

# (–)-Epicatechin Gallate (ECG) Stimulates Osteoblast Differentiation via Runt-related Transcription Factor 2 (RUNX2) and Transcriptional Coactivator with PDZ-binding Motif (TAZ)-mediated Transcriptional Activation\*

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**Background:** Catechins in green tea have a beneficial effect in bone formation, but the detailed mechanism is not fully understood.

**Results:** ECG, a major compound of green tea, stimulates TAZ- and RUNX2-mediated osteogenic gene transcription through PP1A.

**Conclusion:** ECG stimulates osteoblast differentiation through a transcriptional activation.

**Significance:** A novel mechanism for green tea-stimulated osteoblast differentiation is revealed.

Osteoporosis is a degenerative bone disease characterized by low bone mass and is caused by an imbalance between osteoblastic bone formation and osteoclastic bone resorption. It is known that the bioactive compounds present in green tea increase osteogenic activity and decrease the risk of fracture by improving bone mineral density. However, the detailed mechanism underlying these beneficial effects has yet to be elucidated. In this study, we investigated the osteogenic effect of (–)-epicatechin gallate (ECG), a major bioactive compound found in green tea. We found that ECG effectively stimulates osteoblast differentiation, indicated by the increased expression of osteoblastic marker genes. Up-regulation of osteoblast marker genes is mediated by increased expression and interaction of the transcriptional coactivator with PDZ-binding motif (TAZ) and Runt-related transcription factor 2 (RUNX2). ECG facilitates nuclear localization of TAZ through PP1A. PP1A is essential for osteoblast differentiation because inhibition of PP1A activity was shown to suppress ECG-mediated osteogenic differentiation. Taken together, the results showed that ECG stimulates osteoblast differentiation through the activation of TAZ and RUNX2, revealing a novel mechanism for green tea-stimulated osteoblast differentiation.

Osteoporosis is a bone disease that leads to reduced bone mineral density and an increased risk of fracture. Bone mineral

density is maintained by the balance between osteoclastic bone resorption and osteoblastic bone formation. Therefore, compounds that increase osteoblastic activity or decrease osteoclastic activity have been considered as potential drug candidates for osteoporosis (1, 2).

Green tea is a popular beverage in Asian countries, and it is made from the leaves of *Camellia sinensis*. Green tea contains several polyphenolic compounds called catechins, including (–)-epigallocatechin-3-gallate (EGCG),<sup>4</sup> (–)-epigallocatechin, (–)-epicatechin-3-gallate (ECG), and (–)-epicatechin (3). Previous studies have reported a correlation between tea consumption and the prevention of age-related bone loss in the elderly human population (4). Green tea polyphenols have been shown to improve bone mass and microarchitecture in various animal models, including ovariectomized aged female rats (5). Among the green tea catechins, EGCG is the most abundant, and its biological relevance in osteoblast differentiation and bone formation has been evaluated by several investigators (6). In mesenchymal stem cells, EGCG induces alkaline phosphatase activity and stimulates the expression of osteoblast marker genes, such as *Runx2* (Runt-related transcription factor 2), *osterix*, and *osteocalcin* (7). EGCG was shown to increase mineralized bone module formation in human osteoblast-like SaOS-2 cells (8). Further, studies show that EGCG has a vital role in osteogenic induction and inhibits the SAPK/JNK pathway by suppressing transforming growth factor- $\beta$  (TGF $\beta$ ) and the prostaglandin D2-mediated induction of HSP27 (9, 10). EGCG may also stimulate osteogenesis by increasing the synthesis of prostaglandin F2-induced vascular endothelial growth factor (6). However, the mechanism of action of other catechin compounds on the osteogenesis of bone has yet to be investigated.

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<sup>4</sup> The abbreviations used are: EGCG, (–)-epigallocatechin-3-gallate; ECG, (–)-epicatechin gallate; hMSCs, human mesenchymal stem cells; PP1A, protein phosphatase 1A; qRT-PCR, quantitative RT-PCR.

Osteoblast differentiation is critical for osteogenesis, and osteoblast-specific gene products regulate the differentiation process (11, 12). RUNX2 is a key transcription factor and a central regulator of bone formation, which mediates the temporal activation or repression of cell growth and phenotypic genes that regulate osteoblast-specific target genes, such as osteocalcin, during osteoblast differentiation (13, 14). *Runx2* knock-out mice show a complete lack of ossification (13, 14). RUNX2 interacts with a spectrum of transcription factors and coregulatory proteins that modulate its functions (15).

TAZ (transcriptional coactivator with PDZ-binding motif) is a 14-3-3-binding protein that regulates cell differentiation, proliferation, and stem cell renewal. TAZ functions as a transcriptional co-regulator and interacts with several transcription factors, including RUNX2 and PPAR $\gamma$  (16–24). TAZ stimulates osteogenic differentiation through RUNX2-mediated gene transcription while inhibiting adipogenic differentiation through the suppression of PPAR $\gamma$ -mediated gene transcription (16). The nuclear localization of TAZ is important for its binding with transcription factors and the activation of target genes. Extracellular signals, including Hippo, TGF $\beta$ , and Wnt pathways, regulate the localization and activity of TAZ. The Hippo signal regulates TAZ-mediated cell proliferation and tumorigenesis (25, 26). The canonical Wnt signal regulates osteogenic and adipogenic differentiation through the stabilization of TAZ (27). In this study, we report that ECG activates TAZ and stimulates the RUNX2-mediated gene transcription during osteoblast differentiation.

## MATERIALS AND METHODS

**Reagents and Cell Lines**—(–)-Epicatechin gallate, ascorbic acid,  $\beta$ -glycerophosphate, fast blue BB salt, and naphthol AS-MX phosphate were purchased from Sigma-Aldrich. C3H10T1/2 cells were purchased from the American Type Culture Collection, and bone marrow-derived human mesenchymal stem cells (hMSCs) were obtained from Lonza.

**Cell Culture and Osteoblast Differentiation**—C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. For the osteogenic differentiation of C3H10T1/2 cells,  $8 \times 10^4$  cells/well were seeded in 12-well culture plates. After 2 days, the culture medium was replaced with DMEM containing 50  $\mu$ g/ml ascorbic acid, 10 mM  $\beta$ -glycerophosphate, and 10% FBS (osteoblast differentiation medium). For the osteogenic differentiation of hMSCs, cells were plated in 12-well culture plates at a density of  $3 \times 10^4$  cells/cm<sup>2</sup>, and 48 h later, the medium was replaced with osteoblast differentiation medium containing 0.5  $\mu$ M dexamethasone. The differentiation medium was replaced every 2 days for differentiation of C3H10T1/2 and hMSCs.

**Alkaline Phosphatase Staining and Enzyme Activity Assay**—For alkaline phosphatase staining, differentiated osteoblast cells were fixed with 3.7% formaldehyde at room temperature for 10 min and then stained with 0.1 mg/ml naphthol AS-MX phosphate, 0.5% *N,N*-dimethylformamide, 2 mM MgCl<sub>2</sub>, 0.6 mg/ml fast blue BB salt, and 0.1 M Tris-HCl (pH 8.5) for 30 min at room temperature. To determine alkaline phosphatase enzyme activity, cells were lysed in 25 mM HEPES (pH 7.6), 0.1% Triton X-100, and 0.9% NaCl, and the cell lysates were incu-

bated with *p*-nitrophenyl phosphate substrate solution for 1 h at 37 °C, and 3 M NaOH solution was added to the reaction mixture to stop the reaction. The absorbance of alkaline phosphatase activity was measured at 405 nm using a microplate reader (Bio-Rad model 680).

**Immunoblot Analysis**—Immunoblot analysis was performed using the following primary antibodies: TAZ (28), RUNX2 (MBL, D130-3), HA (Covance),  $\alpha$ -tubulin (Abfrontier, LF-PA0146), Lamin B1 (Santa Cruz Biotechnology, Inc., sc-20682), Erk1/2 (Cell Signaling Technology, catalog no. 9102), phospho-Erk1/2 (Cell Signaling Technology, catalog no. 9101), JNK (Cell Signaling Technology, catalog no. 9252), phospho-JNK (Cell Signaling Technology, catalog no. 9251), p38 MAPK (Cell Signaling Technology, catalog no. 9212), phospho-p38 MAPK (Cell Signaling Technology, catalog no. 9211), AKT (Santa Cruz Biotechnology, sc-8312), phospho-AKT (Thr-308) (Cell Signaling Technology, catalog no. 9275), GSK-3 $\beta$  (Santa Cruz Biotechnology, sc-9166), phospho-GSK-3 $\beta$  (Ser-9) (Cell Signaling Technology, catalog no. 9323), and  $\beta$ -actin (Sigma-Aldrich, A5441).

**Stable Cell Lines**—Phoenix cells were transfected using the calcium phosphate-mediated transfection method with pBabepuro (Bp), pBabepuro-TAZ (T), or pBabepuro-mTAZ  $\Delta$ WW (T $\Delta$ WW) expression plasmids, which have been described previously (16). Viral supernatants were harvested and added to the C3H10T1/2 cells cultured in DMEM containing 4  $\mu$ g/ml Polybrene. The cells were further incubated with 2  $\mu$ g/ml puromycin for 6 days to eliminate the uninfected cells. The stable cell lines were used in follow-up experiments. The expression of TAZ was analyzed by immunoblot analysis.

**Quantitative Real-time PCR Analysis**—Total RNA was isolated from differentiated osteogenic cells using TRIzol reagent (Invitrogen), and cDNA was generated using RevertAid reverse transcriptase (Thermo Scientific). Expression of osteogenic marker genes, including osteocalcin (*Oc*), osteopontin (*Opn*), TAZ, *Runx2*, and *PPIA*, was analyzed by quantitative RT-PCR. Expression of mRNA was normalized using *GAPDH*. Quantitative RT-PCR was performed using the primers described in Table 1.

**Chromatin Immunoprecipitation**—Differentiated osteoblast cells were cross-linked with 0.75% formaldehyde and lysed in buffer containing 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. The immunoprecipitation, reverse cross-linking, and DNA purification were performed as described previously (29). The promoter region of the osteocalcin gene was amplified using the following primers: osteocalcin promoter F, 5'-CTGAACTGGGCAAATGAGGACA-3'; osteocalcin promoter R, 5'-AGGGGATGCTGCCAGGACTAAT-3'.

**Fractionation**—C3H10T1/2 cells were differentiated with or without 10  $\mu$ M ECG for 6 days and harvested with hypotonic buffer (20 mM HEPES, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, and protease inhibitor) for 5 min, and the lysed cells were centrifuged at 10,000  $\times$  g for 10 min. After that, soluble supernatants were collected for cytosolic protein, and then the pellets were lysed with radioimmune precipitation buffer for nuclear protein. Fractionated protein was analyzed by immunoblot analysis.

**TABLE 1**  
qRT-PCR primers

| Gene               | Forward primer                | Reverse primer                  |
|--------------------|-------------------------------|---------------------------------|
| Mouse <i>Oc</i>    | 5'-CTGACCTCACAGATGCCAAGC-3'   | 5'-TGGTCTGATAGCTCGTCACAAG-3'    |
| Mouse <i>Opn</i>   | 5'-GATTGCTTTTGGCTGTTGG-3'     | 5'-TGAGCTGCCAGAATCAGTCACT-3'    |
| Mouse <i>Taz</i>   | 5'-GTCACCAACAGTAGTCAGATC-3'   | 5'-GGACACTGTAGCACCCCTAACCCA-3'  |
| Mouse <i>Runx2</i> | 5'-CGGCCCTCCCTGAACCTCT-3'     | 5'-CGGTGGGGAAGACTGTGCCTG-3'     |
| Mouse <i>Pp1a</i>  | 5'-GACAGTTGGTGACACTCTTCTC-3'  | 5'-CTTGAGGATCTGGAAGGAACAC-3'    |
| Mouse <i>Gapdh</i> | 5'-GCTTGTTCATCAACGGGAAG-3'    | 5'-GATGTTAGTGGGGTCTCG-3'        |
| Human <i>TAZ</i>   | 5'-CCTCTCAATGATGTAGAGTCTGC-3' | 5'-AGTGATTACAGCCAGGTTAGAAAAG-3' |
| Human <i>MSX2</i>  | 5'-CTACCCGTTCCATAGACCTGT-3'   | 5'-GAGAGGGAGAGGAAACCCCTTT-3'    |
| Human <i>DLX5</i>  | 5'-CTACAACCGCGTCCCAAG-3'      | 5'-GCCATTACCATTTCTCACCT-3'      |
| Human <i>RUNX2</i> | 5'-AGAGGTACCAGATGGGACTGT-3'   | 5'-GGTAGCTACTTGGGGAGGATT-3'     |
| Human <i>GAPDH</i> | 5'-GACCCCTTCATTGACCTCAAC-3'   | 5'-GGTGCCATGGAATTTGCCATG-3'     |

**Luciferase Assay**—The 293T cells were transfected with 6XOSE2-luciferase reporter, RUNX2, and TAZ plasmids using X-tremeGene 9 DNA transfection reagent (Roche Applied Science). 6XOSE2-luciferase reporter and RUNX2 plasmids were a gift from R. Derynck (University of California, San Francisco, CA) and Yoshiaki Ito (Institute of Molecular and Cell Biology, Singapore), respectively. The culture medium was replaced with DMEM containing 10 μM ECG 24 h post-transfection. Luciferase activity was measured with the luciferase assay system (Promega, E1501), using a luminometer (Promega, Glomax®).

**PP1A siRNA Transfection**—C3H10T1/2 cells were seeded at a density of 4 × 10<sup>4</sup> cells/cm<sup>2</sup> in 12-well culture plates, transfected with PP1A siRNA duplex using Lipofectamine 2000 (Invitrogen), and, 24 h after transfection, incubated with osteogenic differentiation medium in the presence or absence of 10 μM ECG for 8 days. PP1A siRNA target sequences were as follows: mouse PP1A siRNA target sequence 1, 5'-CCATTCTTCTGGAGCTTGA-3'; mouse PP1A siRNA target sequence 2, 5'-TCTCCAGACTTGCAATCCA-3'.

**Immunocytochemistry**—C3H10T1/2 cells were plated on 12-mm coverglasses and treated with or without 10 μM ECG for 24 h. Then cells were fixed with 4% paraformaldehyde, permeabilized with 0.05% Triton X-100, and incubated with TAZ-specific antibody (prepared with the TAZ-specific peptide SSGGHPGPRLAGGA at Covance (Boston, MA)) overnight at 4 °C. After primary antibody incubation (1:100 dilution), cells were washed three times with 1× PBS containing 0.05% BSA and further incubated with FITC-conjugated secondary antibody. The green fluorescent TAZ signal was observed by confocal microscopy (Carl Zeiss LSM 510 Meta confocal microscope).

**Mitogen-activated Protein Kinase (MAPK) Activity Assay**—For the kinase activity assay, C3H10T1/2 cells were plated at a density of 2 × 10<sup>5</sup> cells/well with 6-well plate, and 24 h later, the culture medium was replaced with DMEM containing 0.1% FBS for 16 h. After the serum starvation, the cells were treated with 2 ng/ml EGF (R&D Systems) or 10 μM ECG for 30 min and lysed with 2× SDS sample buffer (125 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 15% glycerol, 0.01% bromphenol blue, 4% SDS). MAPK activities were analyzed by immunoblot analysis.

**Endogenous RUNX2 Immunoprecipitation**—C3H10T1/2 cells were incubated in osteoblast differentiation medium with or without 10 μM ECG for 6 days. The differentiated cells were lysed with radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and protease inhibitors), and immunoprecipitated with 1 μg of immunoglobulin G (IgG)

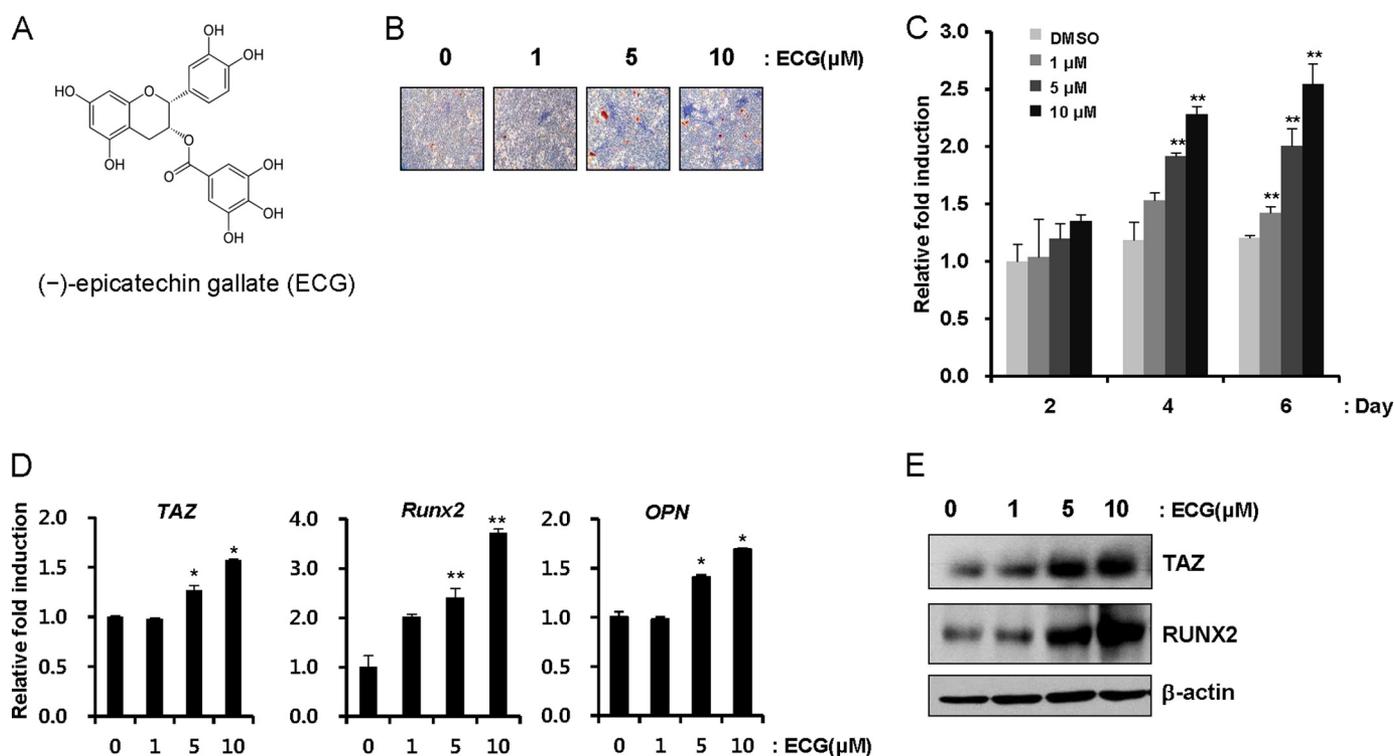
or TAZ antibody (BD Biosciences, catalog no. 560235) overnight. Endogenous immune complexes were analyzed by immunoblot analysis.

**RESULTS**

**ECG Stimulates Osteoblast Differentiation**—To study the effect of ECG (Fig. 1A) on osteoblast differentiation, C3H10T1/2 cells were cultivated in osteogenic differentiation medium with increasing concentrations of ECG. To assess differentiation, alkaline phosphatase activity, an osteogenic marker, was analyzed at 2, 4, and 6 days. As shown in Fig. 1, B and C, a dose-dependent induction of alkaline phosphatase activity was observed. To study the activity of ECG further, expression of osteoblast marker genes, including *TAZ*, *Runx2*, and osteopontin, was analyzed after 6 days of differentiation using qRT-PCR. As shown in Fig. 1D, a dose-dependent increase in the expression of the marker genes was observed (Fig. 1D). We also observed a significant, dose-dependent increase in the expression of the TAZ and RUNX2 proteins during osteogenic differentiation (Fig. 1E). Thus, the results suggest that ECG stimulates osteogenic differentiation by inducing the expression of TAZ and RUNX2.

**ECG Stimulates RUNX2-mediated Gene Transcription**—TAZ physically interacts with RUNX2 and activates RUNX2-mediated gene transcription (16) of osteoblastic marker genes, including osteocalcin. Thus, to study whether ECG stimulates RUNX2-mediated gene transcription through TAZ, luciferase reporter constructs containing RUNX2-binding sites (6xOSE2-luc) were used. The reporter plasmids were transfected into 293T cells with the RUNX2 and TAZ expression plasmids in the presence of ECG. As shown in Fig. 2A, RUNX2 and TAZ stimulated reporter activity, and the presence of ECG further increased reporter activity by 50%, suggesting that ECG stimulates RUNX2 and that TAZ mediates the transcription of osteoblastic genes.

Next, to understand the mechanism of ECG-induced reporter activity, we investigated the physical interaction between TAZ and RUNX2 in the presence of ECG. Tagged TAZ and RUNX2 plasmids were transfected into 293T cells, and the physical interaction of TAZ and RUNX2 was analyzed by immunoprecipitation. As shown in Fig. 2B, ECG significantly enhanced the physical interaction of TAZ and RUNX2. We further investigated the interaction. Endogenous TAZ proteins were immunoprecipitated, and the bound proteins were analyzed with RUNX2 antibody. We observed that endogenous TAZ interacts with RUNX2, and ECG facilitates the interaction (Fig. 2C).



**FIGURE 1. ECG stimulates the differentiation of osteoblasts.** *A*, structure of ECG. *B*, ECG increases alkaline phosphatase activity in a dose-dependent manner. C3H10T1/2 cells were incubated in osteogenic differentiation medium in the presence of ECG at the indicated concentration. At 6 days after differentiation, alkaline phosphatase activity was visualized by staining to determine the osteogenic potential of ECG. A blue color indicates increased alkaline phosphatase activity. *C*, alkaline phosphatase activity in *B* was analyzed at the indicated time points. DMSO was used as a vehicle. \*\*,  $p < 0.05$  by Student's *t* test. *D*, ECG stimulates the expression of osteoblastic marker genes. C3H10T1/2 cells were incubated in osteoblastic differentiation medium in the presence of 10  $\mu\text{M}$  ECG. After 6 days of differentiation, the cells were harvested, and total RNA was obtained. Using qRT-PCR, the expression of *TAZ*, *Runx2*, and *Opn* was analyzed. Their relative expression was calculated after normalization to the GAPDH level. \*,  $p < 0.01$ ; \*\*,  $p < 0.05$  by Student's *t* test. *E*, C3H10T1/2 cells were treated with ECG and induced to differentiate for 6 days. Whole-cell extracts were harvested, resolved by SDS-PAGE, and analyzed for TAZ, RUNX2, and  $\beta$ -actin levels using immunoblotting. Error bars, S.D.

These results suggest that the increased interaction between TAZ and RUNX2 stimulates RUNX2-mediated osteogenic gene transcription.

Next, to study whether TAZ induces osteogenic marker genes through an endogenous RUNX2-binding site in the presence of ECG, chromatin immunoprecipitation analysis was performed with cells overexpressing FLAG-tagged TAZ or WW domain-deleted TAZ, which cannot interact with RUNX2 (Fig. 2, *D* and *E*). The chromatin fragments that co-precipitated with the FLAG-tagged proteins were analyzed with PCR primers spanning the RUNX2-binding site of the osteocalcin promoter. As shown in Fig. 2, *F* and *G*, FLAG-TAZ was recruited into the RUNX2-binding site of the osteocalcin gene promoter, and a 2-fold increase in the recruitment of FLAG-TAZ was observed in the presence of ECG. However, WW-deleted TAZ was not recruited into the promoter (Fig. 2, *F* and *G*). These results showed that ECG stimulates osteocalcin expression through the recruitment of TAZ at the endogenous RUNX2-binding site of osteocalcin promoter in differentiating cells.

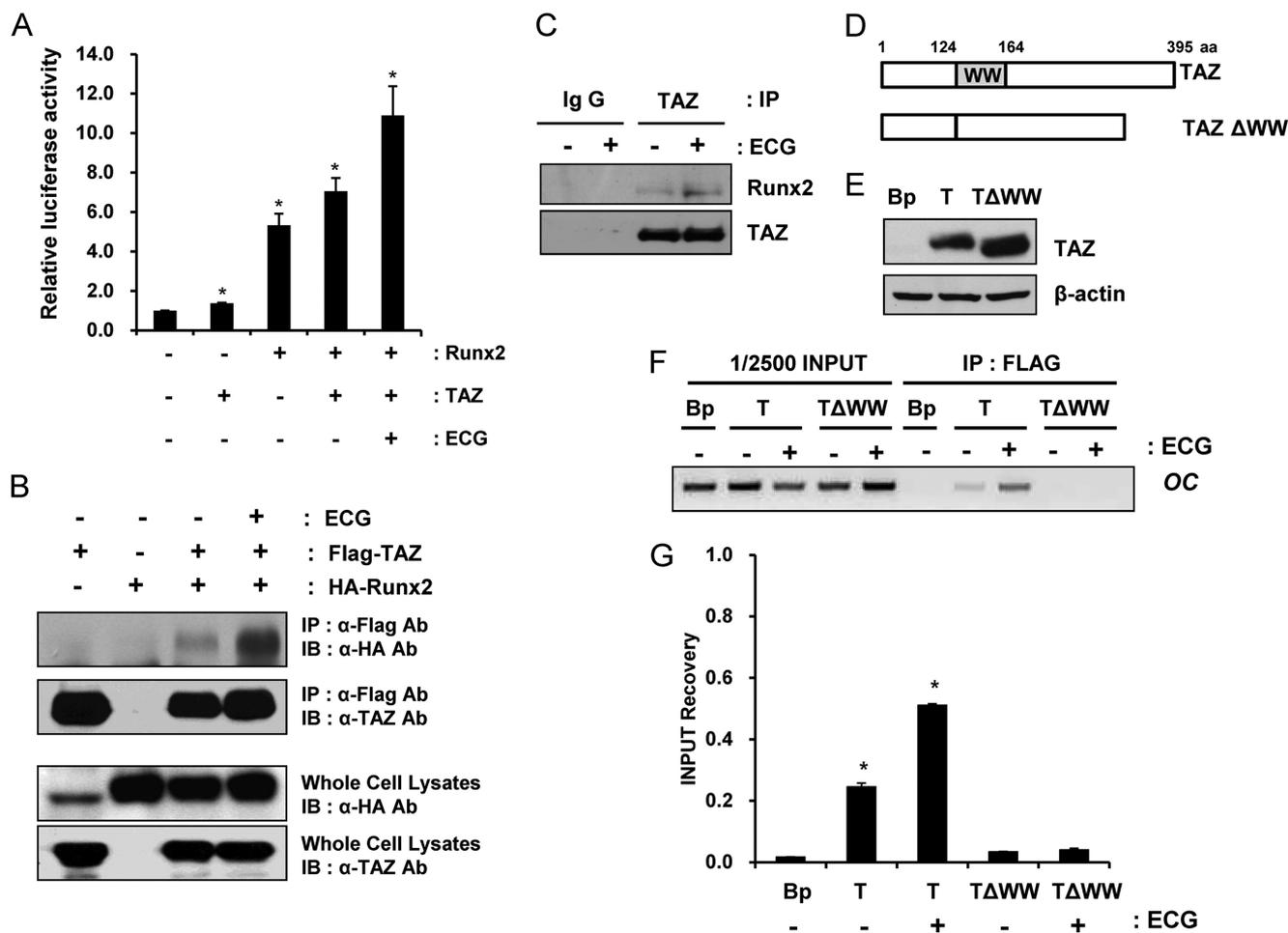
**ECG Stimulates Nuclear Localization of TAZ**—Increased recruitment of TAZ at the endogenous osteocalcin promoter indicated that TAZ preferentially localizes to the nucleus in the presence of ECG. The cellular distribution of TAZ after ECG treatment was evaluated using immunocytochemistry. As shown in Fig. 3*A*, ECG facilitated nuclear localization compared with the control, and predominant nuclear localization of

TAZ was observed in over 50% of cells. To further analyze the nuclear localization of TAZ, ECG-treated cells were prepared, and cytosolic and nuclear protein fractions were isolated. As in Fig. 3*B*, a significant increase in TAZ was observed in the nucleus of ECG-treated cells. We also observed that RUNX2 expression was significantly increased in nuclei after ECG treatment (Fig. 3*C*). Thus, these results suggest that ECG increases nuclear localized TAZ and RUNX2 and stimulates TAZ-mediated gene transcription.

The subcellular localization of TAZ is determined by its phosphorylation status (28). The phosphorylation of TAZ at serine 89 induces 14-3-3 binding and cytosolic sequestration of TAZ, whereas dephosphorylated TAZ is not subjected to 14-3-3 binding and localizes to the nucleus (16, 28). Therefore, we studied the phosphorylation status of TAZ at serine 89 using a phospho-specific antibody. There was a significant increase in total TAZ protein levels, although phosphorylation at serine 89 was reduced in the presence of ECG (Fig. 3, *D* and *E*). These results suggest that ECG may regulate the phosphorylation status of TAZ at serine 89.

**ECG Stimulates PPIA Expression and Stimulates Dephosphorylation of TAZ**—Hippo signal regulates organ size by modulating cell proliferation, cell death, and cell differentiation (30, 31). Lats kinase is a core component of the Hippo signaling pathway and has been reported to phosphorylate human TAZ (25) at serine 311 and the mouse TAZ at serine 306. Casein

## ECG Stimulates Osteogenic Differentiation

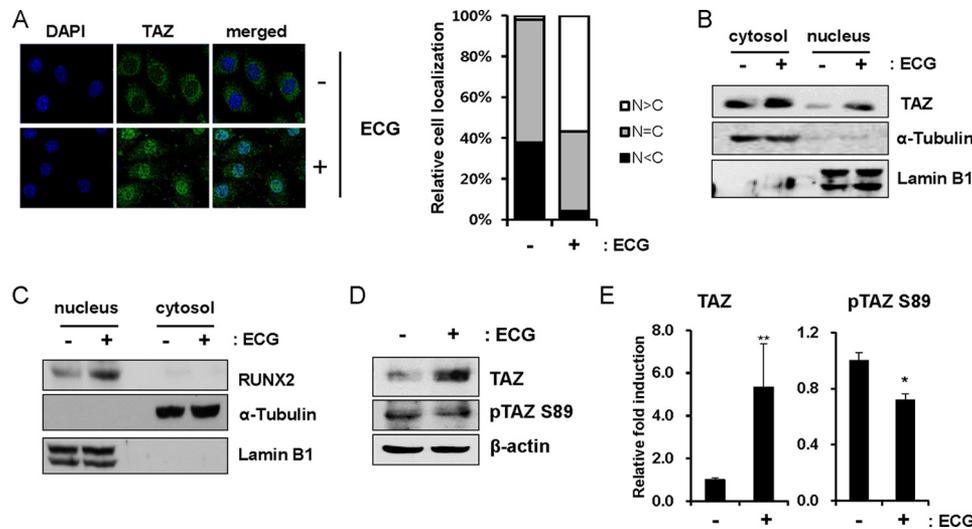


**FIGURE 2. ECG stimulates RUNX2-mediated osteoblast differentiation.** *A*, stimulation of RUNX2-driven gene expression by ECG. 293T cells were transfected with the RUNX2 expression plasmid (0.005  $\mu$ g/well) and the 6xOSE2-luciferase reporter construct (0.05  $\mu$ g/well), which contains six copies of the RUNX2-binding site in the osteocalcin promoter. After 24 h of transfection, the cells were incubated with 10  $\mu$ M ECG. After 24 h, cell lysates were prepared to analyze the luciferase activities. Differences in the transfection efficiency were adjusted by normalizing the firefly luciferase activity to that of *Renilla* luciferase. The luciferase activity was calculated and expressed as -fold induction. \*,  $p < 0.01$ ,  $t$  test. *B*, 293T cells were transfected with HA-tagged RUNX2 and/or FLAG-tagged TAZ expression plasmids and incubated with 10  $\mu$ M ECG for 24 h. Whole-cell lysates (WCL) were precipitated (IP) with FLAG-M2-agarose beads. The precipitates and whole-cell lysates were analyzed by immunoblot analysis (IB) with antibodies against HA and TAZ. *C*, C3H10T1/2 cells were incubated in the presence or absence of 10  $\mu$ M ECG, and the cell lysates were immunoprecipitated with an IgG or anti-TAZ antibody. Endogenous TAZ bound to RUNX2 was analyzed by immunoblot analysis. *D*, TAZ structures. TAZ WW domain, which interacts with RUNX2, is deleted in TAZ  $\Delta$ WW. aa, amino acids. *E*, FLAG-tagged TAZ wild type (T) and TAZ  $\Delta$ WW-expressing (T $\Delta$ WW) C3H10T1/2 cells were prepared by retrovirus. The TAZ expression of stable cell lines was analyzed by immunoblot analysis. The expression of  $\beta$ -actin was analyzed as a loading control. Bp, control cells. *F*, ECG increases recruitment of TAZ, not TAZ  $\Delta$ WW, at the endogenous osteocalcin promoter. The above cells were treated for 4 days with osteogenic differentiation medium in the presence of 10  $\mu$ M ECG, and enriched DNA immunoprecipitated using anti-FLAG antibodies was analyzed for osteocalcin promoter occupancy by PCR. ECG increases TAZ wild type recruitment, not TAZ  $\Delta$ WW, into the osteocalcin promoter. *G*, the recruited TAZ in *F* were quantitatively analyzed by qRT-PCR. Recovered DNAs from input DNAs were quantified by analyzing the Ct value. \*,  $p < 0.01$  by Student's  $t$  test. Error bars, S.E.

kinase-1 further phosphorylates TAZ and induces its ubiquitin-mediated proteolytic degradation (25). The phosphorylation of TAZ is a reversible process, and dephosphorylation occurs by PP1A phosphatase (32). PP1 is a eukaryotic Ser/Thr protein phosphatase involved in diverse cellular functions (33). PP1A is a catalytic subunit of PP1. Therefore, we studied whether PP1A activity is important for ECG-induced TAZ expression. First, to study whether ECG can stabilize TAZ, C3H10T1/2 cells were incubated with ECG in the presence of cycloheximide, a protein synthesis inhibitor. As shown in Fig. 4A, we observed that TAZ was more stable in the presence of ECG than control, suggesting that ECG plays a role in the stabilization of TAZ. Next, C3H10T1/2 cells were treated with a PP1A inhibitor, okadaic acid, for 3 h. As shown in Fig. 4B, we observed that okadaic acid significantly decreased the TAZ expression that was induced by

ECG. However, the decrease in TAZ expression was not caused by the inhibition of transcription because TAZ mRNA expression was not affected by the 3-h okadaic acid treatment (Fig. 4B). These results indicate that okadaic acid regulates TAZ expression at a post-transcriptional level. To further analyze the effect of PP1A activity, we depleted endogenous PP1A protein using siRNAs. Fig. 4C shows that PP1A was depleted by the two siRNAs tested, which resulted in a significant decrease in the expression of TAZ, suggesting that PP1A plays an important role in ECG-induced TAZ expression.

It is noteworthy that ECG induced the expression of PP1A by about 3-fold (Fig. 4, B and C), suggesting that the PP1A induction plays an important role in ECG-mediated osteogenic stimulation. Next, to study the effect of PP1A on ECG-mediated osteogenic differentiation, the control and PP1A siRNA-treated cells were



**FIGURE 3. ECG increases nuclear localization of TAZ.** *A*, C3H10T1/2 cells were incubated with 10  $\mu\text{M}$  ECG. After 24 h, the cells were fixed, and the cellular location of TAZ was analyzed by immunocytochemistry. An FITC-conjugated secondary antibody was used for the green fluorescence signal. DAPI staining indicates the nuclei of the cells. The *right-hand panel* shows quantitative analysis of TAZ localization. The cellular distribution of TAZ was analyzed based on whether TAZ levels were higher in the nucleus ( $N > C$ ), higher in the cytoplasm ( $N < C$ ) or evenly distributed between the nucleus and cytoplasm ( $N = C$ ). The percentage of cells in each category was determined after observing cells in five different microscopic fields. *B* and *C*, C3H10T1/2 cells were incubated with vehicle or 10  $\mu\text{M}$  ECG for 24 h, and the cell lysates were prepared and fractionated into cytosol and nuclear extracts according to the indicated methods. TAZ (*B*) and RUNX2 (*C*) expression was analyzed by immunoblot analysis. *D*, C3H10T1/2 cells were treated for 2 days with osteogenic differentiation medium in the absence or presence of 10  $\mu\text{M}$  ECG; cell lysates were prepared, and the phosphorylation status of TAZ at serine 89 was analyzed with a phospho-specific TAZ antibody, which was prepared with TAZ phosphopeptides (CHVRSHpSSPASL) at AbFrontier (Seoul, Korea). *E*, quantitative analysis of total and phosphorylated TAZ. The amount of total and phosphorylated TAZ at serine 89 from three independent experiments of *D* was analyzed with a densitometer, and relative-fold induction is shown here. \*,  $p < 0.01$ ; \*\*,  $p < 0.05$  by Student's *t* test. Error bars, S.E.

maintained in osteogenic differentiation medium. Fig. 4*D* indicates that ECG enhanced the osteogenic potential of C3H10T1/2 cells, as evidenced by the increased alkaline phosphatase activity. However, PP1A depletion using siRNAs led to marked reduction of osteogenic potential. The results were verified by analyzing the expression of osteogenic marker genes in parallel. As shown in Fig. 4*E*, ECG up-regulated osteopontin, *Runx2*, *TAZ*, and osteocalcin expression, whereas PP1A depletion significantly reduced expression of osteogenic markers, suggesting that PP1A is an important mediator of ECG-mediated osteogenesis.

**ECG Stimulates p38 MAPK**—MAPKs are serine/threonine kinases that are involved in osteogenic differentiation (34). To study whether ECG activates the MAPKs, active kinases were analyzed using phospho-specific antibodies. As shown in Fig. 5*A*, p38 MAPK, not ERK and JNK, is significantly activated after ECG treatment. We also observed that ECG weakly stimulates the AKT activity and phosphorylates GSK3 $\beta$  for its inhibition (Fig. 5*B*). Thus, these results suggest that ECG may activate osteogenic differentiation through p38 MAPK.

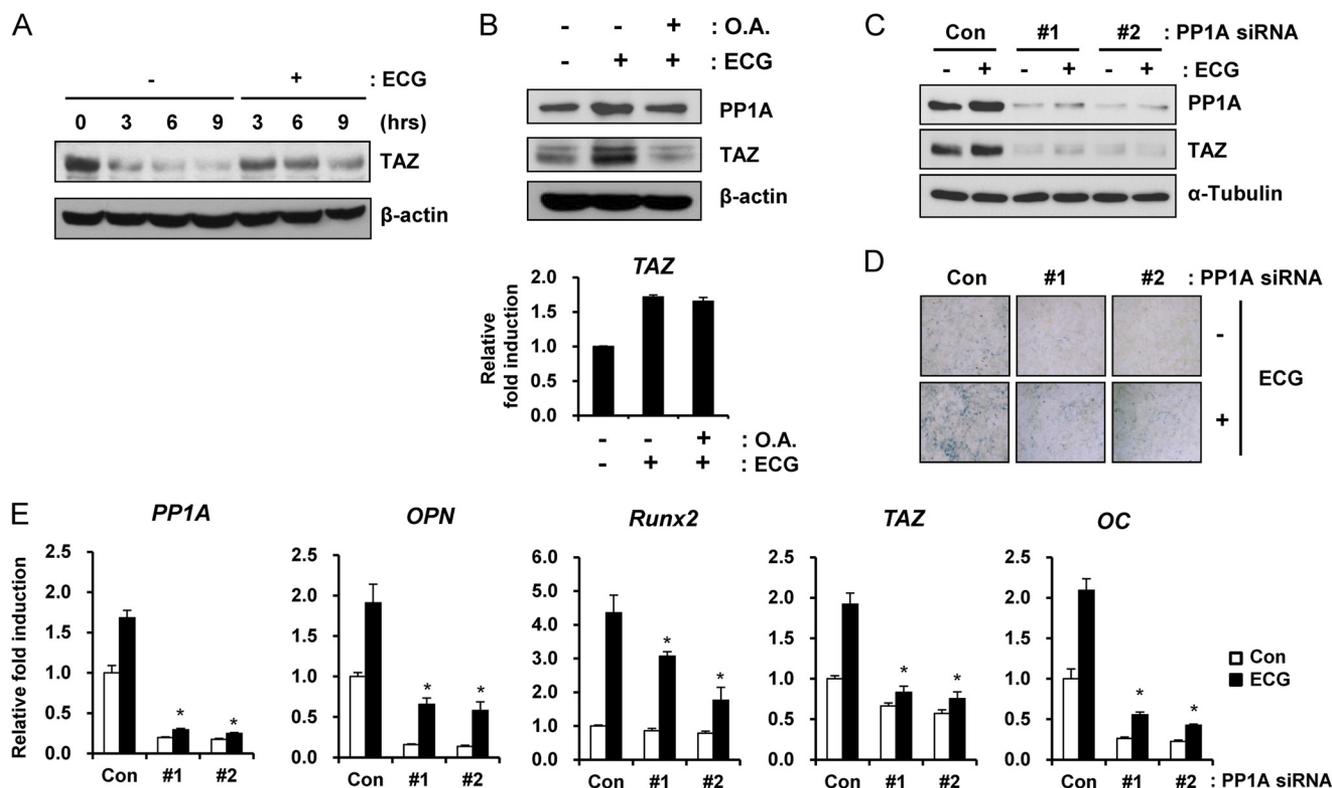
**ECG Stimulates the Differentiation of Human Mesenchymal Stem Cells into Osteoblasts**—hMSCs are of mesodermal origin and are multipotent cells that can differentiate into osteoblasts, chondrocytes, and adipocytes. Here, we investigated the potential of ECG to stimulate differentiation of hMSCs into osteoblasts. As shown in Fig. 6*A*, ECG stimulated alkaline phosphatase activity in a dose-dependent manner. In addition, the osteogenic marker genes *DLX5*, *MSX2*, *Runx2*, and *TAZ* were up-regulated in a dose-dependent manner (Fig. 6*B*). *TAZ* and RUNX2 protein expression was also increased after ECG treatment (Fig. 6*C*). These results suggest that ECG holds promise as

a therapeutic compound for induction of osteogenesis in osteoporosis patients.

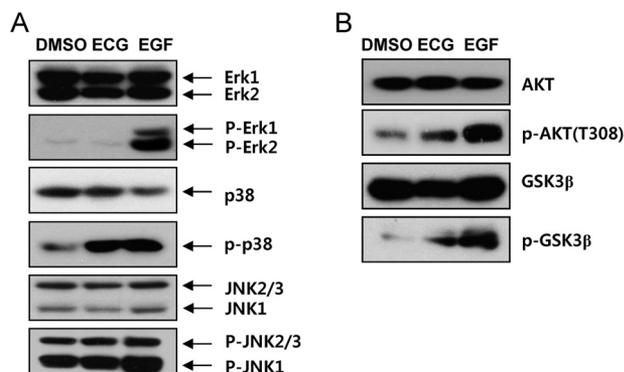
## DISCUSSION

Epidemiological studies have established a correlation between green tea consumption and the prevention of age-related bone loss (4). Among the components of green tea, catechins have received much attention for their potentially beneficial effects on osteogenesis (5). However, detailed functional mechanisms underlying the role of catechins in osteogenesis have yet to be understood. In this study, we observed that ECG, one of the major catechins found in green tea, stimulates osteoblast differentiation through a mechanism mediated by RUNX2, the master regulator of osteoblast marker gene transcription. Further, ECG enhances the transcriptional and post-transcriptional expression of TAZ, a transcriptional coregulator involved in osteogenesis (Fig. 7). During osteogenesis, TAZ mRNA expression is up-regulated, and the resultant TAZ protein is stabilized by PP1A phosphatase-mediated dephosphorylation (Figs. 1 and 4). The proteolytic degradation of TAZ is dependent on its phosphorylation status. When the phosphodegron motif at its C terminus is phosphorylated by Lats and casein kinase-1, it is recognized by proteasomal complexes and ubiquitinated for degradation (25). Dephosphorylation of TAZ is accomplished by the PP1A phosphatase, which, in turn, increases its nuclear localization. Interestingly, ECG also decreases the phosphorylation of TAZ at serine 89, stimulating its nuclear localization and, therefore, its transcriptional activity (Fig. 3). On the contrary, PP1A knockdown led to decreased TAZ expression and osteogenic potential (Fig. 4). Thus, these results show that ECG regulates osteogenic differentiation through PP1A.

## ECG Stimulates Osteogenic Differentiation



**FIGURE 4. ECG stimulates TAZ expression through PP1A.** *A*, ECG stabilizes TAZ. C3H10T1/2 cells were incubated with 20  $\mu\text{g}/\text{ml}$  cycloheximide in the presence or absence of 10  $\mu\text{M}$  ECG. At the indicated time points, cell lysates were prepared, and the expression of TAZ was analyzed by immunoblot analysis. *B*, the PP1A inhibitor, okadaic acid, inhibits ECG-induced TAZ protein expression. C3H10T1/2 cells were treated with 10  $\mu\text{M}$  ECG for 24 h, and 50 ng/ml okadaic acid was added for 3 h. TAZ protein and mRNA expression levels were analyzed using immunoblot and qRT-PCR, respectively (*bottom*). *C*, PP1A depletion leads to a decreased level of TAZ. C3H10T1/2 cells were transfected with 100  $\mu\text{mol}$  of scrambled control siRNA (Con), mouse PP1A siRNA 1, or mouse PP1A siRNA 2 for 24 h and subsequently incubated in 10  $\mu\text{M}$  ECG containing differentiation medium for 2 days. Next, cell lysates were prepared, and PP1A and TAZ expression levels were examined by immunoblot analysis.  $\alpha$ -Tubulin expression was analyzed as a loading control. *D*, PP1A depletion decreases ECG-induced osteogenic differentiation. C3H10T1/2 cells in *C* were differentiated into osteoblasts, and alkaline phosphatase activity was analyzed at 8 days after differentiation. *E*, PP1A depletion decreases ECG-induced expression of osteogenic marker genes. After 8 days of differentiation, C3H10T1/2 cells in *D* were harvested, and total RNA was obtained. Using qRT-PCR, the expression of *PP1A*, *Opn*, *Runx2*, *TAZ*, and *Oc* were analyzed. Their relative expression was calculated after normalization to the *GAPDH* level. \*,  $p < 0.01$  by Student's *t* test. Error bars, S.E.



**FIGURE 5. ECG stimulates p38 MAPK for osteoblast differentiation.** *A*, C3H10T1/2 cells were incubated with a vehicle DMSO, 10  $\mu\text{M}$  ECG, and 2 ng/ml EGF. After 30 min, the cells were lysed, and the activity of cellular ERK, p38 MAPK, and JNK was analyzed by immunoblot analysis. The activation status of the kinases was analyzed using their phospho-specific (*p*-) antibodies. *B*, in the above condition, the activity of cellular AKT and GSK3 $\beta$  were analyzed by immunoblot analysis using phospho-specific antibodies. Error bars, S.E.

Alteration of TAZ expression has profound effects on osteogenic differentiation; deletion of TAZ suppresses osteogenic differentiation in mesenchymal stem cells (16). Osteoblast-specific overexpression of TAZ increases bone mass *in vivo* (35). Also, a recent report has shown that the canonical Wnt signal

stabilizes TAZ and stimulates osteogenic differentiation (27). Thus, these results strengthen the importance of TAZ expression in ECG-induced osteogenic differentiation.

Previously, it was shown that catechins stimulate osteogenesis by enhancing the activity of protein phosphatase 2A, another type of phosphoprotein phosphatase (36). The study suggests that catechin induces protein phosphatase 2A and down-regulates ERK activity. EGCG, another catechin compound, stimulates osteoblast differentiation in several cells, including mesenchymal stem cells. It suppresses HSP27 induction through inhibition of the SAPK/JNK or p44/p42 MAPK pathways (9, 10). Therefore, we investigated whether ECG regulates the MAPK pathway. Interestingly, we observed that ECG stimulates p38 MAPK, not ERK and JNK MAPK (Fig. 5), suggesting that p38 MAPK is a key signaling kinase for ECG-induced osteogenic differentiation. At this point, it is notable that p38 kinase is critical for osteoblast differentiation (37).

Bone formation is induced by increased osteogenic differentiation and decreased osteoclast differentiation. It was shown that green tea and its catechin compounds suppress osteoclast differentiation. EGCG increases apoptosis of osteoclasts by stimulating the DNA damage response or caspase-3 activation (38, 39). EGCG also decreases the survival of osteoclasts by

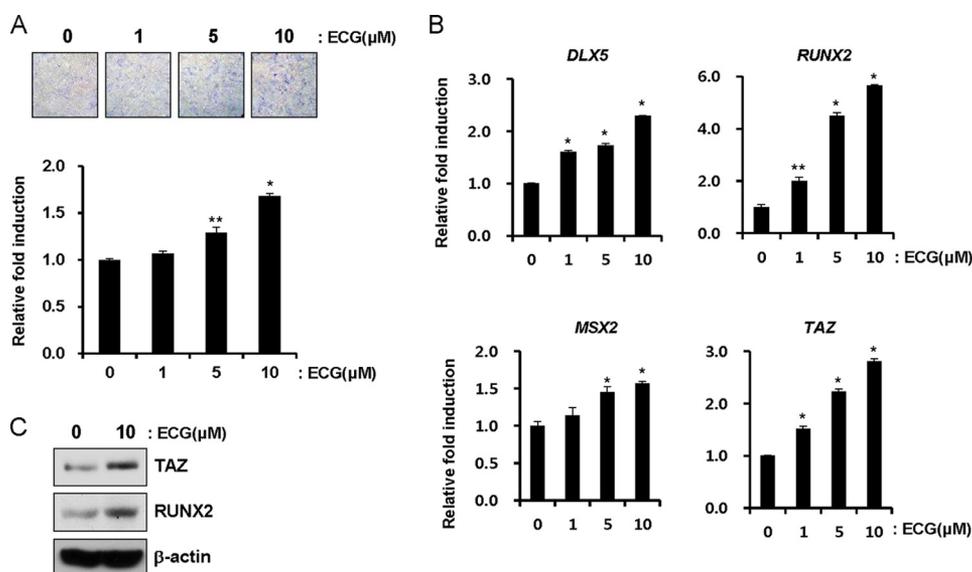


FIGURE 6. **ECG stimulates the osteogenic differentiation of human mesenchymal stem cells.** A, bone marrow-derived human mesenchymal stem cells were treated with the indicated concentrations of ECG to induce osteoblast differentiation. After 12 days of differentiation, alkaline phosphatase activity was visualized to determine the osteogenic potential of ECG. The bottom panel shows quantitative alkaline phosphatase activity. B, qRT-PCR analysis of expression of the osteoblastic marker genes *DLX5*, *Runx2*, *MSX2*, and *TAZ* using total RNA prepared from the cells in A. \*,  $p < 0.01$ ; \*\*,  $p < 0.05$ , Student's *t* test. C, hMSCs were incubated for 6 days with osteogenic differentiation medium in the presence of 10  $\mu\text{M}$  ECG, and the expression of *RUNX2* and *TAZ* was analyzed by immunoblot analysis. Error bars, S.E.

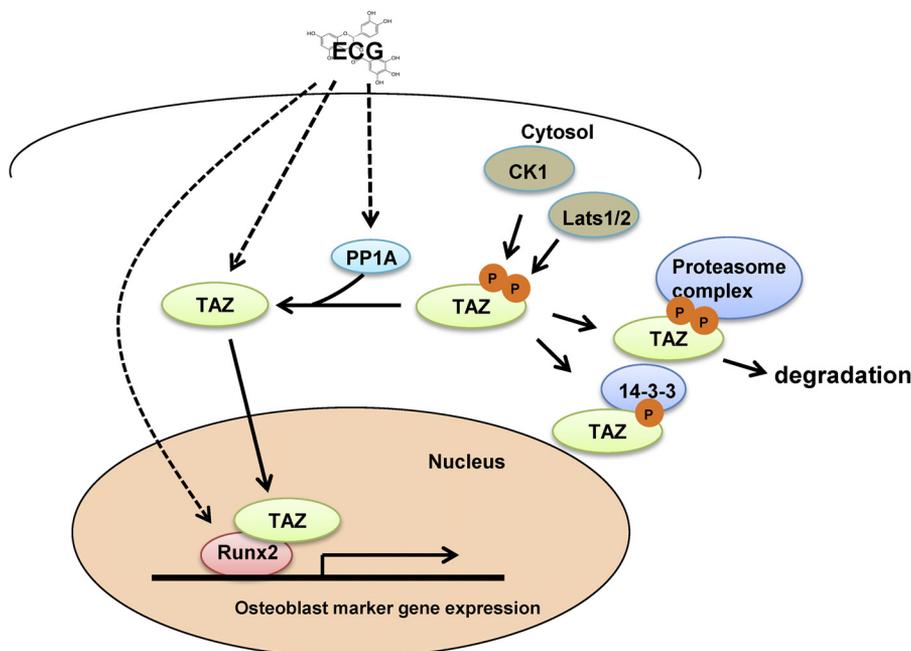


FIGURE 7. **Experimental model.** ECG increases *TAZ*, *Runx2*, and *PP1A* expression. Increased *PP1A*, a phosphatase, facilitates dephosphorylation of *TAZ*, which inhibits 14-3-3 binding and proteasomal degradation, and facilitates the nuclear localization of *TAZ*. Under the activation of Hippo signal, the signaling component, *Lats1/2* kinase, and casein kinase 1 can phosphorylate *TAZ* at serines 306 and 309, which can be recognized by proteasome complexes, and it induces the proteolytic degradation of *TAZ*. The phosphorylation of *TAZ* at serine 89 induces its interaction with 14-3-3, a scaffold protein, and the complexes are sequestered at the cytosol. When *TAZ* is dephosphorylated by *PP1A*, it moves into the nucleus and interacts with *RUNX2* and stimulates the transcription of osteoblastic marker genes.

decreasing RANKL-induced NF- $\kappa$ B activation (40, 41). Thus, it would be interesting to test whether ECG regulates osteoclast differentiation.

In our study, ECG stimulates the differentiation of osteoblast in C3H10T1/2 cells and hMSCs (Fig. 6). The results indicate that ECG may stimulate osteogenic lineage determination and that the osteogenic effect of ECG is not a cell type-specific response. Thus, the results suggest that ECG is one of the ben-

eficial compounds present in green tea that is capable of improving human bone mineral density.

Interestingly, we observed that ECG stimulates *DLX5* and *MSX2* expression in addition to *RUNX2* and *TAZ* in hMSCs (Fig. 6). It was shown that *DLX5* and *MSX2* play critical roles in bone development. *DLX5* knock-out mice exhibit craniofacial abnormalities, a delayed ossification of the roof of the skull and abnormal osteogenesis (42). Femurs of *DLX5* knock-out mouse

## ECG Stimulates Osteogenic Differentiation

embryos exhibit a reduction in both total and trabecular bone volume (43). *MSX2* knock-out mice have defects in skull ossification and endochondral bone formation (44). Haploinsufficiency of the *MSX2* in humans causes defects in skull ossification (45). In this study, we did not assess the induction mechanism for *DLX5* and *MSX2* expression, but it will be interesting to investigate it in the near future. In summary, we report a novel stimulator of osteogenesis, catechin ECG, which acts through the induction of TAZ and activation of *RUNX2*-mediated transcription of osteoblast differentiation marker genes.

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**(-)-Epicatechin Gallate (ECG) Stimulates Osteoblast Differentiation via Runt-related Transcription Factor 2 (RUNX2) and Transcriptional Coactivator with PDZ-binding Motif (TAZ)-mediated Transcriptional Activation**

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