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# TGF $\beta$ 1 Suppressed Matrix Mineralization of Osteoblasts Differentiation by Regulating SMURF1–C/EBP $\beta$ –DKK1 Axis

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**Abstract:** Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) is a major mediator in the modulation of osteoblast differentiation. However, the underlying molecular mechanism is still not fully understood. Here, we show that TGF $\beta$ 1 has a dual stage-dependent role in osteoblast differentiation; TGF $\beta$ 1 induced matrix maturation but inhibited matrix mineralization. We discovered the underlying mechanism of the TGF $\beta$ 1 inhibitory role in mineralization using human osteoprogenitors. In particular, the matrix mineralization-related genes of osteoblasts such as osteocalcin (OCN), Dickkopf 1 (DKK1), and CCAAT/enhancer-binding protein beta (C/EBP $\beta$ ) were dramatically suppressed by TGF $\beta$ 1 treatment. The suppressive effects of TGF $\beta$ 1 were reversed with anti-TGF $\beta$ 1 treatment. Mechanically, TGF $\beta$ 1 decreased protein levels of C/EBP $\beta$  without changing mRNA levels and reduced both mRNA and protein levels of DKK1. The degradation of the C/EBP $\beta$  protein by TGF $\beta$ 1 was dependent on the ubiquitin–proteasome pathway. TGF $\beta$ 1 degraded the C/EBP $\beta$  protein by inducing the expression of the E3 ubiquitin ligase Smad ubiquitin regulatory factor 1 (SMURF1) at the transcript level, thereby reducing the C/EBP $\beta$ -DKK1 regulatory mechanism. Collectively, our findings suggest that TGF $\beta$ 1 suppressed the matrix mineralization of osteoblast differentiation by regulating the SMURF1-C/EBP $\beta$ -DKK1 axis.

**Keywords:** osteoblast differentiation; mineralization; TGF $\beta$ 1; SMURF1; C/EBP $\beta$ ; DKK1

## 1. Introduction

Bone is a living tissue that undergoes constant degeneration and rebuilding throughout life. This bone remodeling is orchestrated by a dynamic relationship between specialized cells and their mediators [1]. Dysregulation of these coordinated processes can lead to bone diseases such as

osteoporosis, renal osteodystrophy, and Paget's disease [2]. Despite the importance of researching bone metabolism to improve the understanding of bone disease pathogenesis, the molecular mechanisms underlying bone remodeling are largely unknown.

In the complex crosstalk in bone remodeling, osteoblasts, differentiated from osteoprogenitors, play a key role in bone formation by synthesizing new collagenous extracellular matrix and then inducing mineralization [1]. The specific markers for the differentiation stages of osteoblast differentiation, alkaline phosphates (ALP), type 1 collagen (COL1), and osteonectin (ON), and osteocalcin (OCN), are expressed at a high level in extracellular matrix maturation and mineralization, respectively [3–5].

Interestingly, C/EBP $\beta$ , a critical determinant for osteoblast differentiation as a transcriptional factor, is known to regulate receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) or Runt-related transcription factor 2 (RUNX2) expression in osteoblasts as well as induce the expression of OCN by directly binding within its promoter [6–8]. Wnt/ $\beta$ -catenin is also a key regulator of osteoblast differentiation and is regulated by several inhibitors including Dickkopf (DKK) [9]. However, a previous study has shown that DKK2 has a role in matrix mineralization rather than only playing a negative role in the Wnt/ $\beta$ -catenin signaling pathway [10]. Moreover, we have reported that the expression of C/EBP $\beta$  and DKK1 mediated by vitamin D3 stimulation is required for the mineralization process of osteoblast differentiation [11].

One of the other major mediators that modulates osteoblast differentiation is the transforming growth factor-beta 1 (TGF $\beta$ 1). TGF $\beta$ 1 had been considered simply as a bone growth stimulant in some early studies [12,13]. However, accumulated data have shown variable results of TGF $\beta$ 1 on bone metabolism [14]. Therefore, it has been widely accepted that TGF $\beta$ 1 has a broad range of effects on bone metabolism: TGF $\beta$ 1 acts in different roles according to the different types of osteoblastic lineage cells or different stages of bone formation. In particular, TGF $\beta$ 1 promotes osteoprogenitor proliferation and early differentiation through the Smad2/3 pathways, whereas TGF $\beta$ 1 inhibits mineralization in the late stage of osteoblast differentiation by the degradation of TGF $\beta$  type I receptor via the induction of Smad ubiquitination regulatory factor (SMURF1) 1 and SMURF2 [15–18]. However, the underlying mechanism is not fully understood.

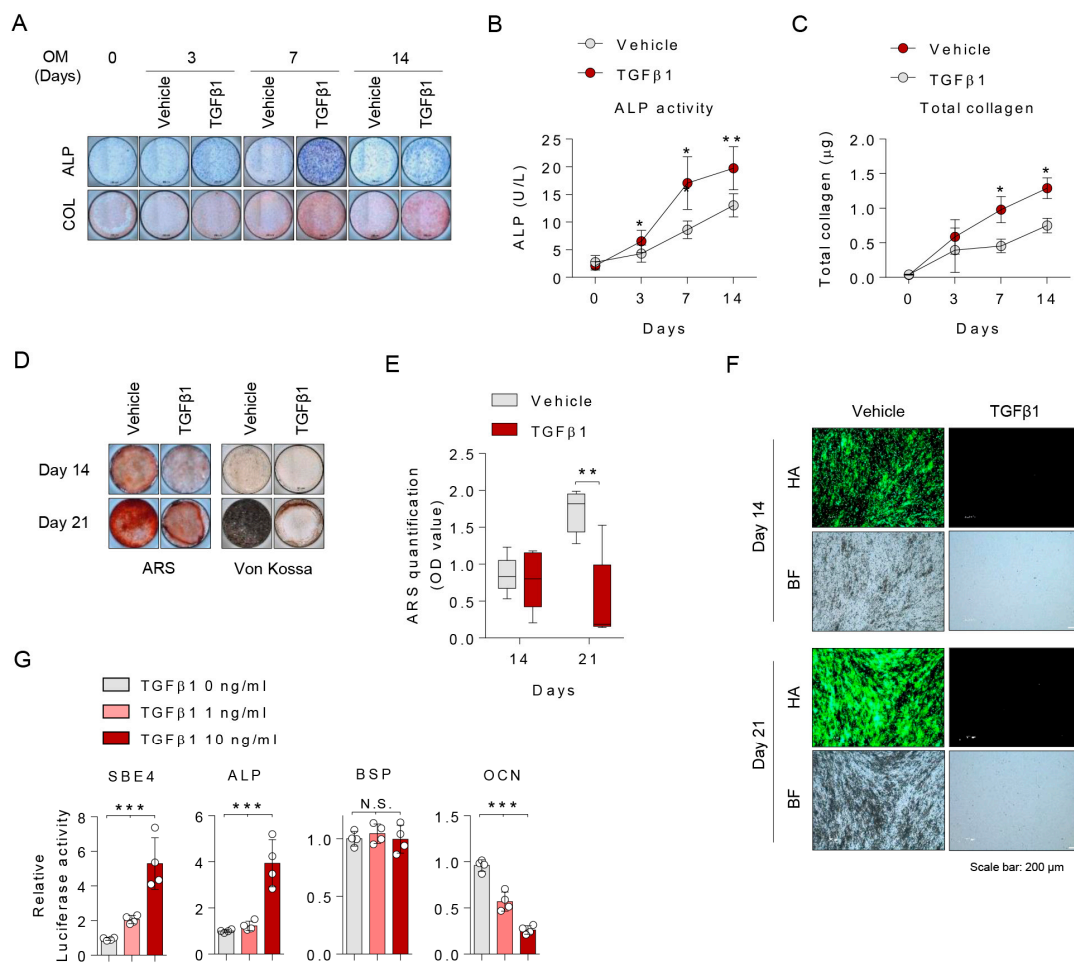
Here, we used human osteoprogenitors to demonstrate that TGF $\beta$ 1 suppressed the mineralization of osteoblast differentiation by regulating the SMURF1-C/EBP $\beta$ -DKK1 axis. Considering that the misregulation of the TGF $\beta$ 1 signaling pathways is frequently associated with various human diseases [19], and several diseases or pathologic conditions can lead to secondary osteoporosis or abnormal bone formation [2], we might provide a better understanding of the link between them, as well as new insights into the molecular mechanisms underlying normal bone remodeling.

## 2. Results

### 2.1. TGF $\beta$ 1 Suppressed Extracellular Matrix Mineralization of Osteoblast Differentiation, but Not Matrix Maturation

Firstly, we optimized the osteoprogenitors treatment of various TGF $\beta$ 1 dose for 3 days to determine the optimal dose of TGF $\beta$ 1 in our system. Cell viability and toxicity were investigated, and then we decided to treat 10 ng/mL of TGF $\beta$ 1 (Figure S1). To determine the effect of TGF $\beta$ 1 on human osteoblast differentiation, human osteoprogenitors were induced to mature osteoblast with differentiation conditional medium in the presence of TGF $\beta$ 1 continually for 21 days. As shown in Figure 1A–C, we observed gradual and time-dependent increases of ALP and collagen deposits until 14 days, indicating that TGF $\beta$ 1 promotes extracellular matrix maturation in the osteoblast differentiation stage (0–14 days). However, in the late stage (14–21 days), extracellular matrix mineralization was suppressed by TGF $\beta$ 1 treatment as indicated by the weaker intensity of Alizarin red staining (ARS), Von Kossa, and hydroxyapatite (HA) staining and lower ARS concentration than those of the vehicle controls (Figure 1D–F). Moreover, treatment with TGF $\beta$ 1 in osteoprogenitors significantly upregulated SBE4 and ALP promoter activities, but it downregulated the OCN promoter in a dose-dependent manner and had no effect on BSP (Figure 1G). Collectively, these data suggest that TGF $\beta$ 1 promotes extracellular

matrix maturation in the early stage of osteoblast differentiation while inhibiting extracellular matrix mineralization in the late stage.

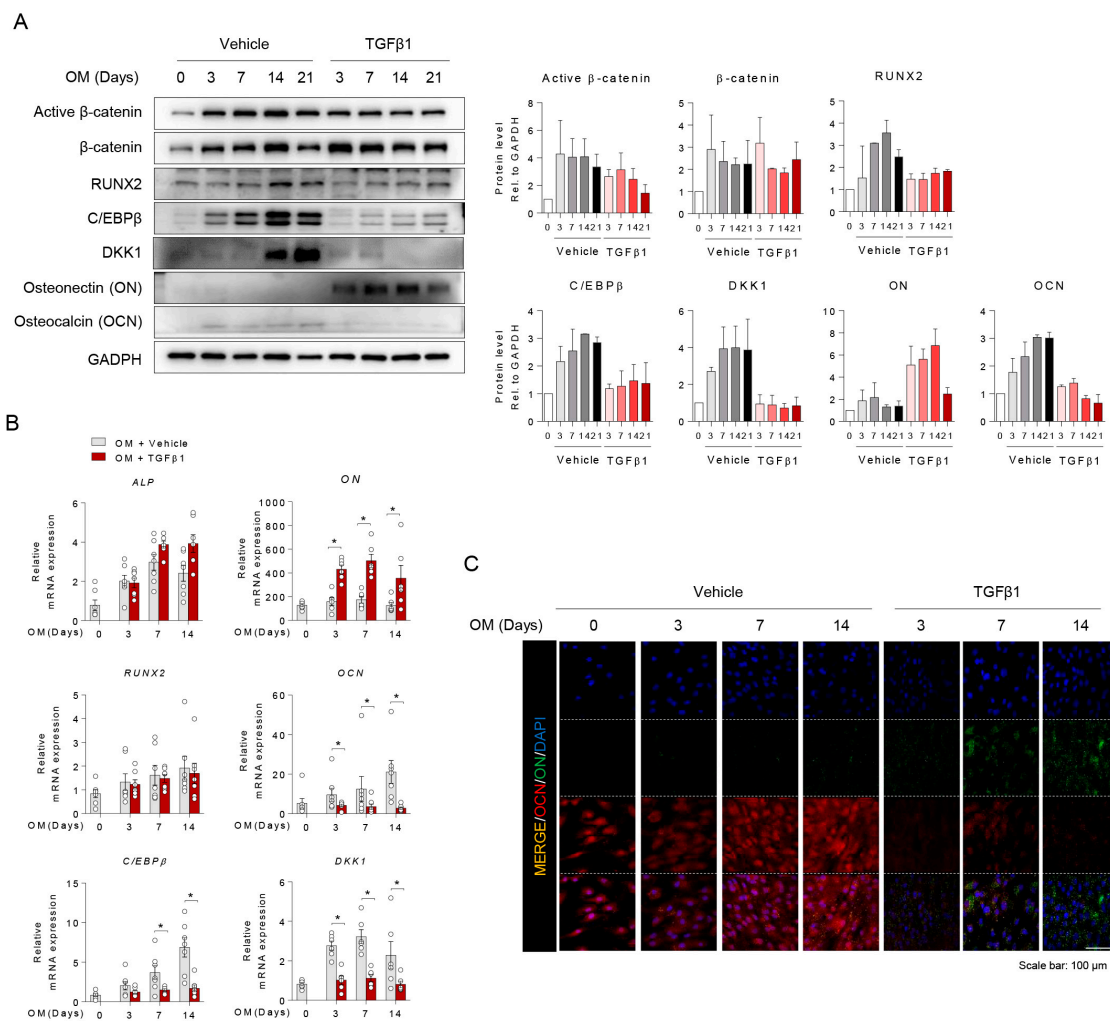


**Figure 1.** Transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) promotes extracellular matrix maturation in the early stage and suppresses mineralization in the late stage of osteogenic differentiation. Osteoprogenitors were differentiated into osteoblasts and continually stimulated by Vehicle or TGF $\beta$ 1 (10 ng/mL) during osteoblasts differentiation. At the indicated days, osteogenic differentiation activity was assessed by (A) alkaline phosphates (ALP) and collagen (COL) staining; scale bar is 200  $\mu$ m, (B) ALP activity of (A), (C) total collagen contents of (A), (D) Alizarin red staining (ARS) and von Kossa staining, (E) ARS quantification of (D), and (F) hydroxyapatite (HA) staining. All images are representative from the five independent experiments. (G) 293T cells were transfected with the promoter plasmids indicated for 48 h, treated with 1 and 10 ng/mL TGF $\beta$ 1 for 24 h, and then analyzed with a luciferase assay ( $n = 4$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; N.S. Not significant. Values are expressed as the mean  $\pm$  SEM (Student's two-tailed  $t$ -test).

## 2.2. TGF $\beta$ 1 Downregulated C/EBP $\beta$ and DKK1 Expression during Osteoblast Differentiation

Previously, we reported that C/EBP $\beta$  and DKK1 play critical regulatory roles in the matrix mineralization of osteoblast differentiation [11]. Therefore, we investigated the protein levels of C/EBP $\beta$  and DKK1 as well as other well-known osteoblast markers such as RUNX2 and OCN; extracellular matrix mineralization marker and ON; and extracellular matrix maturation marker. As expected, the protein levels of C/EBP $\beta$ , DKK1, RUNX2, and OCN were gradually increased in a time-dependent manner during osteoblast differentiation, whereas the protein levels of C/EBP $\beta$ , DKK1, RUNX2, and OCN were decreased by TGF $\beta$ 1 treatment (Figure 2A). Protein and mRNA expression showed that TGF $\beta$ 1 downregulated OCN but upregulated ON and COL1A during osteoblast differentiation, indicating that

TGF $\beta$ 1 sustained the matrix maturation stage but inhibited matrix mineralization. To further explore the physiological differences in osteoblast differentiation, we showed that TGF $\beta$ 1 increased and sustained ON expression as a matrix maturation marker during differentiation but inhibited OCN expression as a matrix mineralization marker using immunofluorescence (Figure 2B). Taken together, we identified the effect of TGF $\beta$ 1 on the molecular mechanism of osteoblast differentiation: TGF $\beta$ 1 promoted the extracellular matrix maturation via upregulating ON and suppressed extracellular matrix mineralization via downregulating C/EBP $\beta$ , DKK1, and OCN (Figure 2C).

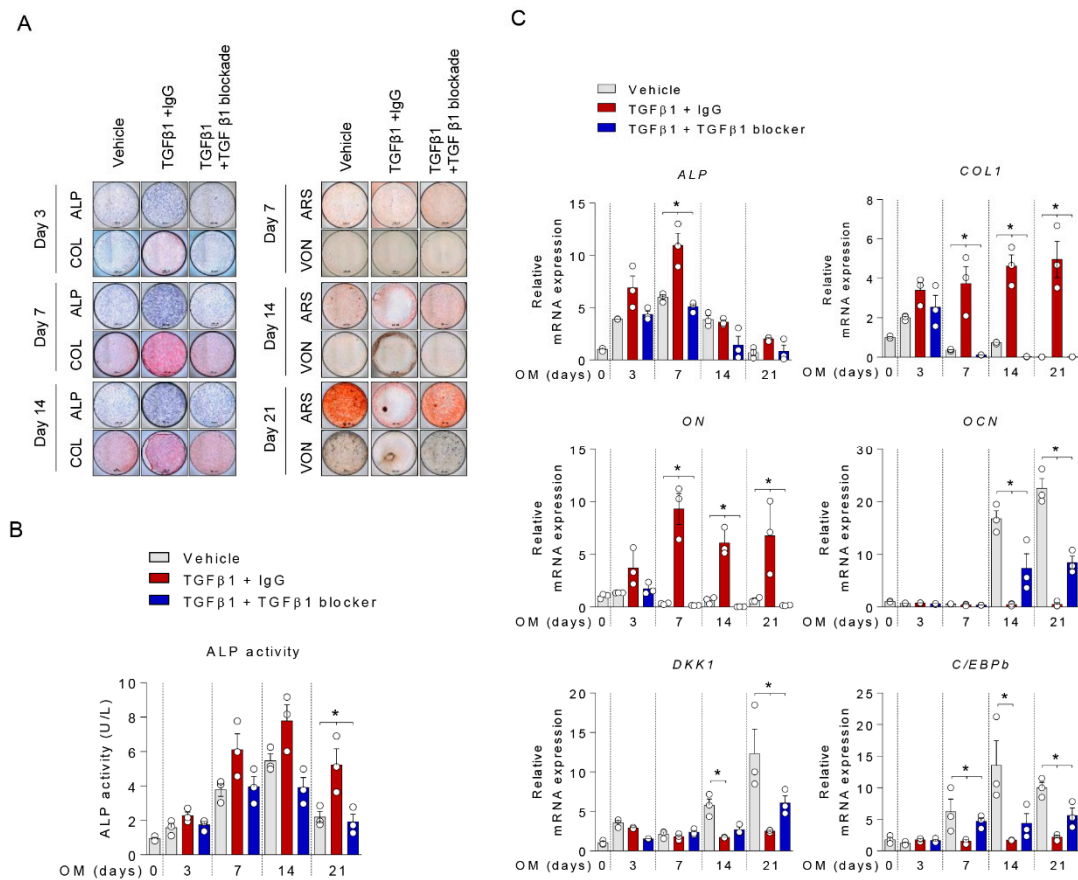


**Figure 2.** TGF $\beta$ 1 downregulated CCAAT/enhancer-binding protein beta (C/EBP $\beta$ ) and Dickkopf 1 (DKK1) expression in osteoblast differentiation. Osteoprogenitors were differentiated into osteoblasts with vehicle or TGF $\beta$ 1 (10 ng/mL) for the indicated days. At the days indicated, osteogenic differentiation activity was analyzed. (A) Immunoblotting with active  $\beta$ -catenin,  $\beta$ -catenin, RUNX2, CCAAT/enhancer-binding protein beta (C/EBP $\beta$ ), DKK1, ON, OCN, and GAPDH were analyzed and quantified with Image J and normalized to GAPDH. (B) RT-qPCR with ALP, COL1A1, ON, OCN, RUNX2, C/EBP $\beta$ , and DKK1 were analyzed and normalized to GAPDH ( $n = 5$ ). (C) Immunostaining with OCN (red), ON (green), and DAPI (blue) were analyzed and immunofluorescence images are representative from the three independent experiments; scale bar is 100  $\mu$ m. Values are expressed as the mean  $\pm$  SD. \*  $p < 0.05$ .

### 2.3. Anti-TGF $\beta$ 1 Antibody Reversed TGF $\beta$ 1-Mediated Suppression of Mineralization

Next, we performed additional experiments using anti-TGF $\beta$ 1 antibody. Consistent with the above results, the addition of TGF $\beta$ 1 suppressed mineralization during osteoblasts differentiation. Anti-TGF $\beta$ 1 treatment rescued the suppressive effect of TGF $\beta$ 1 on mineralization (Figure 3A). TGF $\beta$ 1 induced

expressions of ALP and COL1, but anti-TGF $\beta$ 1 blocked these effects (Figure 3B,C). As shown in Figure 3C, qPCR results showed that anti-TGF $\beta$ 1 antibody reversed the TGF $\beta$ 1-mediated reductions of C/EBP $\beta$  and DKK1 expression.

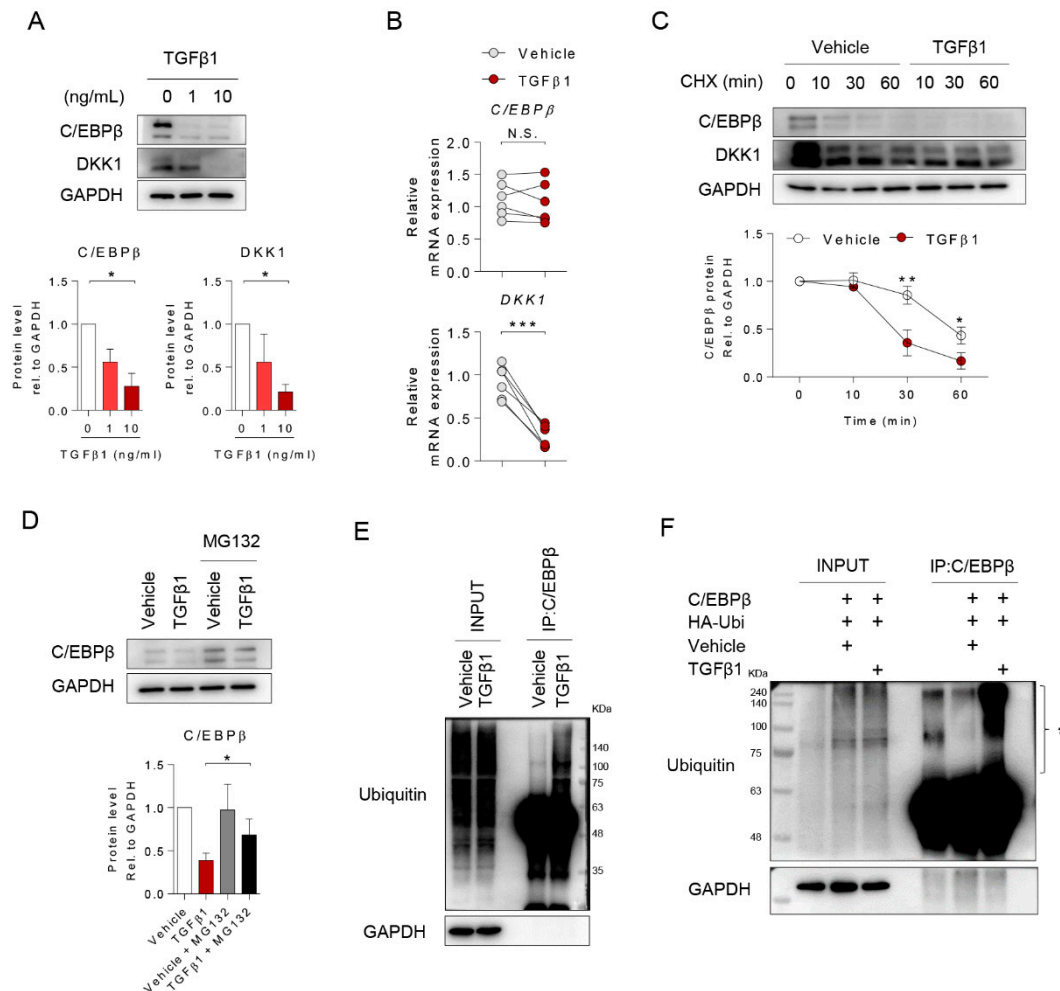


**Figure 3.** Anti-TGF $\beta$ 1 antibody blocked the suppressive effect of TGF $\beta$ 1 on mineralization. Osteoprogenitors were differentiated into osteoblasts in the presence of 10 ng/mL TGF $\beta$ 1 and Anti-TGF $\beta$ 1 antibody for the indicated days and analyzed by (A) ALP, COL, ARS, and von Kossa staining; scale bar is 200  $\mu$ m, (B) intracellular ALP activity, and (C) the mRNA levels for the osteogenic differentiation-related factors ( $n = 3$ ). All images of (A) are representative from the three independent experiments. Values are expressed as the mean  $\pm$  SD. \*  $p < 0.05$ .

#### 2.4. TGF $\beta$ 1 Inhibited DKK1 Expression by Inducing Ubiquitination of C/EBP $\beta$ Protein

To identify the mechanism underlying the downregulation of C/EBP $\beta$  and DKK1 by TGF $\beta$ 1 in osteoblast differentiation, we treated osteoprogenitors with TGF $\beta$ 1 and investigated the expression of C/EBP $\beta$  and DKK1 at both the protein and gene levels. TGF $\beta$ 1 decreased the protein level of C/EBP $\beta$  and DKK1 dose-dependently (Figure 4A). Interestingly, the mRNA level of C/EBP $\beta$  did not significantly change in the presence of TGF $\beta$ 1, while that of DKK1 decreased (Figure 4B). In the presence of Actinomycin D (Actino. D), a transcription blocking agent, there were no significant differences in the C/EBP $\beta$  mRNA expression level with and without TGF $\beta$ 1 treatment (Figure S2). We next verified the differences in the cellular mechanisms regulating C/EBP $\beta$  and DKK1 protein synthesis using cycloheximide (CHX), a protein synthesis inhibitor, and MG132, a proteasome degradation inhibitor. CHX treatment led to a significant decrease in the protein levels of C/EBP $\beta$  and DKK1 in the presence of TGF $\beta$ 1 compared to vehicle controls (Figure 4C). Exposure to TGF $\beta$ 1 effectively reduced the C/EBP $\beta$  protein but accumulated the C/EBP $\beta$  protein in MG132-treated cells (Figure 4D). Interestingly, TGF $\beta$ 1 triggered the overall ubiquitination of both endogenous and exogenous C/EBP $\beta$  protein (Figure 4E,F). The reduction of exogenous C/EBP $\beta$  protein in 293T cells by TGF $\beta$  was consistent

with our results of the alteration of endogenous C/EBP $\beta$  protein in osteoprogenitors (Figure S3). Notably, we observed that TGF $\beta$ 1 induced poly-ubiquitinated C/EBP $\beta$  protein, suggesting that the downregulation of C/EBP $\beta$ -DKK1 by TGF $\beta$ 1 might be controlled by post-translational mechanisms of C/EBP $\beta$ .

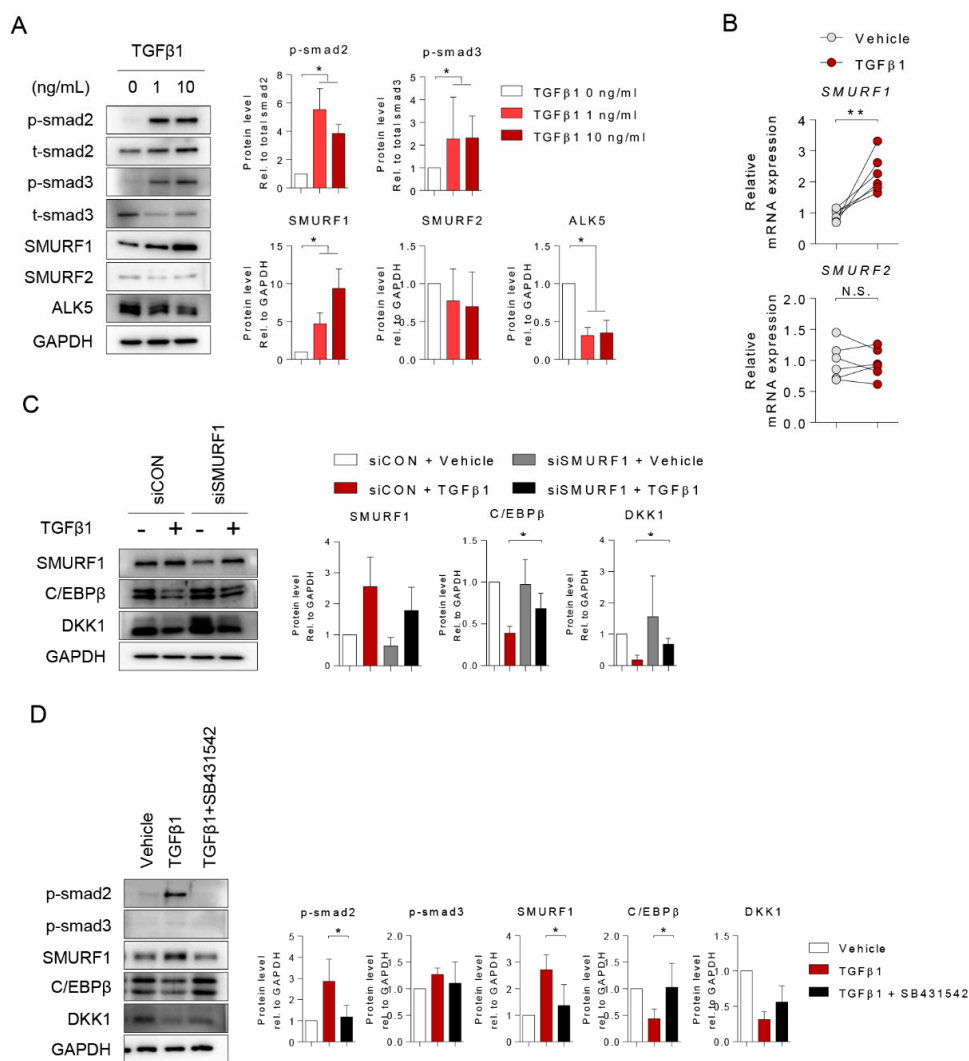


**Figure 4.** TGF $\beta$ 1 inhibited DKK1 expressions by ubiquitin-mediated C/EBP $\beta$  protein degradation. Osteoprogenitors were stimulated with 0, 1, and 10 ng/mL TGF $\beta$ 1 for 24 h, lysed, and subjected to analysis for (A) immunoblotting (upper) and quantification of the immunoblot images (lower,  $n = 3$ ), and (B) qPCR with C/EBP $\beta$  and DKK1 expressions ( $n = 5$ ). (C) Osteoprogenitors were pretreated with cycloheximide (CHX), protein synthesis inhibitor, for 30 min, followed by treatment with 10 ng/mL TGF $\beta$ 1 for the times indicated, and analyzed by immunoblotting (upper) and quantification of the immunoblot images (lower,  $n = 5$ ). (D) Osteoprogenitors were pretreated with MG132, proteasome inhibitor, for 30 min, followed by treatment with 10 ng/mL TGF $\beta$ 1 for 24 h and analyzed by immunoblotting and quantified with Image J ( $n = 3$ ; normalized to GAPDH). (E) Osteoprogenitors were stimulated with 10 ng/mL TGF $\beta$ 1 for 24 h, followed by IP with C/EBP $\beta$  antibody and analyzed by immunoblotting ( $n = 4$ ). (F) 293T cells were co-transfected with C/EBP $\beta$  and HA-Ubi plasmids for 24 h, followed by treatment with 10 ng/mL TGF $\beta$ 1 for 24 h, IP with C/EBP $\beta$  antibody, and analyzed by immunoblotting ( $n = 3$ ). Asterisk indicated poly-ubiquitin with C/EBP $\beta$  protein. Values are expressed as the mean  $\pm$  SD. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; N.S. Not significant.

### 2.5. TGF $\beta$ 1 Induced SMURF1 to Regulate C/EBP $\beta$ -DKK1 Expressions

Since SMURF expression in osteoblasts was induced by TGF $\beta$ 1 and the expression of SMURF1 was responsible for osteoblastic activity and bone formation, we confirmed alterations in expression

after TGFβ1 treatment [20,21]. Upon TGFβ1 treatment, both mRNA and protein expression of SMURF1 were induced and altered the expression levels of TGFβ-inducible proteins such as phosphor (p)-smad2, p-smad3, and ALK5 in osteoprogenitors (Figure 5A,B). The induction of SMURF1 promoter by TGFβ1 treatment also were observed (Figure S4). To investigate whether SMURF1 regulates the C/EBPβ protein in osteoprogenitors, we investigated the SMURF1 knockdown osteoprogenitor using two types of SMURF1 siRNA mixed in further experiments (Figure S5). Intriguingly, SMURF1 knockdown (siSMURF1) and TGFβ1 specific inhibitor (SB431542) were sufficient to attenuate TGFβ1-mediated C/EBPβ and DKK1 protein degradation (Figure 5C,D). These results suggest that the upregulation of SMURF1 by TGFβ1 might overlap with previously known smad2/3-related mechanisms and could associate with C/EBPβ protein degradation.



**Figure 5.** TGFβ1 induced Smad ubiquitin regulatory factor 1 (SMURF1) to regulate C/EBPβ-DKK1 expression. Osteoprogenitors were stimulated with 0, 1, and 10 ng/mL TGFβ1 for 24 h, lysed, and subjected to analysis for (A) immunoblotting (left) and quantification of the immunoblot images (right,  $n = 3$ ), and (B) qPCR with SMURF1 and SMURF2 expressions ( $n = 6$ ). (C) Osteoprogenitors were transfected with sicontrol (siCON) or two siSMURF1 #1 and #2, followed by treatment with 10 ng/mL TGFβ1 for 24 h, and analyzed by immunoblotting and quantified with ImageJ. (D) Osteoprogenitors were pretreated with SB411542 for 30 min, followed by treatment with 10 ng/mL TGFβ1 for 24 h, and analyzed by immunoblotting and quantified with ImageJ. Values are expressed as the mean  $\pm$  SD. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; N.S. Not significant.

### 3. Discussion

In the present study, we replicated a previously reported finding that the C/EBP $\beta$ -DKK1 axis may play a dual stage-dependent role in osteoblast differentiation: C/EBP $\beta$  and DKK1 expression levels are decreased in extracellular matrix maturation but increased in the mineralization phase of osteoblast differentiation [11]. Conversely but similarly, TGF $\beta$ 1 induced ALP and collagen synthesis in osteoprogenitors during the matrix maturation stage but suppressed mineralization. Moreover, we discovered the underlying mechanism of the inhibitory role of TGF $\beta$ 1 in mineralization. It was accompanied by the downregulation of DKK1 following the degradation of the C/EBP $\beta$  protein by the ubiquitin–proteasome pathway via inducing SMURF1 expression. Collectively, TGF $\beta$ 1 suppressed the mineralization of osteoblasts differentiation by regulating the SMURF1–C/EBP $\beta$ –DKK1 axis.

TGF $\beta$ 1 is a pleiotropic cytokine ubiquitously expressed in most tissues. Since TGF $\beta$ 1 was first identified in 1983 [22], accumulated data and experience have established its regulatory role in a broad range of biological processes including immune responses, angiogenesis, and bone formation [16,23,24]. Parallel with the wide range of roles, the dysregulation of TGF $\beta$ 1 expression and activity contributes to a number of disease states, including many cancers, cardiovascular disease, and musculoskeletal disease [19]. Additionally, the bifunctional and context-dependent nature of TGF $\beta$  has also been documented [25]. In bone metabolism, we reported previously that TGF $\beta$ 1 has both inhibitory and stimulatory effects on osteoclast differentiation mediated by SMAD1 and 3 signaling [26].

We investigated the regulatory role of TGF $\beta$ 1 in osteoblast differentiation. Not surprisingly but interestingly, TGF $\beta$  treatment for the first 3 days significantly promoted ALP expression and collagen synthesis in the matrix maturation phase. Moreover, this early and transient exposure of TGF $\beta$ 1 exhibited a subsequent increase in mineralization (Data not shown). However, continuous exposure to TGF $\beta$ 1 suppressed bone mineralization. These results are consistent with previous studies [15,18,20]. Data from several in vitro experiments have reported the dual, stage-dependent role of TGF $\beta$ 1 on osteoblast differentiation. TGF $\beta$ 1 increases bone formation mainly by recruiting progenitors and stimulating their proliferation, and promoting the early stages of differentiation (bone matrix maturation). Conversely, it blocks the later phases of differentiation and mineralization [15,18,27]. These data derived from in vitro studies might reflect in vivo responses of the bone remodeling mechanism; TGF $\beta$ 1 serum concentrations significantly increased during the early healing period in patients with bone fracture, and they decreased continuously during the late healing period [28].

Moreover, TGF $\beta$ 1 appeared to act differently according to doses even in the same stage. In the early stage of osteoblast differentiation, ALP activity significantly increased with increasing doses of TGF $\beta$ 1 (1 ng/mL to 10 ng/mL). However, cell death occurred when the TGF $\beta$ 1 dose was increased even higher, over 50 ng/mL (Figure S1). Previous studies have resulted in similar data regarding the effect of TGF $\beta$ 1 on ALP activity using human osteoblasts, but there were controversies in the optimal doses of TGF $\beta$ 1. ALP activity markedly increased with 1 and 2 ng/mL TGF $\beta$ 1 treatment compared to negative control and 0.5 and >5 ng/mL TGF $\beta$ 1 treatment [29]. These variable results might depend on cell density and the timing of TGF $\beta$ 1 treatment.

To focus on the pathologic mechanism of TGF $\beta$ 1 on the inhibition of mineralization, we observed that continuous TGF $\beta$ 1 treatment induced the retention of the matrix maturation phase by sustaining collagen production, leading to impaired mineralization. A similar mechanism was observed with Activin A, which is a member of the TGF $\beta$  superfamily. Activin A has an inhibitory role in bone formation by altering the extracellular matrix composition such as ALP and collagen [30] or without ALP and collagen. [30,31]. Furthermore, OCN is known to be a marker for bone mineralization, but it increased in bone formation in OCN knockout mice [32]. However, two recent independent studies showed the functional role of OCN; OCN knockout mouse exhibits a reduction of bone mineralization, strength, and its related metabolism [33,34]. These results support our conclusion that TGF $\beta$ 1 diminished the process of bone mineralization induced by osteoblasts through OCN reduction. Further experiments are needed to demonstrate that TGF $\beta$ 1 inhibited OCN expression during osteoblast differentiation.



With regard to molecular mechanisms, our results indicate that the effects of TGF $\beta$ 1 on bone mineralization may involve DKK1, which is a secreted protein originally located within the endoplasmic reticulum (ER) in the cytoplasm. Several studies have found that ER stress induces DKK1 secretion, eventually leading to cell apoptosis [35–37]. Osteoblasts can either transform when they are embedded in bone as osteocytes or undergo apoptosis for bone mineralization at the end phase of bone formation [38]. Therefore, most osteoblasts become a mineralized component of bone with apoptosis rather than osteocytes, and DKK1 upregulation seems indispensable in achieving the terminal feature of bone. Our data show how TGF $\beta$ 1 can regulate the mineralization of osteoblast differentiation. TGF $\beta$ 1 suppressed mineralization by the downregulation of DKK1 following the degradation of the C/EBP $\beta$  protein via SMURF1-induced ubiquitination.

The present study has a few limitations. First, the function of TGF $\beta$ 1-induced ON in bone metabolism is unclear and needs further study. The ON is known for its vital role in bone mineralization [39] but did not alter osteoblast differentiation in our system (Figure S6). Second, we investigated only TGF $\beta$ 1 among three TGF $\beta$  isoforms; TGF $\beta$ 1, TGF $\beta$ 2, and TGF $\beta$ 3. When considering the promoter regions of the genes encoding different TGF $\beta$  isoforms [40], different TGF $\beta$  isoforms might have different roles in bone metabolism. However, we focused on TGF $\beta$ 1, because it is the dominant species in human bone [41]. Third, we found that TGF $\beta$ 1 treatment reduced active  $\beta$ -catenin protein expression in osteoprogenitors during osteoblast differentiation, but the negative regulatory signaling of  $\beta$ -catenin remains unknown. Despite these limitations, our data support the dose stage-dependent role of TGF $\beta$ 1 in osteoblast differentiation using human osteoprogenitors. We provide novel insight into the molecular mechanisms underlying the inhibitory role of TGF $\beta$ 1 in mineralization. Given that the components of TGF $\beta$  signaling are often found to be deregulated in a variety of bone-related disease, understanding how TGF $\beta$ 1 works in mineralization is important not only for the fundamental understanding of bone metabolism but also for opening up new opportunities for drug development.

## 4. Materials and Methods

### 4.1. Human Bone Sample Collection

Human bone specimens were collected from patients with primary osteoarthritis (OA) who underwent total knee replacement at the Hanyang University Guri Hospital. Surgical knee bone samples were collected from 25 patients with OA (mean ages  $74.32 \pm 7.13$  years). OA was diagnosed according to the clinical classification criteria developed by the American College of Rheumatology [42], and patients with OA secondary to other diseases including connective tissue diseases were excluded. Surgical spinal bone samples were collected from 9 patients with non-inflammatory spinal disease (mean ages  $68.11 \pm 9.01$  years). The present study was approved by the Institutional Review Board of Hanyang University Guri Hospital (IRB file No. 2018-07-024), Hanyang University Seoul Hospital (IRB file No. 2017-05-003), and was carried out in accordance with the Declaration of Helsinki. All patients provided written informed consent, and all data were de-identified and anonymous.

### 4.2. Isolation of Human Primary Osteoprogenitors and Differentiation of Osteoblasts

Cancellous bones from surgical knee bone samples and spinal bones were cut into small pieces (bone chips) using rongeur and operating scissors. These bone chips were vigorously washed by vortexing for 10 min at least 3 times with phosphate-buffered saline (PBS) containing antibiotics to remove non-adherent bone marrow cells completely. These bone chips were placed in cell culture plates and incubated in Dulbecco's modified eagle medium (DMEM) growth medium containing 10% fetal bovine serum (FBS) and 1% antibiotics to isolate osteoprogenitors using outgrowth methods [43–45]. Osteoprogenitors were stimulated with conditioned medium containing osteogenic supplements including 50  $\mu$ M ascorbic acid (AA), 10 mM  $\beta$ -glycerolphosphate, and 100 nM dexamethasone to induce osteoblast differentiation, as described in previous studies [46–48].

#### 4.3. Assessment of Osteoblast Differentiation

We investigated the impact of TGF $\beta$ 1 on osteoblast differentiation according to the following two stages: matrix maturation (initial stage, 0–7 days) and matrix mineralization (late stage, 14–21 days). After examining various doses of TGF $\beta$ 1 to find an optimal dose on osteoblast differentiation in our system (Figure S1), TGF- $\beta$  was continually maintained at a constant concentration (10 ng/mL) throughout culture.

In the matrix maturation stage of osteoblast differentiation (initial stage), we assessed the expression of ALP and collagen. ALP was assessed using an ALP activity colorimetric assay kit (K412; Biovision, Milpitas, CA, USA) and ALP staining (85L2; Sigma-Aldrich, St. Louis, MO, USA). The extent of the collagen deposition was assessed with collagen staining (ab150681; abcam, Cambridge, UK) and quantified with a total collagen assay according to the manufacturers' instructions (K218–100; Biovision). For the assessment of the matrix mineralization phase (late stage), calcium deposition was visualized using several staining methods including Alizarin red staining (ARS; A5533; Sigma-Aldrich) for calcium deposition, Von Kossa staining using 5% aqueous silver nitrate solution (S7179; Sigma-Aldrich) for calcium phosphate, and hydroxyapatite staining (HA; PA-1503; Lonza, Basel, Switzerland). ARS and HA stains for the mineralization stage were assessed and quantified. For ARS quantification, stained wells were extracted with absolute acetic acid at 37 °C for 30 min followed by centrifugation. The supernatant was transferred to a white 96-well plate and read at an excitation wavelength of 405 nm with an ELISA plate reader. For Von Kossa quantification, stained images were analyzed by Image J. For HA quantification, stained cells were read at an excitation wavelength of 492 nm and an emission wavelength of 550 nm.

#### 4.4. Total Collagen Assay

Total collagen was assessed with the supernatant of each well according to the manufacturers' instructions (K218-100, Biovision). By adding 12 M HCl and incubating at 120 °C for 3 h, acidic hydrolysis takes place and hydroxyprolines are formed. After homogenization, the sample was clarified by adding 4 mg of activated charcoal. The hydroxyprolines react in an oxidative reaction to a chromophore with a specific absorbance at 560 nm, correlating to the collagen content of the sample.

#### 4.5. Luciferase Assay

293T cells or MG63 were co-transfected with each Smad binding element-4 (SBE4), ALP, bone sialoprotein (BSP), OCN, (1  $\mu$ g/well), SMURF1 promoter and Renilla (0.5  $\mu$ g/well) using Lipo3000 (L3000015; Thermo Fisher, Waltham, MA, USA). Two days after transfection, the cells were reseeded, treated with TGF $\beta$ 1 for 24 h, and lysed to conduct a luciferase assay (E1500; Promega, Madison, WI, USA), according to the manufacturer's protocol. The luciferase activity was measured with a Luminometer (Berthold, Bad Wildbad, Germany) and normalized to Renilla luciferase.

#### 4.6. Constructs, Transfection, and Reagents

Recombinant human TGF $\beta$ 1 (100-21; Peprotech, Rocky Hill, NJ, USA), MG132 (474791; EMD Millipore, Burlington MA, USA), CHX (C4859; Sigma-Aldrich), Actinomycin D (A9415; Sigma-Aldrich), and TGF $\beta$ 1 blocker (MAB240-100; R&D Systems, Minneapolis, MN, USA) were obtained. DKK1 cDNA plasmid (HG10170-CY) and empty plasmid (CV013) were purchased from Sino Biological (Wayne, PA, USA). SBE4-Luc (#16495) was purchased from Addgene (Watertown, MA, USA). C/EBP $\beta$  and empty vector were kindly given by Dr. Yun Jong Lee (Division of Rheumatology, Department of Internal Medicine, Seoul National University Bundang Hospital, Korea) [49,50]. Bone-related promoters such as ALP, BSP, and OCN were kindly given by Dr. Kwang Yeol Lee (College of Pharmacy, Chonnam National University, Korea) [51]. HA-Ubi plasmid, MG63, and 293T cell lines were kindly given by Dr. Heekyoung Chung (Department of Pathology, College of Medicine, Hanyang University, Korea) [52]. SMURF1 promoter was kindly given by Dr. Jeong-Tae Koh (School of

Dentistry, Chonnam National University, Korea) [53]. Small interfering RNA (siRNA) oligos were obtained from Genolution Inc. (Seoul, Korea). The siSMUR1 siRNA oligos were as follows:

siControl: 5'-CCUCGUGCCGUUCCAUCAGGUAGUU-3';  
 siSMURF1#1: 5'-CCAGUAUUCUACGGACAAU-3';  
 siSMURF1#2: 5'-CAUGAAAUGCUGAAUCCUU-3';  
 siSMURF1#3: 5'-CCAGCACUAUGAUCUAUAUUU-3';  
 siSMURF1#4: 5'-GUGCCAUGAAAUGCUGAAUUU-3';  
 siSMURF1#5: 5'-GUCCGGUUGUAUGUAAACUUU-3'.

The transfection of primary osteoprogenitors was carried out using Lipofectamine 3000 (L3000015, Thermo Fisher) according to the manufacturer's protocol.

#### 4.7. Immunoblot and RT-qPCR

Protein level and RNA expression were analyzed using immunoblotting and RT-qPCR, respectively, with standard and basic methods as used in a previous study [54]. RT-qPCR was performed on a CFX96 Real-Time PCR detection system (BR18B-5200, Bio-Rad Laboratories, Hercules, CA, USA). The expression of each target gene was normalized to GAPDH. Normalized expression values were averaged, and then average fold changes were calculated. Primers used for PCR were as follows: ALP\_F-ACGAGCTGAACAGGAACAACGT; ALP\_R-CACCAGCAAGAAGAAGCCTTTG; COL1\_F-AGTGGTTTGGATGGTGCCAA; COL1\_R-GCACCATCATTTCACGAGC; ON\_F-GGATGAGAACAACACCCCA; ON\_R-TTTGCAAGGCCGATGTAGT; OCN\_F-AGCCACCGAGACCACTGAGA; OCN\_R-CTCCTGAAAGCCGATGTGGTC; RUNX2\_F-GTGGCCTTCAAGGTGGTAG; RUNX2\_R-ACTCTTGCCTCGTCCACTC; C/EBP $\beta$ \_F-CGACGAGTACAAGATCCGGC; C/EBP $\beta$ \_R-TGCTGGAACAAGTTCGCAG; DKK1\_F-CACACCAAAGGACAAGAAGG; DKK1\_R-CAAGACAGACCTTCTCCACA; SMURF1\_F-CCCCAGGATACCAAGAGACCT; SMURF1\_R-GGCTCCTTGAGTTGGACT; SMURF2\_F-CCTGACAGTACTCTGTGCAAAA; SMURF2\_R-ATTGCCAGATCCATCAACCA.

The primary antibodies for C/EBP $\beta$  (sc-7962), DKK1 (sc-374574), ON (sc-73472), and SMURF1 (sc-100616) were from Santa Cruz Biotechnology (Dallas, TX, USA). Non-phospho (Active)  $\beta$ -catenin (Ser45) (19807),  $\beta$ -catenin (9562), RUNX2 (12556), p-SMAD2 (3101), SMAD2 (3122), p-SMAD3 (9523), SMAD3 (9520), SMURF2 (12024),  $\beta$ -actin (4970), and GAPDH (2118) were from Cell Signaling Technology (Danvers, MA, USA). ALK-5 (AF3025) was from R&D Systems (Minneapolis, MN, USA). OCN (ab12320) was from Abcam. Secondary antibodies for goat anti-rabbit (111-035-003) and goat anti-mouse (115-035-003) were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

#### 4.8. Immunofluorescence (IF)

The differentiated osteoprogenitors were washed twice with PBS and fixed with 10% formalin for 15 min. This was followed by permeabilization with PBS containing 0.1% Triton X-100 and 1% BSA for 1 h and incubation with primary antibody overnight. Then, they were washed with PBS and incubated with Cy3 or Alexa 488-conjugated secondary antibody for 1 h. The stained cells were washed with distilled water and mounted with DAPI (H1200, Vector, Burlingame, CA, USA). To visualize stained cells, immunofluorescence images were obtained using a confocal microscope (Leica Microsystems, Wetzlar, Germany). Antibodies used in IF were as follows: ON (sc-73472; Santa Cruz Biotechnology, Dallas, TX, USA), OCN (sc-365797; Santa Cruz, TX, USA), Alexa-488 (A11001; Thermo Fisher), and Cy3 (A10520, Thermo Fisher).

#### 4.9. Immunoprecipitation (IP)

293T cells were co-transfected with HA-Ubi (1  $\mu$ g) and C/EBP $\beta$  (3  $\mu$ g) and incubated for 24 h, and then treated with TGF $\beta$ 1 or Vehicle for 8 h. The stimulated cells were lysed by non-SDS lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 10% glycerol, and 1% Triton X-100) containing proteinase and phosphatase inhibitors. For IP, C/EBP $\beta$  antibody (5  $\mu$ g) was added to protein lysates (1  $\mu$ g) and

subjected to incubation on a rotator for overnight at 4 °C. The next day, protein A/G agarose beads were added to the mixed lysates to incubate at 4 °C for 1 h and then washed three times with ice-cold lysis buffer containing protease inhibitors. Immunoprecipitated proteins were dissolved in 2× Laemmli sample buffer and analyzed by immunoblotting.

#### 4.10. Statistical Analysis

Statistical analysis and the graphical representation of data were performed using GraphPad Prism 6 software. A two-tailed *t*-test was used to compare data between two unpaired groups. Values are presented as mean ± standard deviation (SD) or standard error of the mean (SEM) from at least three independent experiments.

## 5. Conclusions

TGFβ1 induced ALP and collagen synthesis in the extracellular matrix maturation stage but suppressed the mineralization of osteoblast differentiation by the downregulation of DKK1 following the degradation of the C/EBPβ protein via SMURF1-induced ubiquitination.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1422-0067/21/24/9771/s1>, Figure S1. Effect of TGFβ1 on osteoprogenitors, Figure S2 TGFβ1 had no effect on C/EBPβ transcription level in osteoprogenitors, Figure S3. TGFβ1 decreased C/EBPβ protein expression, Figure S4. TGFβ1 induced SMURF1 promoter, Figure S5. Suppression efficiency of siSMURF1 in osteoprogenitors, Figure S6. Osteonectin did not affect osteoblastic differentiation.

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## Abbreviations

TGFβ1	Transforming growth factor β1
DKK1	Dickkopf 1
C/EBPβ	CCAAT/enhancer-binding protein β
SMURF1	Smad ubiquitin regulatory factor 1
SMURF2	Smad ubiquitin regulatory factor 2
ALP	Alkaline phosphates
BSP	Bone sialoprotein
COL1	Type 1 collagen
ON	Osteonectin
OCN	Osteocalcin
RANKL	Receptor activator of nuclear factor-κB ligand
RUNX2	Runt-related transcription factor 2
OA	Osteoarthritis
PBS	Phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
AA	Ascorbic acid
ARS	Alizarin red S
HA	Hydroxyapatite
BF	Bright Field
siRNA	Small interfering RNA
Actino D	Actinomycin D
CHX	Cycloheximide

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