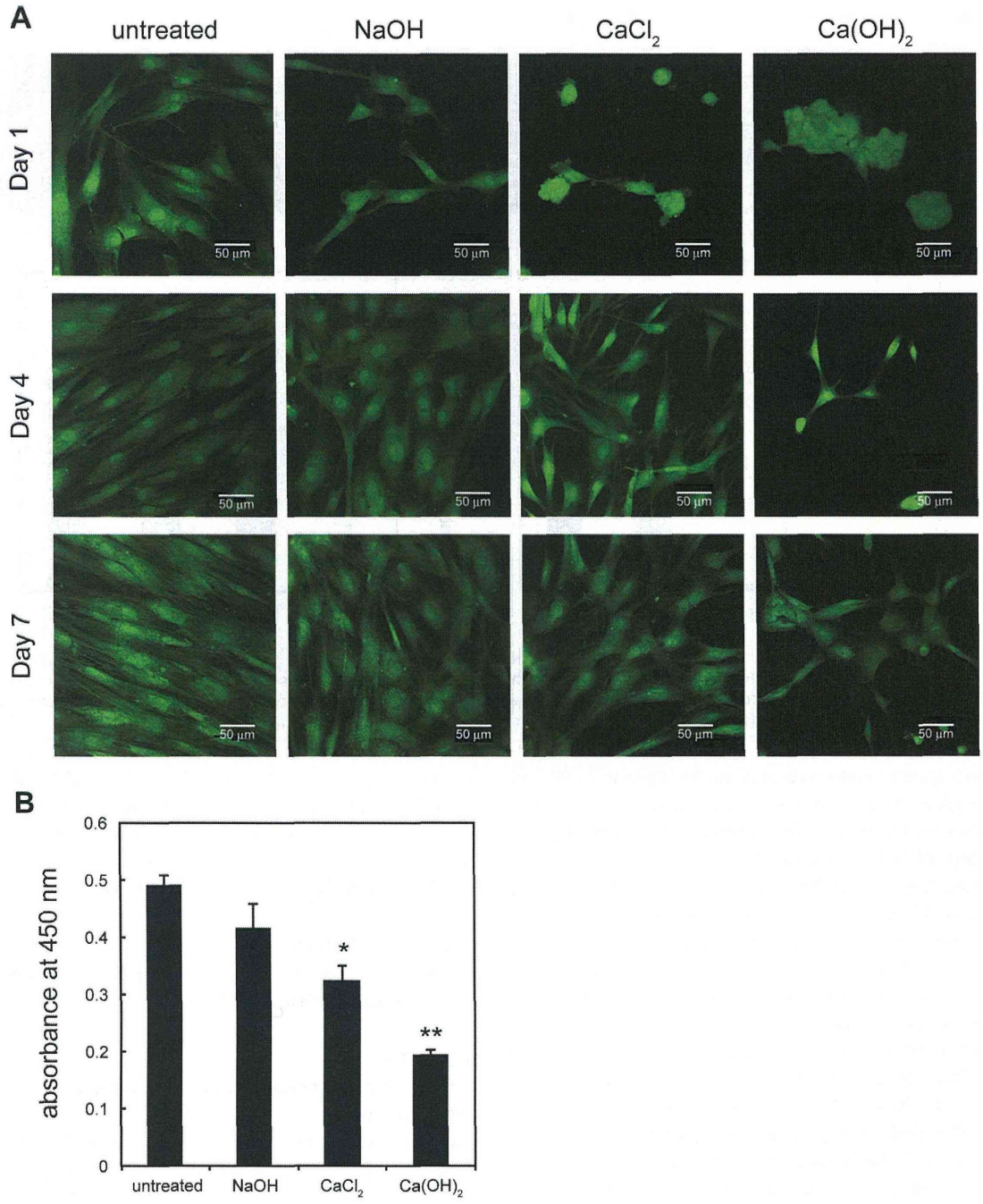

FIGURE 2. Confocal microscopy of hMSCs cultured on untreated, NaOH, $CaCl_2$, and $Ca(OH)_2$ treated titanium disks for 1, 4, and 7 days (A). Images are representative of five independent experiments. Cells were stained with CellTrackerTM Green Fluorescent Probe (Lonza). Proliferation of hMSCs for 7 days was determined with the cell proliferation assay reagent TetraColor One (B). The optical density of each well was measured at 450 nm ($n = 3$). Significant differences in comparison to untreated titanium are shown as $^*p < 0.05$ and $^{**}p < 0.01$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

soaked in Hanks' Balanced Salt Solution for 7 days. The amount of Ca^{2+} on titanium disks treated with NaOH, $CaCl_2$, and Ca(OH) was directly correlated with apatite formation. Apatite formation was slightly but significantly greater on $CaCl_2$-treated disks than on NaOH-treated disks [Fig. 1(C)]. Furthermore, $Ca(OH)_2$-treated titanium disk caused significantly greater apatite formation than the NaOH- and $CaCl_2$-treated disks [Fig. 1(C)].

Cell morphology and proliferation

To understand cell adhesion, we investigated the morphologies of hMSCs cultured on chemically modified titanium disks. We labeled the cells with CellTracker (Lonza) and observed at 1, 4, and 7 days after seeding. Confocal microscopy revealed cells on the modified disks showed smaller spread areas 1 day after seeding, although cells on the untreated disks exhibited normal cell morphology [Fig. 2(A)

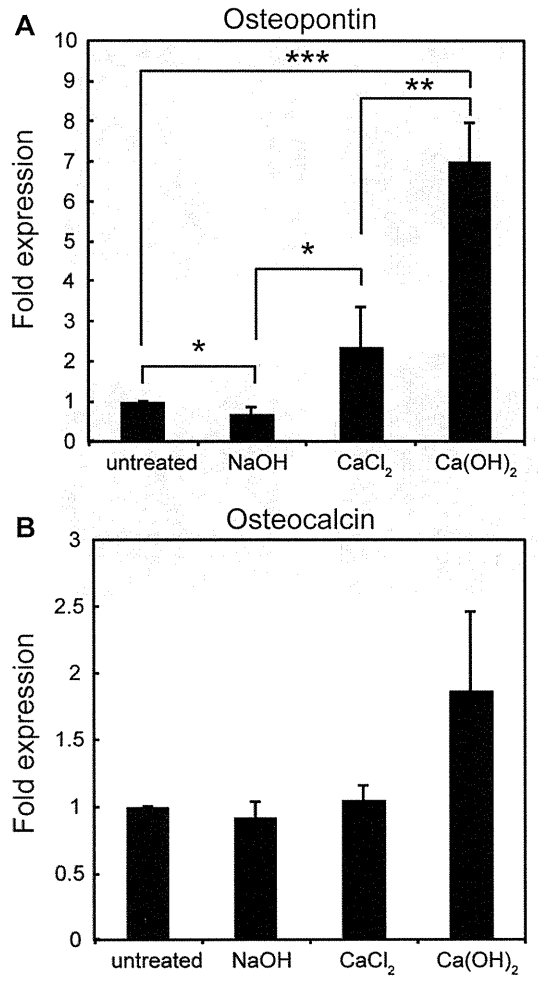

FIGURE 3. Relative expression of osteopontin (A) and osteocalcin (B) were analyzed by real-time PCR at 7 days after hMSC seeding ($n = 3$). The value was normalized to GAPDH. Significant differences between groups are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

upper panels]. Cells grown on $CaCl_2$- or $Ca(OH)_2$-treated disks were round in shape. Four days after seeding, the cells on NaOH- or $CaCl_2$-treated disks were comparable to cells cultured on untreated disks, whereas the cells on the $Ca(OH)_2$-treated disk were not. hMSCs on $Ca(OH)_2$-treated disks were comparable in shape at 7 days after seeding [Fig. 2(A) middle and lower panels]. hMSC proliferation for 7 days was also investigated. Culture on chemically modified titanium surfaces decreased hMSC cell numbers [Fig. 2(B)].

Cell differentiation and quantitative real-time PCR

To investigate the osteogenic differentiation of hMSCs cultured on chemically modified titanium disks, OPN and OCN transcripts were characterized by real-time PCR after culture for 7 days (Fig. 3). The transcript levels of OPN in hMSCs cultured on $CaCl_2$-treated titanium disks were significantly higher than those on NaOH-treated disks [Fig. 3(A)]. OPN expression was significantly greater on $Ca(OH)_2$-treated disks than on un-, NaOH-, and $CaCl_2$-treated disks [Fig. 3(A)]. OCN expression on $Ca(OH)_2$-treated disks was slightly higher than on the other treated disks [Fig. 3(B)].

We also examined OCN protein expression by immunofluorescence. We observed hMSCs cultured on chemically modified titanium for 7, 14, 21 days (data not shown), and 28 days [Fig. 4(A)]. Although we did not detect expression on all cells until 21 days after seeding (data not shown), only hMSCs on the $Ca(OH)_2$-treated disks showed OCN expression 28 days after seeding [Fig. 4(B)]. The fluorescence intensity of hMSCs cultured on $Ca(OH)_2$-treated titanium was significantly stronger than on the other treated disks [Fig. 4(C)].

Microarray and pathway analysis

To investigate the mechanism of osteogenic induction in hMSCs by chemically modified titanium, we performed DNA microarray analysis. We analyzed mRNA extracted from hMSCs cultured on chemically modified titanium for 7 days. The top 30 genes significantly upregulated on treated versus untreated titanium disks are summarized in Tables I–III. A significant change was defined as a difference of more than twofold relative to the control. Differentially expressed genes significantly upregulated on NaOH versus nontreated disks are shown in Table I. Interleukin 6 receptor (IL6R) increases osteoblast differentiation, and integrin, beta 1 (ITGB1) plays a critical role in the process; both were significantly upregulated on NaOH-treated disks (Table I). Genes significantly upregulated by $CaCl_2$ or $Ca(OH)_2$ treatments versus the untreated control are shown in Tables II and III. SPP1 (OPN) and MMP13 (matrix metallopeptidase 13), involved in normal bone remodeling,[35] and ectonucleotide pyrophosphatase (ENPP1) increase osteoblast differentiation[36]; all were significantly upregulated by $CaCl_2$ or $Ca(OH)_2$ treatment (Tables II and III). In addition, $Ca(OH)_2$ treatment significantly upregulated the expression of IL6R and integrin, alpha 2 (ITGA2), which plays a critical role in osteoblast differentiation, bone morphogenetic protein 2 (BMP2), and parathyroid hormone-like hormone (PTHLH), which also affects osteoblast differentiation (Table III). To differentiate the effect of different calcium surface treatments on gene expression, we compared gene profiles in hMSCs cultured on $CaCl_2$ and on $Ca(OH)_2$. The expression of 94 genes in hMSCs cultured on $Ca(OH)_2$ disks were upregulated more than twofold in comparison to cells grown on $CaCl_2$ (data not shown). Six of these genes significantly ($p = 3.96 \times 10^{-4}$) overlapped with the genes associated with "formation of bone" in the IPA. The genes were SPP1, PTHLH, FGF1 (fibroblast growth factor 1), BMP2, PTGS1 (cyclooxygenase 1), and PTGS2 (cyclooxygenase 2; Cox2) (Table IV).

To determine the significance of the effects of different titanium treatments on osteogenic differentiation in hMSCs, we used IPA. Differentially expressed genes that were significantly influenced by NaOH, $CaCl_2$, or $Ca(OH)_2$ treatment were compared with untreated controls in the canonical pathway defined by IPA in osteoblast and are shown in Figures 5–7. Genes that were not detected in untreated controls, but were detected in hMSCs cultured on chemically modified titanium are represented as "induced genes" in these figures. Genes that were detected in untreated controls but not in hMSCs grown on chemically modified titanium are indicated as "suppressed genes". The NaOH-treated titanium surface induced

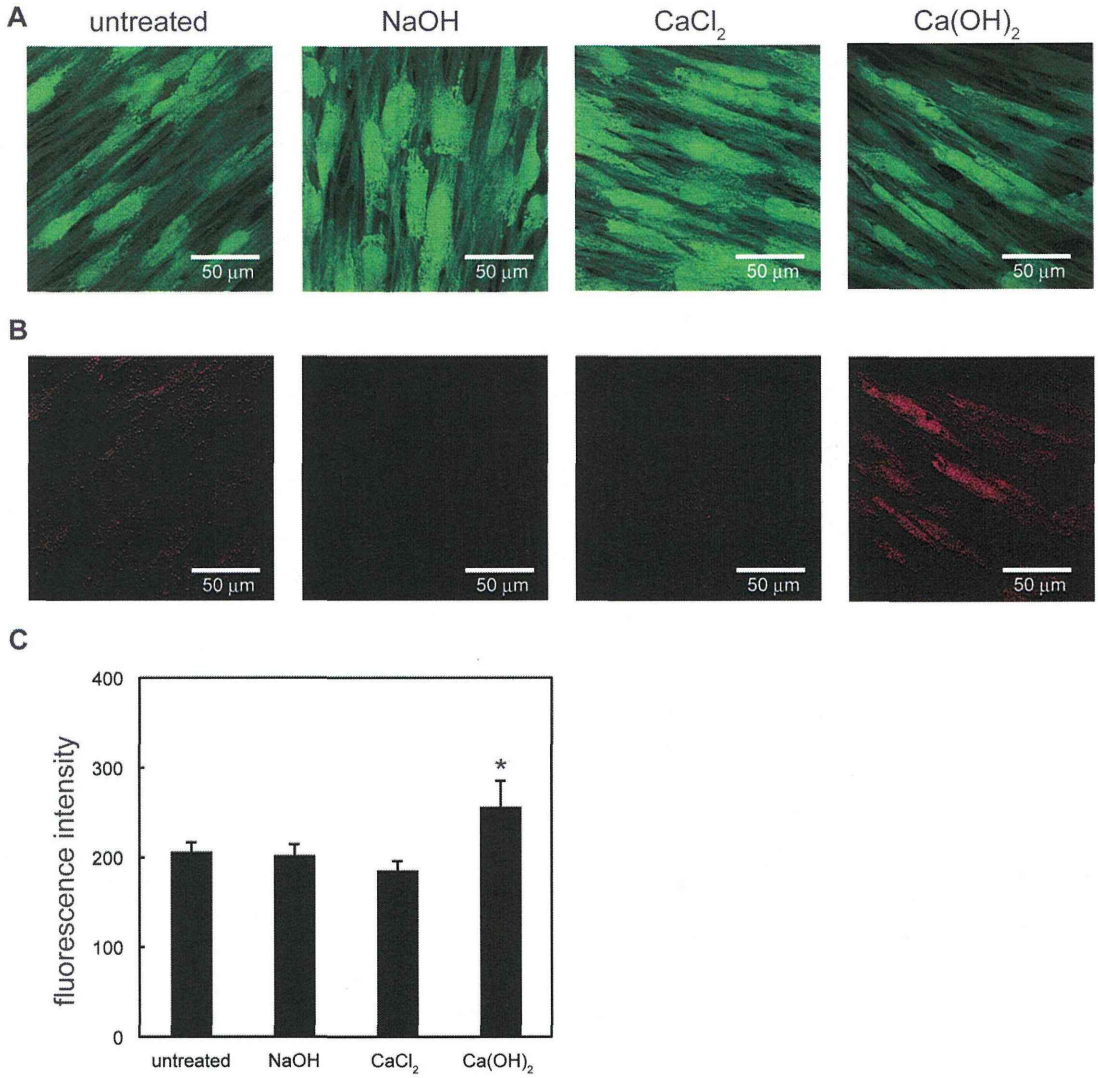

FIGURE 4. Confocal microscopy of hMSCs (A) and osteocalcin in hMSCs (B) cultured on chemically modified titanium disks for 28 days. Green: CellTrackerTM Green Fluorescent Probe; red: Alexa Fluor 647 donkey anti-mouse. Images representative of three independent experiments are shown. Fluorescence intensity of osteocalcin in cells on five random areas was measured with the microscope software (B) (FV10-ASW, Olympus). Significant differences versus cells on untreated titanium are shown as $^*p < 0.05$.

expression of the osteogenic promoter WNT and its cell surface receptor Frizzled. Furthermore, NaOH treatment induced expression of Axin and adenomatous polyposis coli (APC), scaffold proteins that bind to intracellular Wnt/β-catenin signaling molecules. The receptor activator of nuclear factor-kappaB ligand (RANKL) decoy receptor osteoprotegerin (OPG) was upregulated by NaOH treatment (Fig. 5). $CaCl_2$ treatment induced expression of Frizzled, Axin, APC, and osteogenic markers BMP and IGF-1. Bone matrix protein OPN expression was upregulated by $CaCl_2$ treatment. Expression of integrin β3 was also induced following OPN upregulation by $CaCl_2$ (Fig. 6). $Ca(OH)_2$ treatment induced LRP5/6 and essential coreceptors of Wnt ligands for canonical β-catenin-dependent signal transduction, in addition to WNT, Frizzled, Axin, and APC. RANKL, which functions as a key factor for osteoclast differentiation and activation, was induced by $Ca(OH)_2$, in addition to BMP, IGF-1, and integrin β3. OCN expression was also upregulated by $Ca(OH)_2$ (Fig. 7).

DISCUSSION

The surface characteristics and chemical composition of titanium are critical in determining biocompatibility. The topography of titanium affects protein adsorption[1,2] and cell–material interactions and regulates osteointegration.[12,23,24,37,38] In this study, we evaluated the influence of chemically modified titanium surface topography on the incorporation of calcium ions on osteogenic differentiation in hMSCs. Sodium hydrogen titanate is formed on the titanium surface by alkali (NaOH) treatment; subsequently, apatite formation is initiated on the chemically treated surface when it is soaked in simulated body fluid.[18,19] Apatite formation is slightly improved on titanium surfaces treated with $CaCl_2$ after NaOH treatment to incorporate calcium ions into the surface.[26] Therefore, we focused on calcium ion incorporation into the titanium surface and investigated the influence of that modification on hMSC differentiation toward an

TABLE I. Significantly Upregulated Genes in Cells Grown on NaOH-Treated Titanium Disks (Top 30)

Symbol	Entrez Gene Name	Fold Change
TFPI2	Tissue factor pathway inhibitor 2	4.990
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	4.821
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	4.387
PRSS1/PRSS3	Protease, serine, 1 (trypsin 1)	4.290
SERPINB2	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	3.989
IL6R	Interleukin 6 receptor	3.612
PAPPA	Pregnancy-associated plasma protein A, pappalysin 1	3.434
AOX1	Aldehyde oxidase 1	3.223
CSGALNACT 1	Chondroitin sulfate N-acetylgalactosaminyltransferase 1	3.197
YTHDC1	YTH domain containing 1	3.169
GPR56	G protein-coupled receptor 56	3.155
OASL	2′-5′-Oligoadenylate synthetase-like	3.091
C10orf18	Family with sequence similarity 208, member B	3.056
ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	2.996
HIST1H2BD	Histone cluster 1, H2bd	2.992
GALNTL2	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-Acetylgalactosaminyltransferase-like 2	2.911
IL17RC	Interleukin 17 receptor C	2.892
TNFSF13B	Tumor necrosis factor (ligand) superfamily, member 13b	2.736
FAM65B	Family with sequence similarity 65, member B	2.655
RIN3	Ras and Rab interactor 3	2.653
CCL2	Chemokine (C-C motif) ligand 2	2.647
FRAT1	Frequently rearranged in advanced T-cell lymphomas	2.645
CENPM	Centromere protein M	2.634
TPD52	Tumor protein D52	2.624
TNFSF15	Tumor necrosis factor (ligand) superfamily, member 15	2.597
SFTPA2	Surfactant protein A2	2.574
NR4A2	Nuclear receptor subfamily 4, group A, member 2	2.571
IFI6	Interferon, alpha-inducible protein 6	2.567
SMCHD1	Structural maintenance of chromosomes flexible hinge domain containing 1	2.541
PLCL2	Phospholipase C-like 2	2.488

osteogenic lineage. We also compared calcium ion modification by $CaCl_2$ and $Ca(OH)_2$.

Experimental titanium disks were treated with aqueous NaOH, $CaCl_2$, and $Ca(OH)_2$ solutions. hMSC morphology was altered and proliferation was significantly reduced by calcium surface treatment of titanium disks [Fig. 2(A,B)]. Several studies have demonstrated a link between cell shape and osteogenic differentiation in hMSCs.[39–42] In addition, incorporation of calcium ions and apatite formation on the titanium surface treated with $Ca(OH)_2$ were significantly greater than on $CaCl_2$-treated titanium disks [Fig. 1(B,C)]. To investigate the osteogenic differentiation of hMSCs cultured on chemically modified titanium disks, the expression of structural genes for the bone matrix OPN and the marker of bone formation OCN and OCN protein expression were examined (Figs. 3 and 4). OPN transcription in hMSCs was significantly upregulated by the calcium surface treatment, and was significantly on $Ca(OH)_2$-treated disks than on $CaCl_2$-treated disks. OCN protein expression was also upregulated by $Ca(OH)_2$ treatment; however, $CaCl_2$ treatment had no effect on the transcript and protein expression of OCN. We conclude that calcium surface treatment of the titanium disks influenced the osteogenic differentiation of hMSCs, and $Ca(OH)_2$ treatment of the titanium surface induced osteogenic differentiation, whereas $CaCl_2$ treatment had a limited effect.

To investigate the mechanism of osteogenic induction in hMSCs by calcium ions, comparative gene expression profiles were assessed by DNA microarray and pathway analyses. Several genes involved in osteogenic differentiation and bone metabolism were significantly upregulated by the chemical modifications of titanium. IL6R and ITGB1 were significantly upregulated by NaOH (Table I). SPP1 (OPN), MMP13, and ENPP1 were significantly upregulated by $CaCl_2$ and $Ca(OH)_2$ (Tables II and III). $Ca(OH)_2$ treatment also significantly upregulated the expression of IL6R, ITGA2, BMP2, and PTHLH (Table III). In addition, to differentiate the effect of two kinds of calcium surface treatments on gene expression, we compared $CaCl_2$ and $Ca(OH)_2$ treatments and found that $Ca(OH)_2$ treatment significantly upregulated expression of BMP2, PTGS2 (Cox2), PTHLH, and SPP1 (OPN) in hMSCs (Table IV). Previous studies have demonstrated Cox2 function is essential for bone formation in rats[43] and osteoblast differentiation is stimulated through induction of Cox2 in

TABLE II. Significantly Upregulated Genes in Cells Grown on $CaCl_2$-Treated Titanium Disks (Top 30)

Symbol	Entrez Gene Name	Fold Change
SPP1 (OPN)	Secreted phosphoprotein 1	6.252
PRSS1/PRSS3	Protease, serine, 1 (trypsin 1)	4.009
MMP13	Matrix metallopeptidase 13 (collagenase 3)	3.882
GPR56	G protein-coupled receptor 56	3.640
C13orf15	Regulator of cell cycle	3.578
IGFBP1	Insulin-like growth factor binding protein 1	3.540
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	3.500
TFPI2	Tissue factor pathway inhibitor 2	3.406
YTHDC1	YTH domain containing 1	3.371
CENPM	Centromere protein M	3.235
COL15A1	Collagen, type XV, alpha 1	3.174
AQP1	Aquaporin 1 (Colton blood group)	3.149
RPL18A	Ribosomal protein L18a	3.098
DUSP4	Dual specificity phosphatase 4	3.095
DIXDC1	DIX domain containing 1	3.016
TMEM158	Transmembrane protein 158 (gene/pseudogene)	2.791
C16orf57	Chromosome 16 open reading frame 57	2.787
SMC1A	Structural maintenance of chromosomes 1A	2.761
ARID5B	AT rich interactive domain 5B (MRF1-like)	2.655
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	2.648
IL17RC	Interleukin 17 receptor C	2.624
DCTN4	Dynactin 4 (p62)	2.605
ING3	Inhibitor of growth family, member 3	2.557
NOL6	Nucleolar protein family 6 (RNA-associated)	2.555
PLTP	Phospholipid transfer protein	2.555
SLC29A1	Solute carrier family 29 (nucleoside transporters), member 1	2.507
MCM5	Minichromosome maintenance complex component 5	2.502
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	2.497
ADAMTS16	ADAM metallopeptidase with thrombospondin type 1 motif, 16	2.493
SMURF1	SMAD specific E3 ubiquitin protein ligase 1	2.491

TABLE III. Significantly Upregulated Genes in Cells Grown on $Ca(OH)_2$-Treated Titanium Disks (Top 30)

Symbol	Entrez Gene Name	Fold Change
SPP1 (OPN)	Secreted phosphoprotein 1	17.721
MMP13	Matrix metallopeptidase 13 (collagenase 3)	16.725
RGS2	Regulator of G-protein signaling 2, 24kDa	8.677
TFPI2	Tissue factor pathway inhibitor 2	7.763
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)	7.343
SLC16A6	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)	7.286
IGFBP1	Insulin-like growth factor binding protein 1	6.677
DUSP4	Dual specificity phosphatase 4	6.649
PCDH19	Protocadherin 19	5.943
PTHLH	Parathyroid hormone-like hormone	5.630
GPR56	G protein-coupled receptor 56	5.129
SLC29A1	Solute carrier family 29 (nucleoside transporters), member 1	4.924
C13orf15	Regulator of cell cycle	4.750
ENPP1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	4.717
EREG	Epiregulin	4.712
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	4.436
CXCL6	Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	4.404
TMEM158	Transmembrane protein 158 (gene/pseudogene)	4.403
PRSS1/PRSS3	Protease, serine, 1 (trypsin 1)	4.397
BMP2	Bone morphogenetic protein 2	4.140
COL10A1	Collagen, type X, alpha 1	3.739
FOXQ1	Forkhead box Q1	3.670
MGP	Matrix Gla protein	3.488
PLAU	Plasminogen activator, urokinase	3.364
CENPM	Centromere protein M	3.288
IL6R	Interleukin 6 receptor	3.223
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	3.205
CLU	Clusterin	3.163
FPR1	Formyl peptide receptor 1	3.114
ESCO2	Establishment of cohesion 1 homolog 2 (*S. cerevisiae*)	3.084

TABLE IV. Significantly Upregulated Genes Associated with 'Formation of Bone' on $Ca(OH)_2$-Treated Titanium Disks vs. $CaCl_2$-Trated Disks

Symbol	Entrez Gene Name	Fold Change
SPP1 (OPN)	Secreted phosphoprotein 1 (Osteopontin)	2.835
PTHLH	Parathyroid hormone-like hormone	2.308
FGF1	Fibroblast growth factor 1 (acidic)	2.202
BMP2	Bone morphogenetic protein 2	2.175
PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase 1)	2.098
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase 2)	2.008

These genes significantly overlapped with the genes associated with "formation of bone" by IPA ($p = 3.96 \times 10^{-4}$).

mesenchymal progenitor cells.[44] BMP2 induces Cox2 in osteoblasts[45] and in mesenchymal cells.[46] It was also reported that extracellular calcium increases expression of BMP2.[47,48] Furthermore, the calcium–calcineurin–nuclear factor of activated T-cell signaling pathway has an important role in the PTH induction of Cox2.[49] Taken together, our results suggest that $Ca(OH)_2$ treatment of titanium disks induces osteogenic differentiation in hMSCs via induction of BMP2, Cox2, and PTHLH. In contrast, Smad signaling was downregulated by chemically modified titanium surfaces (Figs. 5–7). A previous study demonstrated that noncanonical BMP signaling regulates Cox2 transcription.[46] These observations suggest noncanonical BMP signaling (independent of Smad signaling) might mediate the osteogenic differentiation of hMSCs on $Ca(OH)_2$-treated titanium.

Postanalysis of microarray data was performed by IPA. NaOH treatment induced the osteogenic promoter WNT and its cell surface receptor Frizzled, as well as Axin and APC,

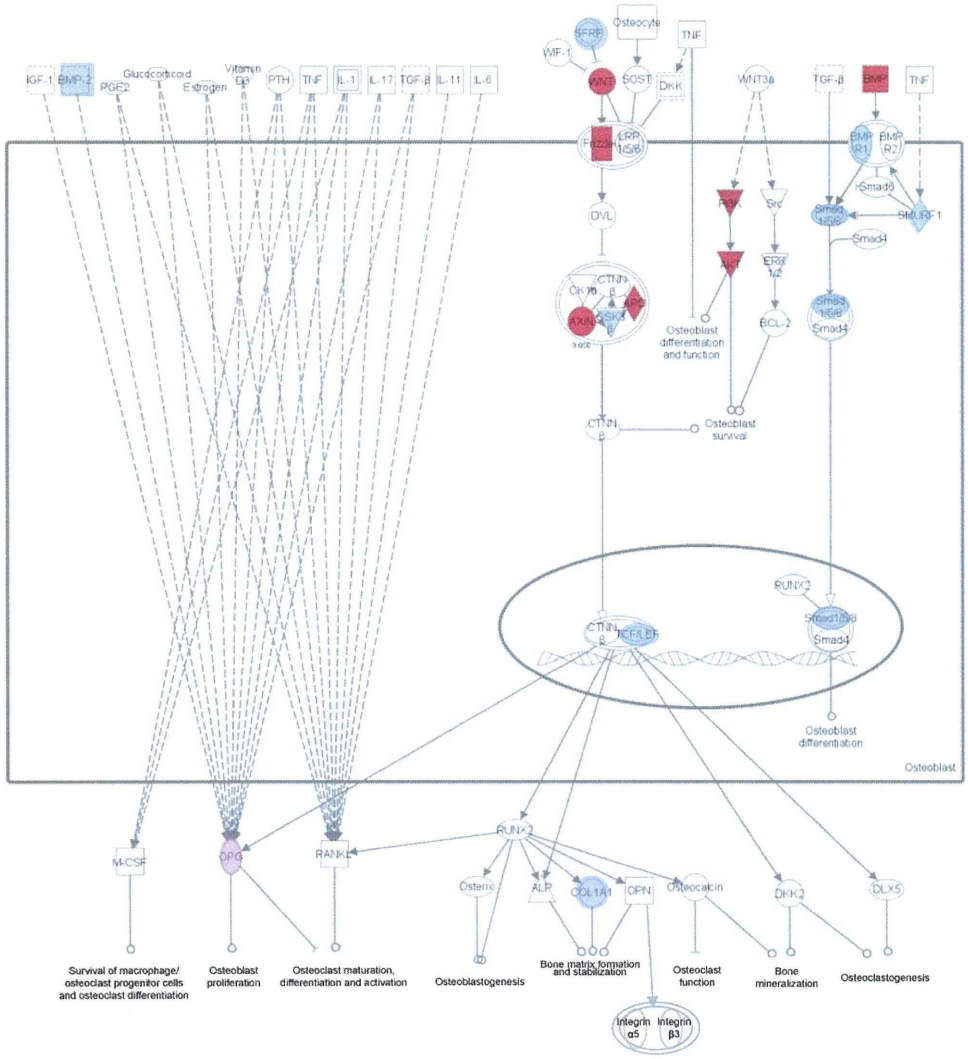

FIGURE 5. Differentially expressed genes in the canonical pathway in osteoblasts were significantly changed by NaOH treatment versus untreated conditions. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

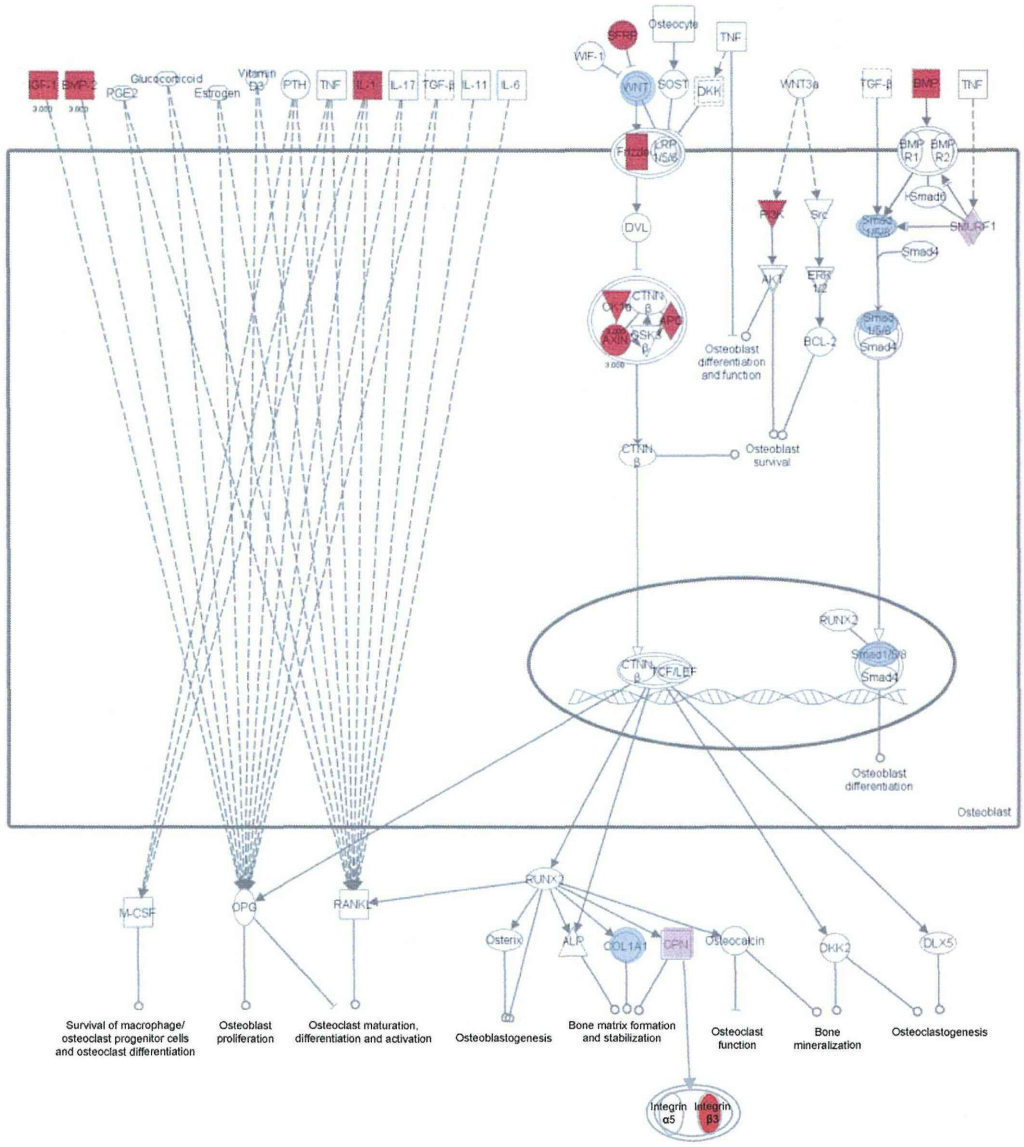

FIGURE 6. Differentially expressed genes in the canonical osteoblast pathway on $CaCl_2$-treated disks versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

scaffolding proteins that bind to intracellular Wnt/β-catenin signaling molecules. RANKL decoy receptor OPG expression was upregulated by NaOH (Fig. 5). $CaCl_2$ treatment induced expression of Frizzled, Axin, and APC as well as of the osteogenic markers BMP and IGF-1. The bone matrix protein OPN was upregulated, and the expression of integrin β3 was induced following OPN upregulation by $CaCl_2$ (Fig. 6). $Ca(OH)_2$ treatment induced LRP5/6 and essential coreceptors of Wnt ligands for canonical β-catenin-dependent signal transduction, in addition to WNT, Frizzled, Axin, and APC. BMP, IGF-1, and integrin β3 were also induced by $Ca(OH)_2$. In addition to OPN, OCN was upregulated by $Ca(OH)_2$ (Fig. 7).

Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast differentiation[50]; surface properties of titanium regulate stem cell fate and induce osteoblast differentiation via the Wnt calcium-dependent pathway and Wnt5a enhanced osteogenesis through positive feedback with integrins.[51] Previous studies have shown the integrin family plays a major role in osteoblastic differentiation on variously modified titanium surfaces.[4,5,10,14] We observed that calcium modification of the titanium surface induced integrin β3 following OPN upregulation. Wnt/β-catenin signaling in hMSCs was also promoted by the calcium modification, more by $Ca(OH)_2$ than $CaCl_2$ treatment. These observations suggest that calcium modification of titanium surfaces induces osteogenic differentiation in hMSCs in the absence of osteogenic factors by activation of Wnt/β-catenin signaling.

In this study, $Ca(OH)_2$ treatment of titanium surface was more effective to osteogenic differentiation in hMSC than $CaCl_2$ treatment, this might be caused by the difference of the amount of calcium ions and apatite formation on the titanium surface between the two kinds of calcium treatments. We

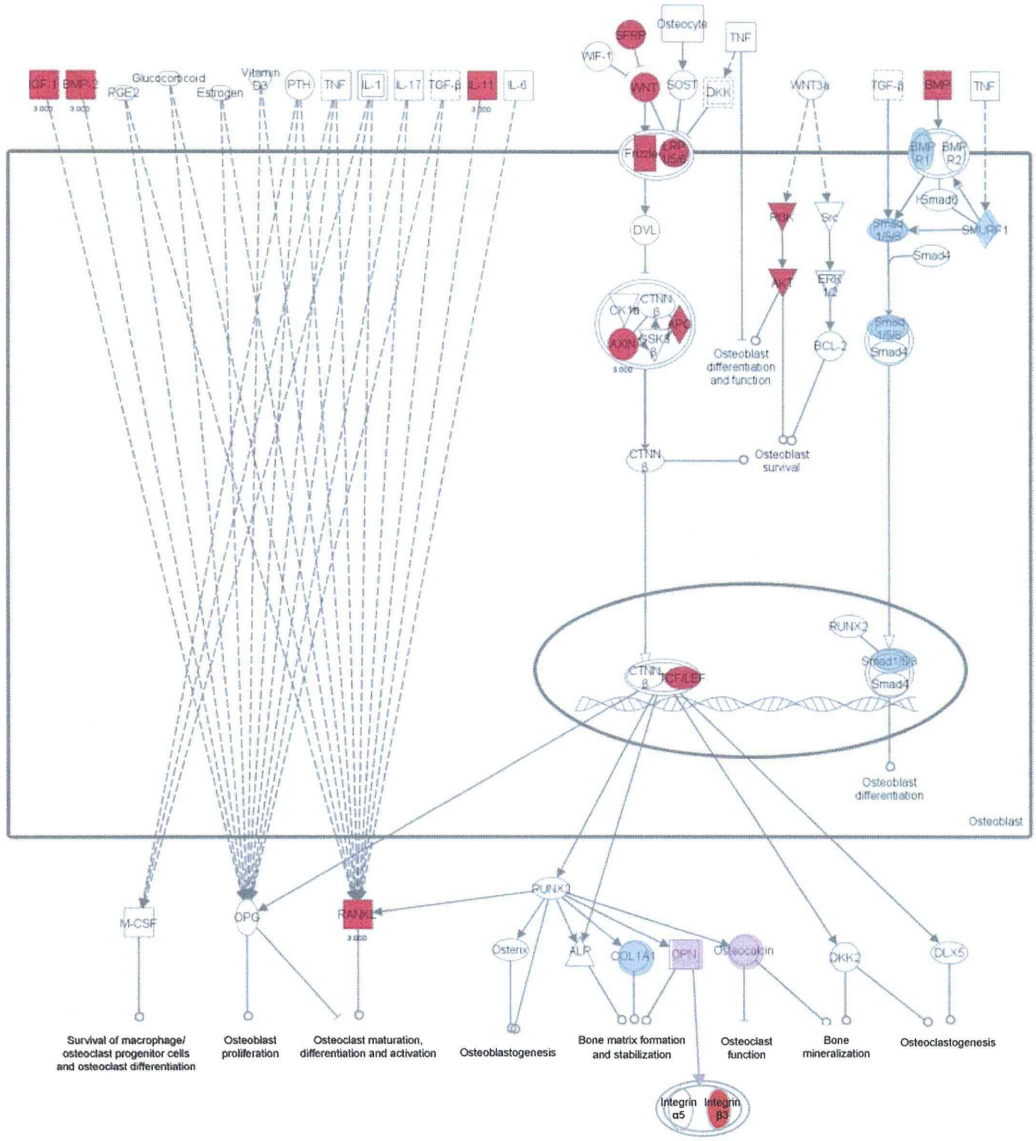

FIGURE 7. Differentially expressed genes in the canonical osteoblast pathway on $Ca(OH)_2$ versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

suggested that $Ca(OH)_2$ treatment of titanium disks induced osteogenic differentiation in hMSCs by the upregulation of BMP2, Cox2, and PTHLH compared with $CaCl_2$ treatment, and the activation of Wnt/β-catenin signaling.

CONCLUSIONS

In this study, we chemically modified titanium surfaces with $CaCl_2$ or $Ca(OH)_2$ after NaOH treatment to alter the surface topology and incorporate calcium ions; subsequently, we investigated the influence of these treatments on osteogenic differentiation in hMSCs in the absence of osteogenic supplements. Calcium modification by $CaCl_2$ or $Ca(OH)_2$ affects cell morphology and molecular responses in hMSCs. Whole genome expression analysis suggested that calcium modification of the titanium surface activates Wnt/β-catenin signaling. In addition, $Ca(OH)_2$ treatment upregulated expression of BMP2, Cox2, and PTHLH. $Ca(OH)_2$ treatment induces osteogenic differentiation in hMSCs, whereas $CaCl_2$ has a limited effect; this may depend on whether there are significant differences between treatments with respect to the amount of calcium ions and apatite formation on the titanium surface.

ACKNOWLEDGMENTS

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