal progenitor cell lines [99]. In the mice model's mandibular condylar cartilage and subchondral bone, PTH administration decreased SOST staining density [101], and Yam et al. showed PTH (1-34) decreased SOST. Similarly, Yam et al. demonstrated that compared to the controls, PTH (1-34) treatment decreased SOST expression in the cartilage by immunohistochemical staining [102]. Moreover, increased serum PTH levels in PHPT mice decreased circulation, SOST levels, and sclerostin expression in calvaria [103]. Taken together, both in vitro and in vivo studies have demonstrated that PTH directly decreases SOST levels. In line with the findings of previous in vitro and in vivo studies, we found that the administration of PTH peptides significantly inhibited the SOST gene expression for 24 hours in UMR106 cells. However, this study underlined the biological activity of PTH is affected by its oxidation, first suggesting only biological forms of PTH downregulate SOST production.

Methionine (Met) residues of proteins can be easily oxidized to form methionine sulfoxide by many oxidants produced in biological systems, including hydroxyl radicals, H_2O_2, chloramine, and hypochlorite. The oxidation of Met caused a loss of protein bioactivity within organisms [128]. As a key hormone regulating calcium and phosphate metabolism, PTH can be oxidized at Met residues 8 and 18 in vivo, resulting in a mixture of three variations such as Met8, Met18, and Met8 together with Met18. In contrast to Met18, Met8 is less easily oxidized due to its location in a hydrophobic pocket [21]. The oxidized Mets within PTH accompany conformational changes of PTH [23]. Previous studies reported that the oxidation at position Met18 has the smallest impact on secondary structure when compared with oxidized Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH, which means the conformation of Met18 PTH is more similar to that of n-oxPTH [23]. In addition, the oxidation of both Mets, namely, Met8, Met18(di-ox)-PTH, gives rise to substantial changes, which are greater than the sum of independently oxidized Met residues [23]. The differences in conformation changes lead to differences in the biological activity of various forms of PTH. Preclinical research

proved that Met18 (ox)-PTH maintains biological action, while Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH possess less or no bioactivities [23]. In this study, we used four types of PTH peptides such as n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH under concentrations of 1 nmol/l, 3 nmol/l, 20 nmol/l, and 30 nmol/l to stimulate UMR106 rat osteoblast-like cells for 24 hours. Our findings showed that both n-oxPTH and Met18 (ox)-PTH peptides at all doses markedly inhibited the production of SOST mRNA through qRT-PCR compared with negative controls. The inhibition of SOST gene expression declined with the increase in PTH peptides. However, the inhibition of SOST production by Met8 (ox)-PTH peptides was the weakest. Only high doses (20 nmol/l, 30 nmol/l) can inhibit SOST expression in vitro. One of our prior studies has observed the effects of different forms of oxidized PTH peptides on FGF23 mRNA expression[36]. Zeng et al. showed that both n-oxPTH and Met18 (ox)-PTH peptides, but not Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH, significantly stimulates FGF23 gene production UMR106 cells[36]. Our in vitro experiments are generally in line with the outcomes of Zeng et al. in terms of the biological effects of n-oxPTH and Met18 (ox)-PTH peptides on SOST expression using qRT-PCR (SYBR Green I Assay). Collectively, our study first found that only the bioactive forms of PTH, including n-oxPTH and Met18 (ox)-PTH, were capable of suppressing SOST gene expression, while other forms of PTH such as Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH did not have this effect. This finding is significant as it helps to further our understanding of the complex molecular mechanisms that regulate bone metabolism and the role that PTH plays in this process. It may also have implications for the development of new therapies for bone disorders such as osteoporosis, which are characterized by an imbalance in bone remodeling due to dysregulation of the SOST gene.

In order to investigate the interplay of n-oxPTH and oxPTH, as well as of different forms of oxPTH, we co-stimulated the UMR106 cells using various forms of PTH peptides. In this study, we found that the addition of oxPTH (Met8, Met18 (di-ox)-PTH) to n-oxPTH did not affect the effect of n-oxPTH on SOST expression with regardless of low or high concentrations in cell cultures. Similarly, Met8, Met18 (di-ox)-PTH did not interfere with the effect of Met18 (ox)-PTH on SOST production. Thus, we assume that there was no direct interaction between n-oxPTH and Met8, Met18 (di-ox)-PTH, also between Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH. The explanation might be that Met8, Met18 (di-ox)-PTH does not compete PTH1R with either n-oxPTH or Met18 (ox)-

PTH. The supportive evidence is based on the study from Hocher and his colleagues [38]. They suggested that oxidized PTH tends to face away from PTH1R. Thus, we assume that oxidized PTH is likely to free up space for biologically active PTH, including n-oxPTH and Met18 (ox)-PTH, to bind PTH1R. Another possible explanation is that the interaction among four forms of PTH relies on the participation of some unknown factors in vivo in the real world. It should be noted that our in vitro study performed the combination of two forms of PTH in vitro, rather than the co-existence of all four forms of PTH in organisms. Therefore, our findings in the part might not be representative to reveal the actual interaction of four forms of PTH in vivo. Consequently, these experimental results only reflect interactions between some of the different PTH forms in vitro. Further, our findings showed that low concentrations of Met18 (ox)-PTH (10 nmol/l) inhibited SOST expression more than high concentrations of Met18 (ox)-PTH (100 nmol/l), which is inconsistent with the findings in the figure 4. The possible reasons are uneven cell counts or technical difficulties.

At present, the detailed mechanisms of inverse interaction between PTH and sclerostin remains unclear. According to previous studies, several assumptions can be made (Figure 3). To begin with, PTH inhibits SOST expression probably by inducing proteasomal degradation of Runx2 [121] - a SOST-dependent gene - and upregulates SOST production [122]. This mechanism explains that continuous PTH stimulation markedly decreases the SOST gene expression and SOST protein in vitro and in vivo osteocytes, suggesting the direct impact of PTH on osteocytes [98]. Second, PTH downregulates osteocyte-specific SOST expression by inhibiting the activity and/or expression of myocyte enhancer factor 2 (MEF2) in osteocytes [104,123], which is important for the transcriptional activation of the SOST bone enhancer [123-125]. Moreover, the expressions of MEF2 and SOST were co-localized in osteoblasts, and the expressions of MEF2 genes, such as MEF2A, MEF2C, and MEF2D, were necessary for endogenous SOST expression in UMR-106 cells [123]. Similarly, in vivo experiments also demonstrated that MEF2C is required to control SOST expression levels [124,125]. Last but not least, many in vitro studies have proven that PTH-induced inhibition of SOST mRNA and sclerostin levels is direct and primarily mediated by the activation of the cAMP / protein kinase A system (PKA) signaling pathway downstream of PTH1R [97,98,104]. To elucidate this mechanism, Keller et al.

pretreated UMR-106 cells for 30 minutes using the protein synthesis inhibitor cycloheximide (CHX) and then treated the cells with 10 nm hPTH (1-34) or not. The researchers found that PTH stably suppresses SOST expression in UMR-106 cells with or without CHX treatment [97]. Moreover, PTH1R can activate the cAMP / PKA signal transduction pathway - the only signaling that has been proven to be associated with anabolic effects in bone [97]. The inhibitory effect of cAMP inducer forskolin on SOST is comparable to that of PTH [97]. These findings supported that the direct activation of cAMP signaling by PTH1R can directly inhibit SOST expression. Overall, while the exact mechanisms of how PTH downregulates SOST gene expression are not fully understood, current research suggests that it likely involves a complex interplay of multiple signaling pathways and regulatory factors. Further studies are needed to fully elucidate the underlying mechanisms of the inverse relationship between PTH and SOST which would be beneficial for the treatment of relevant diseases in clinical practice, such as osteoporosis and hyperparathyroidism.

Several limitations need to be considered in this study when interpreting its findings. To begin with, we observed various effects of n-oxPTH, Met18 (ox)-PTH, Met8 (ox)-PTH and Met8, Met18 (di-ox)-PTH peptides on SOST mRNA expression in cell culture. In contrast, we did not assess the effects of different PTH peptides on the SOST protein expression levels. Secondly, we only used one type of cell lines in the study (UMR106 osteoblast-like cells). More evidence from other independent cell lines such as osteocytic MLO-A5 cells is also warranted which is beneficial to enhance our findings. Moreover, except for in vitro studies, SOST gene expression induced by n-oxPTH- and different forms of oxPTH-peptides appeals further researches on animal models. Third, we examined the effect of n-oxPTH and other forms of oxPTH on SOST production only after 24 hours. We should observe more time points, such as after 6 hours, 12 hours, and 18 hours. Last but not least, considering multiple previous studies focusing on intracellular signaling events such as the cAMP / PKA activation that occur upstream of PTH-induced SOST suppression, our current model lacks data on assessing effects of different PTH peptides on these signaling events. Thus, strengthened studies are needed to explore mechanisms of these upstream signaling events that lead to PTH-induced sclerostin suppression.

To conclude, the results of the present study suggest that the bioactive PTH, such as n-oxPTH and Met18 (ox)-PTH, significantly inhibit SOST gene expression in vitro. In addition, we found that the co-existence of either n-oxPTH and oxPTH (Met8, Met18 (di-ox)-PTH), or Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH did not interact with each other in cultured cells. The significant inhibitory effects of both n-oxPTH and Met18 (ox)-PTH on SOST expression were not affected by the addition of Met8, Met18 (di-ox)-PTH in ex vivo. At present, the actual interaction among different forms of PTH in vivo remains unclear, more researches on mechanisms are needed in the near further.

SUMMARY

SOST is a negative regulator of bone homeostasis. It is mainly produced by osteocytes. PTH acts on bones, intestines, and kidneys to regulate calcium, phosphate and vitamin D metastasis. Previous preclinical and clinical studies have proved that SOST is inversely associated with PTH both in vitro and in vivo. Currently, the specific mechanisms of the negative association between SOST and PTH continue to be incompletely understood.

Two of the amino acids in PTH (positions 8 and 18) are easily oxidized in vivo. Prior studies have demonstrated that the oxidation of the PTH leads to a partial or complete loss of bioactive action, which depends on the specific oxidation position of the PTH. As a consequence, PTH oxidized at Met18 (Met18 (ox)-PTH) remains bioactive, whereas PTH oxidized at Met8 (Met8 (ox)-PTH) or both Met8 and Met18 together (Met8, Met18 (di-ox)-PTH) shows little or no bioactivity. In organisms, four forms of PTH co-exist in the peripheral blood including non-oxidized PTH (n-oxPTH), Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH. Currently, it is unclear how n-oxPTH and different forms of oxPTH induce SOST inhibition, respectively. Moreover, how four types of PTH interact with one another, and how their interplay influences the inhibition of SOST have not been elucidated.

In this study, we evaluated different effects of four forms PTH peptides on the expression of the SOST gene in UMR106 osteoblast-like cells, which were separately activated by n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH for 24 hours. Our in vitro study found both n-oxPTH and Met18 (ox)-PTH at doses of 1 nmol/l, 3 nmol/l, 20 nmol/l, 30 nmol/l significantly inhibit SOST gene expression, while Met8 (ox)-PTH has the weakest inhibition of SOST production. Based on our prior findings above, we primarily hypothesized that n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH can interact with one another, and their interplay can influence the SOST expression. Thus, we stimulated the UMR106 cells using two kinds of combinations of PTH peptides. One combination is n-oxPTH with concentrations of 3 nmol/l, 10 nmol/l,100 nmol/l and Met8, Met18 (di-ox)-PTH with concentrations of 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, 100 nmol/l, respectively. The

aim is to investigate the interaction between n-oxPTH and oxPTH. The other combination is Met18 (ox)-PTH with concentrations of 3 nmol/l, 10 nmol/l and Met8, Met18 (di-ox)-PTH with concentrations of 0 nmol/l, 3 nmol/l, 10 nmol/l, 30 nmol/l, 100 nmol/l, respectively. We aimed to explore the interplay among various forms of oxPTH. However, our primary results in vitro were not inspiring. Our current study did not find the direct interaction either between n-oxPTH and Met8, Met18 (di-ox)-PTH, or between Met18 (ox)-PTH and Met8, Met18 (di-ox)-PTH. To strengthen our conclusions, further research design should be more complete including following improvements: (1) using different types of cell lines, such as osteocytic MLO-A5 cells lines or primary bone-derived cells to enhance our findings; (2) measuring the dynamic SOST gene expression by testing at different time points (such as after 6 hours, 12 hours, and 18 hours) to evaluate the changes of PTH-induced SOST inhibition over time; (3) assessing the effect of different forms of PTH on SOST expression at protein levels, measuring by the enzyme-linked immunosorbent assay or Western blot; (4) building animal models to evaluate the inverse association of n-oxPTH, Met8 (ox)-PTH, Met18 (ox)-PTH, and Met8, Met18 (di-ox)-PTH with SOST in vivo.

To conclude, bioactive PTH, including n-oxPTH and Met18 (ox)-PTH, inhibits SOST production in vitro significantly. Moreover, the addition of Met8, Met18 (di-ox)-PTH peptides to either n-oxPTH or Met18 (ox)-PTH did not affect effects of the n-oxPTH or the Met18 (ox)-PTH on SOST suppression.

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APPENDIX

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PUBLICATION LIST

Published Thesis:

1. **Mei Li**, Zoran Popovic, Chang Chu, Christoph Reichetzeder, Wolfgang Pommer, Bernhard K. Krämer, Berthold Hocher. Impact of Angiopoietin-2 on Kidney Diseases [J] Kidney Dis (Basel), 2023, 9(3): 143-156.
2. **Mei Li**, Zoran Popovic, Chang Chu, Bernhard K. Krämer, Berthold Hocher. Endostatin in Renal and Cardiovascular Diseases [J] Kidney Dis (Basel), 2021, 7(6): 468-481.
3. **Mei Li***, Qin Zhang*, Kaixuan Yang. Role of MRI-Based Functional Imaging in Improving the Therapeutic Index of Radiotherapy in Cancer Treatment [J] Front Oncol, 2021,11:645177. (* means equal contribution)
4. Kaixuan Yang*, **Mei Li***, Jinbing Zhu*, Lei Zeng, Jiangfang Tian, Wenji Xie, Arthur Shou, Yiping Li, Guangjun Li. Nomograms for predicting survival outcomes in IMRT era of nasopharyngeal carcinoma: A study based on Epstein-Barr Virus (EBV) DNA biological responses [J] Head Neck, 2021, 43(6):1838-1847. (* means equal contribution)
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