

Table 6 中胚葉分化に相関のある mRNA および miRNA

正に相関のある mRNA

Gene Symbol	相関係数
<i>LOC400680</i>	0.8667
<i>TRIM4</i>	0.8061
<i>ALPK3</i>	0.7939
<i>KLK5</i>	0.7939
<i>ALPK3</i>	0.7939
<i>ZNF506</i>	0.7455
<i>FLJ34208</i>	0.7212
<i>SH3PXD2A</i>	0.7212
<i>PKIB</i>	0.7091
<i>FGFR4</i>	0.6970
<i>ZNF572</i>	0.6848
<i>ZFP3</i>	0.6848
<i>ZNF506</i>	0.6848
<i>CBLC</i>	0.6727
<i>CIDEB</i>	0.6727
<i>FLJ35024</i>	0.6606
<i>CAT</i>	0.6606
<i>CD47</i>	0.6606
<i>ZNF585B</i>	0.6485
<i>CAT</i>	0.6485
<i>DHDH</i>	0.6485
<i>ATP8B3</i>	0.6485
<i>LOC100128252</i>	0.6485

負に相関のある mRNA

Gene Symbol	相関係数
<i>TNFRSF11B</i>	-0.7939
<i>EN2</i>	-0.7818
<i>AJAP1</i>	-0.7212
<i>FOXG1</i>	-0.6970
<i>ASCL1</i>	-0.6849
<i>SALL3</i>	-0.6606
<i>LOC389906</i>	-0.6606

正に相関のある miRNA

Gene Symbol	相関係数
hp_hsa-mir-516a-2_s_st	0.7212
hsa-miR-518e-star_st	0.7212
hsa-miR-520c-5p_st	0.7212
hsa-miR-526a_st	0.7212
hsa-miR-518c-star_st	0.6848
hsa-miR-519c-5p_st	0.6848
hsa-miR-222-star_st	0.6727
hsa-miR-512-5p_st	0.6606
hsa-miR-519a-star_st	0.6606
hsa-miR-524-5p_st	0.6606
hsa-miR-518d-5p_st	0.6485
hsa-miR-518f-star_st	0.6485

負に相関のある miRNA

Gene Symbol	相関係数
aae-miR-100_st	-0.6970

Table 7 内胚葉分化に相関のある mRNA および miRNA

正に相関のある mRNA

Gene Symbol	相関係数
<i>DDX58</i>	0.842424
<i>C3orf67</i>	0.733333
<i>CRYZ</i>	0.721212
<i>AACSP1</i>	0.684848
<i>SLC25A4</i>	0.684848
<i>EFCAB2</i>	0.660606
<i>ATP8B3</i>	0.648485

負に相関のある mRNA

Gene Symbol	相関係数
<i>TNFRSF11B</i>	-0.89091
<i>NXP2</i>	-0.80606
<i>RAB3B</i>	-0.79394
<i>ST8SIA4</i>	-0.79394
<i>NHS</i>	-0.78182
<i>MED13L</i>	-0.7697
<i>SORBS2</i>	-0.74545
<i>LHFP</i>	-0.73333
<i>LARP4</i>	-0.73333
<i>SPIRE2</i>	-0.72121
<i>BAGE2 /// BAGE3 /// BAGE5 /// MLL3</i>	-0.72121
<i>FBN1</i>	-0.70909
<i>AJAP1</i>	-0.69697
<i>SORBS2</i>	-0.69697
<i>C17orf51</i>	-0.69697
<i>PCDH9</i>	-0.68485
<i>XPNPEP3</i>	-0.68485
<i>KLF12</i>	-0.68485
<i>DCC</i>	-0.68485
<i>CPNE8</i>	-0.68485
<i>AJAP1</i>	-0.67273
<i>C6orf106</i>	-0.66061
<i>GPAM</i>	-0.66061
<i>SALL3</i>	-0.66061
<i>CDK6</i>	-0.64848
<i>OSBPL11</i>	-0.64848
<i>CDK6</i>	-0.64848
<i>APCDD1L</i>	-0.64848

正に相関のある miRNA

Gene Symbol	相関係数
hsa-miR-150-star_st	0.7818
ame-miR-3741_st	0.7455
hp_hsa-mir-516a-2_s_st	0.7455
hsa-miR-367_st	0.7333
hsa-miR-4505_st	0.7091
hsa-miR-515-3p_st	0.7091
hsa-miR-516a-5p_st	0.7091
bdi-miR166a_st	0.6970
hsa-miR-4739_st	0.6848
hsa-miR-4521_st	0.6727
hsa-miR-520g_st	0.6727
bmo-miR-2785_st	0.6606

負に相関のある miRNA

なし

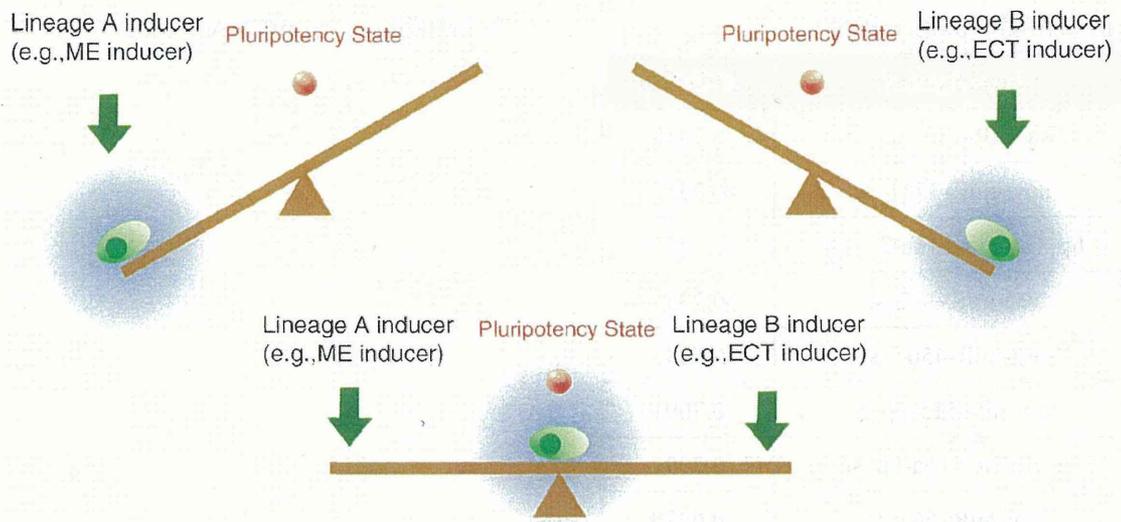


Figure 2 シーソーモデル

iPS 細胞が分化多能性をもつメカニズムの仮説として Shu らの論文 (*Cell*. 2013; 153: 963-975) 中で紹介されたモデル. 青色で示した領域は偶然性 (ノイズ) があると思われる細胞状態, 赤色で示した点は多能性領域を示す. ここでは例として, **Lineage A inducer** が中内胚葉への分化促進遺伝子, **Lineage B inducer** が外胚葉への分化促進遺伝子として示されており, 両者がバランスを保った際に細胞は多能性を獲得するという概念を表している.

Table 8 未分化マーカーにおける iPS 細胞の第一主成分得点ランク

未分化	
iPS細胞株	主成分得点
R-2A	12.011
Ai-103	5.939
Tic	5.821
R-12A	3.695
Ai-100	3.434
409B2	2.106
R-1A	-7.200
253G1	-7.318
201B7	-7.437
mc-iPS	-11.050

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	頁
中島啓行, 安田智, 佐藤陽治	ヒト ES/iPS 細胞に由来する再生医療製品の造腫瘍性をどう見るか?	中辻憲夫, 末盛博文	ES・iPS 細胞実験スタンダード	羊土社	東京	2013	61-68
田埜慶子, 草川森士, 佐藤陽治	細胞・組織加工製品の製造における造腫瘍性評価	技術情報協会	再生医療における臨床研究と製品開発	技術情報協会	東京	2013	
鈴木孝昌	網羅的な発現をみる マイクロアレイ解析との比較を例に	北條浩彦	原理からよくわかるリアルタイム PCR 完全実験ガイド	羊土社	東京	2013	111-121
安田智, 佐藤陽治	再生医療製品の品質関連規制と対応の留意点	技術情報協会	動物細胞の培養を成功させる条件設定集	技術情報協会	東京		印刷中
Kuroda T, Yasuda S, Sato Y.	In vitro detection of residual undifferentiated cells in retinal pigment epithelial cells derived from human induced pluripotent stem cells.	Kioussi C.	<i>Methods in Stem Cells and Tissue Repair</i>	Springer			印刷中

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	頁	出版年
Sawada R, Kono K, Isama K, Haishima Y, Matsuoka A.	Calcium-incorporated titanium surfaces influence the osteogenic differentiation of human mesenchymal stem cells.	<i>J Biomed Mater Res A</i>	101(9)	2573-85	2013
Ito-Nagahata T, Kurihara C, Hasebe M, Ishii A, Yamashita K, Iwabuchi M, Sonoda M, Fukuhara K, Sawada R, Matsuoka A, Fujiwara Y.	Stilbene Analogs of Resveratrol Improve Insulin Resistance through Activation of AMPK.	<i>Biosci Biotechnol Biochem</i>	77(6)	1229-1235	2013
Sato Y, Tsutsumi H, Sawada R, Suzuki T, Yasuda S.	Regulatory science research to facilitate the development of cell/tissue-proceed products.	<i>Bull Natl Inst Health Sci</i>	131	16-19	2013
Kono K, Niimi S, Sawada R.	Cyclin D2 promotes the proliferation of human mesenchymal stem cells.	<i>J Bone Marrow Res</i>			in press
斎藤嘉朗, 前川京子, 齊藤公亮, 佐藤陽治, 鈴木孝昌	タンパク質・内在性代謝物バイオマーカーを利用した医薬品開発の活性化にむけて	国立医薬品食品衛生研究所報告	131	20-24	2013
中村里香, 酒井信夫, 靄島由二, 福井千恵, 鈴木孝昌, 中村亮介, 蜂須賀暁子, 安達玲子, 手島玲子	ショットガンプロテオミクスによる加水分解小麦とその原料であるグルテンに含まれるタンパク質の網羅的解析	国立医薬品食品衛生研究所報告	131	50-57	2013
Suzuki T.	“Scientific Considerations Regarding Radiation Risk” JEMS Open Symposium 2012.	<i>Genes and Environment</i>	35	57-62	2013
Suzuki T.	Unconscious Exposure to Radiation.	<i>Genes and Environment</i>	35	63-68	2013
五十嵐友香, 佐藤陽治	再生医療製品の造腫瘍性・悪性腫瘍形成能の評価	医学のあゆみ	246	1069-70	2013
田埜慶子, 佐藤陽治	再生医療製品の素材としての多能性幹細胞 (ES/iPS 細胞) の品質	レギュラトリーサイエンス学会誌	4	71-7	2014

Kanemura H, Go MJ, Nishishita N, Sakai N, Kamao H, <u>Sato Y</u> , Takahashi M, Kawamata S.	Pigment epithelium-derived factor secreted from retinal pigment epithelium facilitates apoptotic cell death of iPSC.	<i>Sci Rep</i>	3	2334	2013
草川森士, <u>佐藤陽治</u>	再生医療製品の造腫瘍性評価	<i>最新医学</i>			印刷中
<u>佐藤陽治</u>	ヒト iPS 細胞由来移植細胞の製造管理のための <i>in vitro</i> 造腫瘍性評価系の開発	<i>Cytometry Research</i>			印刷中
村岡ひとみ, <u>佐藤陽治</u>	再生医療・細胞治療の臨床研究から実用化までの道のり	<i>Geriatric Medicine (老年医学)</i>			印刷中

Calcium-incorporated titanium surfaces influence the osteogenic differentiation of human mesenchymal stem cells

Rumi Sawada,¹ Ken Kono,¹ Kazuo Isama,² Yuji Haishima,¹ Atsuko Matsuoka¹

¹Division of Medical Devices, National Institute of Health Sciences, Tokyo, Japan

²Division of Environmental Chemistry, National Institute of Health Sciences, Tokyo, Japan

Received 21 September 2012; accepted 13 December 2012

Published online 11 February 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.a.34566

Abstract: In this study, a titanium surface was chemically modified with calcium ions and assessed for its influence on osteogenic differentiation and molecular responses of human mesenchymal stem cells (hMSCs). Titanium disks were treated with NaOH (NaOH treatment), NaOH + CaCl₂ (CaCl₂ treatment), or NaOH + Ca(OH)₂ (Ca(OH)₂ treatment). Ca(OH)₂ treatment caused significantly greater calcium incorporation onto the titanium surface and apatite formation than CaCl₂ treatment. The morphology of hMSCs differed on CaCl₂- and Ca(OH)₂-treated disks. The osteopontin (OPN) expression in hMSCs cultured on CaCl₂-treated titanium was significantly higher than that in cells cultured on NaOH-treated disks; OPN expression was significantly higher in cells cultured on Ca(OH)₂-treated disks than on un-, NaOH-, and CaCl₂-treated disks. Osteocalcin (OCN) protein expression in hMSCs cultured on Ca(OH)₂-treated disks was significantly higher than

that on all the other disks. Comparative expression profiling by DNA microarray and pathway analyses revealed that calcium modification of the titanium surface induced integrin β 3 after OPN upregulation and promoted Wnt/ β -catenin signaling in hMSCs. In addition, Ca(OH)₂ treatment upregulated the expression of bone morphogenetic protein 2, cyclooxygenase 2, and parathyroid hormone-like hormone in comparison to CaCl₂ treatment. These observations suggest that calcium-modified titanium surfaces affect osteogenic differentiation in hMSCs and that Ca(OH)₂ treatment induced osteogenic differentiation in hMSCs, whereas CaCl₂ treatment had a limited effect. © 2013 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 101A: 2573–2585, 2013.

Key Words: surface modification, titanium, calcium, stem cell, osteogenesis, gene expression

How to cite this article: Sawada R, Kono K, Isama K, Haishima Y, Matsuoka A. 2013. Calcium-incorporated titanium surfaces influence the osteogenic differentiation of human mesenchymal stem cells. *J Biomed Mater Res Part A* 2013;101A:2573–2585.

INTRODUCTION

Titanium is widely used in orthopedic and dental implants due to its corrosion and wear resistance, durability, and biocompatible interface. The topography of titanium implants plays a major role in cell–material interaction. Several studies have shown that surface modifications influence protein adsorption,^{1,2} cell–substrate interactions,³ cell adhesion,^{3–5} cell morphology,^{5,6} osteogenic differentiation,^{5,7–10} and, consequently, the tissue integration of titanium implants.^{11,12}

The nanoscale and microscale surface roughness of titanium has been reported to increase osteogenic differentiation in osteoblasts cultured on titanium surfaces^{7,9–12} and promote osteogenic differentiation in human mesenchymal stem cells (hMSCs) in the presence of osteogenic supplements.⁸ Osteoblast response has also been reported to be modulated by a modified microstructured titanium surface with increased wettability due to a polyelectrolyte thin film coating.¹³ Titanium nanopores also affect osteogenic differentiation and hMSC cell morphology.⁵ Integrin is critical for the responses of osteoblasts^{4,10,14} and hMSCs⁵ to some tita-

nium surface modifications. Gene expression studies have shown an increase in the expression of the osteoinductive genes RUNX2, osterix, alkaline phosphatase, bone sialoprotein, and osteocalcin (OCN) in osteoblasts^{6,15} and hMSCs^{16,17} grown on micro- and nanoroughened surfaces in the presence of osteogenic supplements.

Alkali- and heat-treated titanium forms a bone-like apatite surface layer in the body and bonds to the bone via this layer.^{18–22} In addition to chemical and thermal treatments, the porous structure of titanium contributes to its biocompatibility²³ and bone formation²⁴ *in vivo*. Fluoride ion modification of a TiO₂ grit-blasted surface enhances osteoblastic differentiation in hMSCs in the presence of osteogenic supplements.²⁵ Calcium ion incorporation into a titanium surface by CaCl₂ treatment yields slightly better apatite formation than alkali (NaOH) treatment *in vitro*,²⁶ but the influence of calcium ion modifications of titanium surfaces on osteogenic differentiation of hMSCs has not been explored.

hMSCs are pluripotent and can differentiate into cells of mesodermal origin, for example, bone, cartilage, adipose,

Correspondence to: R. Sawada; e-mail: rsawada@nihs.go.jp

Contract grant sponsor: Health and Labour Sciences Research Grants for Research on Regulatory Science of Pharmaceuticals and Medical Devices from the Ministry of Health, Labour and Welfare of Japan; contract grant numbers: H22-IYAKU-IPPAN-009, H24-IYAKU-SHITEI-018

and muscle cells.²⁷⁻³¹ hMSCs also have the capacity to differentiate into myocytes,^{32,33} hepatocytes,^{27,34} and neural cells.²⁹ hMSCs are currently being used with biomedical materials in several clinical studies on bone regeneration.

We aimed to evaluate the influence of CaCl₂- or Ca(OH)₂-modified titanium surfaces on osteogenic differentiation and molecular responses in hMSCs. We examined the amount of calcium ion incorporation and apatite formation on CaCl₂- and Ca(OH)₂-treated surfaces. We investigated the morphology, proliferation, and osteogenic differentiation of hMSCs cultured on the chemically modified titanium. To definitively conclude whether incorporation of calcium ions on a titanium surface induces osteogenic differentiation in hMSCs, osteogenic supplements were not used in this study. Whole genome expression analysis provided a comprehensive understanding of the mechanism of osteogenic induction by chemically modified titanium.

MATERIALS AND METHODS

Chemical treatment of titanium disks

Titanium disks (grade II commercially pure titanium, 33.5 mm diameter, 2-mm thick) were supplied by Nakashima Medical Co. (Okayama, Japan). They contain over 99.3075% of titanium, and their surface roughness (Ra) is 0.4 μm. NaOH treatment was performed by soaking the disks in 5.0 mol/L NaOH aqueous solution at 60°C for 24 h and then washing gently with distilled water. CaCl₂ or Ca(OH)₂ treatments were performed by soaking the NaOH-treated disks in 0.1 mol/L CaCl₂ or 0.01 mol/L Ca(OH)₂ at 60°C for 24 h and then gently washing with distilled water. Untreated titanium disks served as controls.

Scanning electron microscopy

The topography of the chemically modified titanium disks was characterized by Scanning electron microscopy (SEM) performed with a JSM-5800 microscope (JEOL, Tokyo, Japan). Images were recorded with 15 kV accelerating voltage.

Calcium ion incorporation and apatite formation

To investigate apatite formation on the titanium surface, untreated or chemically modified disks were soaked in Hanks' balanced salt solution that included calcium and magnesium (Life Technologies Co., Carlsbad, CA) for 7 days at 37°C. The solution was changed every 2 days.

The incorporated calcium ions were dissolved in nitric acid and their concentration was measured by Agilent 7500ce ORS ICP-MS (inductively coupled plasma mass spectrometry; Agilent, Santa Clara, CA) in helium collision mode with monitoring at *m/z* 43.

Cell culture

hMSCs derived from bone marrow were purchased from Lonza (Walkersville, MD). The hMSCs were cultured in MSCGM BulletKit, a mesenchymal stem cell basal medium with mesenchymal cell growth supplement, L-glutamine, and gentamycin/amphotericin-B (Lonza Walkersville, MD), at 37°C under a 5% CO₂ atmosphere. The medium was changed every 3 days. The cells were detached by mild

treatment with trypsin/ethylenediaminetetraacetic acid solution for hMSCs (Lonza Walkersville, MD) and subcultured when they were just subconfluent (approximately 80–90% confluence). Cell passages 2–4 were used for experiments. hMSCs were cultured on each surface-modified titanium disk for 1, 4, 7, and 28 days.

Cell morphology and immunofluorescence staining

To analyze the morphology of hMSCs cultured on the chemically modified titanium disks, the cells were stained with CellTracker (Lonza) according to the manufacturer's protocol.

To examine the expression of OCN, cells stained with CellTracker were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) for 15 min at room temperature and permeabilized with a blocking solution [10% normal donkey serum (Jackson ImmunoResearch Laboratories, Baltimore, PA), 0.1% Triton X-100, and 0.01% NaN₃ in PBS]. The cells were stained with anti-OCN antibody (Abcam, Cambridge, MA) in blocking solution without Triton X-100 for 16 h at 4°C for primary staining and were secondarily stained with Alexa Fluor 647-conjugated donkey anti-mouse antibody (Jackson ImmunoResearch Laboratories) for 30 min at room temperature.

Images were collected and analyzed by confocal microscopy (FLUOVIEW FV1000; Olympus, Tokyo, Japan).

Cell proliferation

The proliferation of hMSCs on titanium disks for 7 days was determined by using a cell proliferation assay reagent, TetraColor ONE (Seikagaku Co., Tokyo, Japan). Cultures were incubated for 2 h in medium containing the reagent. The absorbance was read at 450 nm (reference at 600 nm) on a plate reader (SH-9000, Corona Electric Co., Ibaraki, Japan).

Preparation of total RNA and real-time polymerase chain reaction

hMSCs were seeded on untreated and chemically modified titanium disks in 35 mm culture dishes at an initial seeding density of 1.2×10^4 cells/cm² and cultured for 7 days before evaluating transcript expression of osteopontin (OPN), OCN, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The housekeeping gene GAPDH was used as a control. Total RNA was extracted from hMSCs with the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA was eluted in RNase-free water and quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Total RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System for real-time polymerase chain reaction (RT-PCR; Life Technologies Co., Carlsbad, CA). Amplification of OPN, OCN, and GAPDH was performed with LightCycler Primer Sets (Roche Applied Science, Basel, Switzerland) and LightCycler Fast Start DNA Master SYBR Green I (Roche Applied Science) in a Roche LightCycler instrument (software version 4.0).

Microarray analysis

Whole genome expression was analyzed after 7-day culture of hMSCs on chemically modified titanium disks. Total RNA

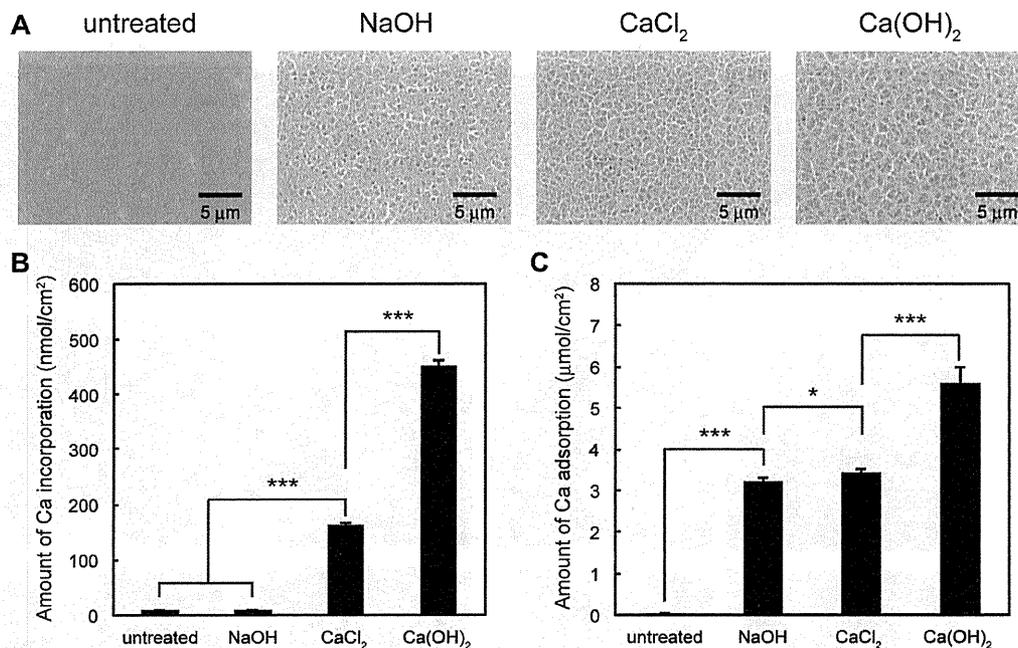


FIGURE 1. SEM images of the surface of untreated, NaOH-, NaOH + CaCl₂ (CaCl₂-), and NaOH + Ca(OH)₂ (Ca(OH)₂-) treated titanium disks (A). Images are representative of three independent experiments. The amount of Ca²⁺ incorporation into the chemically modified titanium disks (B; left) and the amount of Ca²⁺ adsorption onto the modified disks after soaking in Hanks' balanced salt solution including calcium and magnesium at 37°C for 7 days (B; right) were measured by ICP-MS (*n* = 3). Significant differences between groups are shown as **p* < 0.05 and ****p* < 0.001.

quantity and quality were assessed on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA); 100 ng of total RNA was used to generate biotin-modified amplified RNA (aRNA) with the GeneChip 3'IVT Express Kit (Affymetrix, Santa Clara, CA). Reverse transcription of first-strand complementary DNA (cDNA) with a T7 promoter sequence was performed with T7 oligo(dT) primer. Second-strand cDNA synthesis was used to convert the single-stranded cDNA into a double-stranded DNA template. The reaction employed DNA polymerase and RNase H to simultaneously degrade the RNA and synthesize second-strand cDNA. *In vitro* transcription of biotin-modified aRNA with IVT Labeling Master Mix generated multiple copies of biotin-modified aRNA from the double-stranded cDNA templates. The aRNA was purified and quantified; after fragmentation, it was hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix). The arrays were stained with phycoerythrin and washed at the GeneChip Fluidics Station 450 (Affymetrix). The microarrays were scanned and data extracted using GeneChip scanner 3000 7G (Affymetrix), image analysis was performed using the Affymetrix GeneChip Command Console Software and digitized using Affymetrix Expression Console.

Data processing and pathway analysis

Data analysis was performed with GeneSpring GX 11.0 software (Agilent Technologies, Santa Clara, CA). Raw data were normalized to the 50th percentile per chip and the median per gene. Normalized means for the four kinds of titanium disks (un-, NaOH-, CaCl₂-, or Ca(OH)₂-treated) were generated for the experimental interpretation. Differentially

expressed genes were selected and flagged for further analysis. To determine whether particular osteogenic differentiation functions were significantly overrepresented, the data set was analyzed using Ingenuity Pathway Analysis (IPA) 9.0 (Ingenuity Systems, Redwood City, CA).

Statistical analysis

All results are shown as means ± SD. Significance was evaluated by the Student's *t* test.

RESULTS

Surface characterization of chemically modified titanium disks

Titanium disks were treated with NaOH (NaOH treatment), NaOH + CaCl₂ (CaCl₂ treatment), and NaOH + Ca(OH)₂ (Ca(OH)₂ treatment; see Materials and methods section). SEM demonstrated a porous network structure formed on the surface of the disks after chemical treatment [Fig. 1(A)]. There were no significant differences between the chemically treated surfaces.

Calcium ion incorporation and apatite formation on the titanium surface

We measured the amount of calcium ion incorporation on the surface of the modified titanium disks [Fig. 1(B)]. As expected, no calcium ions were incorporated into the untreated or NaOH-treated disks. Significantly more calcium ions were incorporated into the Ca(OH)₂-treated disks than the CaCl₂-treated disks.

To investigate the effects of chemical treatments on apatite formation on the titanium surface, the disks were

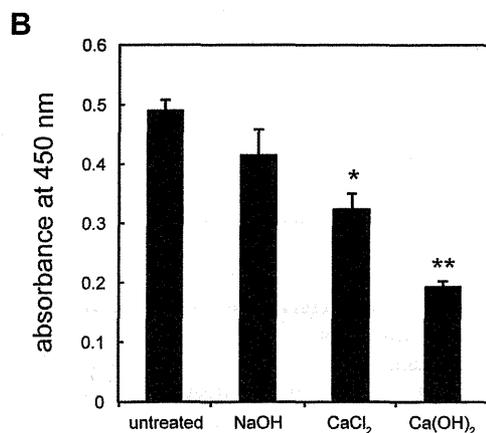
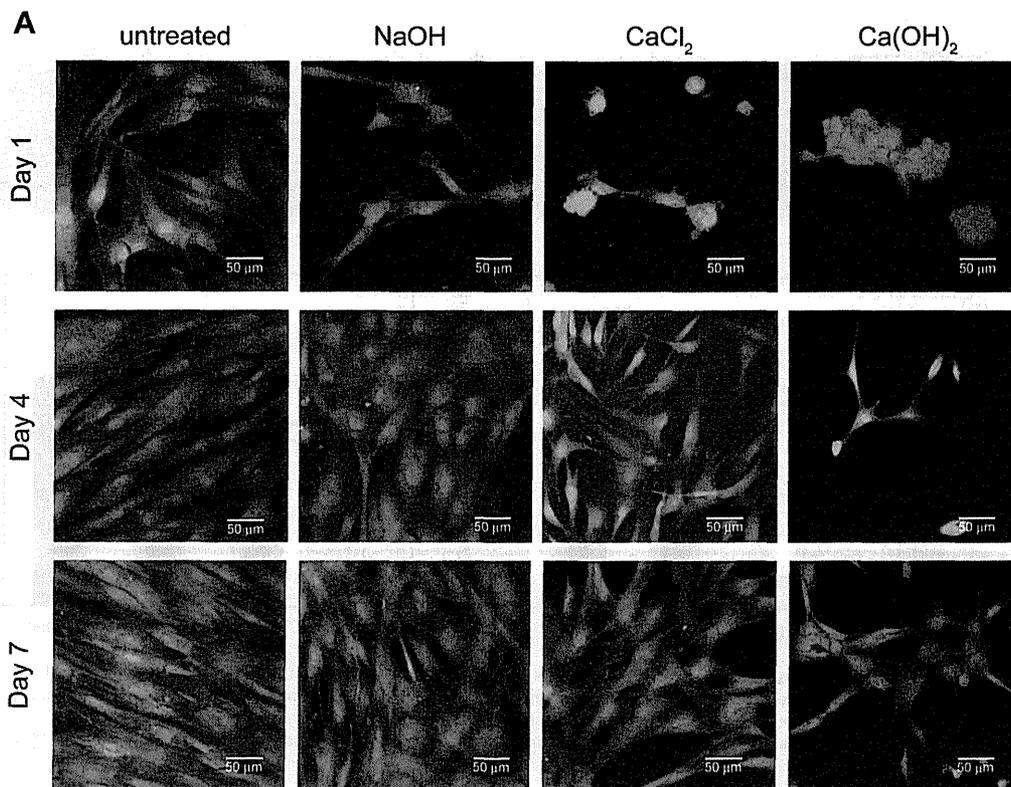


FIGURE 2. Confocal microscopy of hMSCs cultured on untreated, NaOH, CaCl₂, and Ca(OH)₂ treated titanium disks for 1, 4, and 7 days (A). Images are representative of five independent experiments. Cells were stained with CellTracker™ Green Fluorescent Probe (Lonza). Proliferation of hMSCs for 7 days was 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 Ca²⁺ on titanium disks treated with NaOH, CaCl₂, and Ca(OH)₂ was directly correlated with apatite formation. Apatite formation was slightly but significantly greater on CaCl₂-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 CaCl₂-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)]

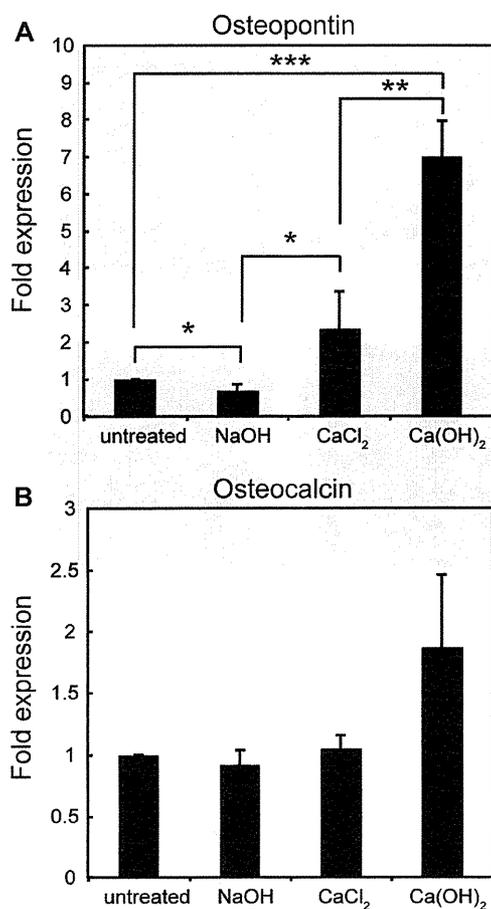


FIGURE 3. Relative expression of osteopontin (A) and osteocalcin (B) were analyzed by real-time PCR at 7 days after hMSC seeding ($n = 3$). The value was normalized to GAPDH. Significant differences between groups are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

upper panels]. Cells grown on CaCl₂- or Ca(OH)₂-treated disks were round in shape. Four days after seeding, the cells on NaOH- or CaCl₂-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 CaCl₂-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 CaCl₂-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 CaCl₂ or Ca(OH)₂ treatments versus the untreated control are shown in Tables II and III. SPP1 (OPN) and MMP13 (matrix metalloproteinase 13), involved in normal bone remodeling,³⁵ and ectonucleotide pyrophosphatase (ENPP1) increase osteoblast differentiation³⁶; all were significantly upregulated by CaCl₂ 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 (PTH1H), 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 CaCl₂ 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 CaCl₂ (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, PTH1H, 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, CaCl₂, 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

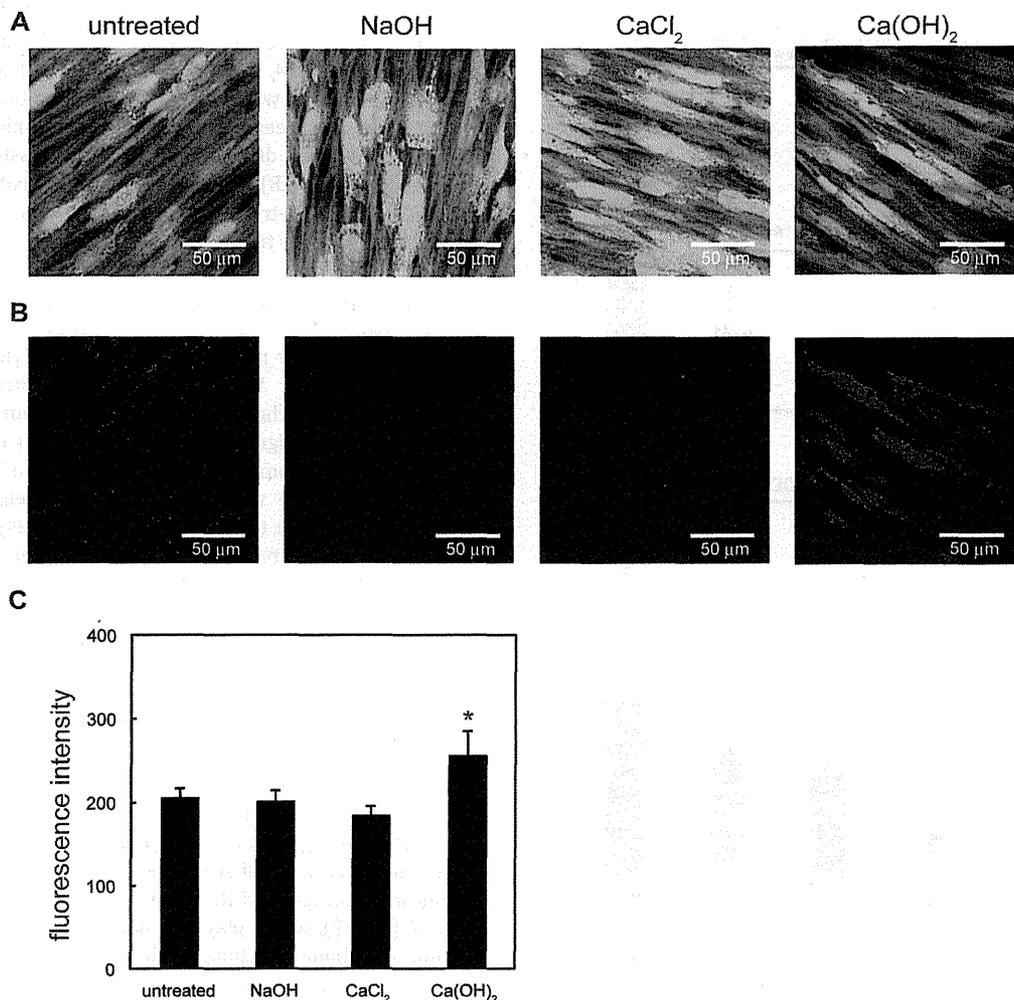


FIGURE 4. Confocal microscopy of hMSCs (A) and osteocalcin in hMSCs (B) cultured on chemically modified titanium disks for 28 days. Green: CellTracker™ Green Fluorescent Probe; red: Alexa Fluor 647 donkey anti-mouse. Images representative of three independent experiments are shown. Fluorescence intensity 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- κ B ligand (RANKL) decoy receptor osteoprotegerin (OPG) was upregulated by NaOH treatment (Fig. 5). CaCl₂ treatment induced expression of Frizzled, Axin, APC, and osteogenic markers BMP and IGF-1. Bone matrix protein OPN expression was upregulated by CaCl₂ treatment. Expression of integrin β 3 was also induced following OPN upregulation by CaCl₂ (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 β 3. 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 CaCl₂ 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 $\text{Ca}(\text{OH})_2$.

Experimental titanium disks were treated with aqueous NaOH, CaCl_2 , and $\text{Ca}(\text{OH})_2$ 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 $\text{Ca}(\text{OH})_2$ 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 $\text{Ca}(\text{OH})_2$ -treated disks than on CaCl_2 -treated disks. OCN protein expression was also upregulated by $\text{Ca}(\text{OH})_2$ 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 $\text{Ca}(\text{OH})_2$ 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 $\text{Ca}(\text{OH})_2$ (Tables II and III). $\text{Ca}(\text{OH})_2$ 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 $\text{Ca}(\text{OH})_2$ treatments and found that $\text{Ca}(\text{OH})_2$ 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 rats⁴³ and osteoblast differentiation is stimulated through induction of Cox2 in

TABLE II. Significantly Upregulated Genes in Cells Grown on CaCl₂-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 metalloproteinase 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 metalloproteinase 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 metalloproteinase 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
PTH1H	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 (<i>S. cerevisiae</i>)	3.084

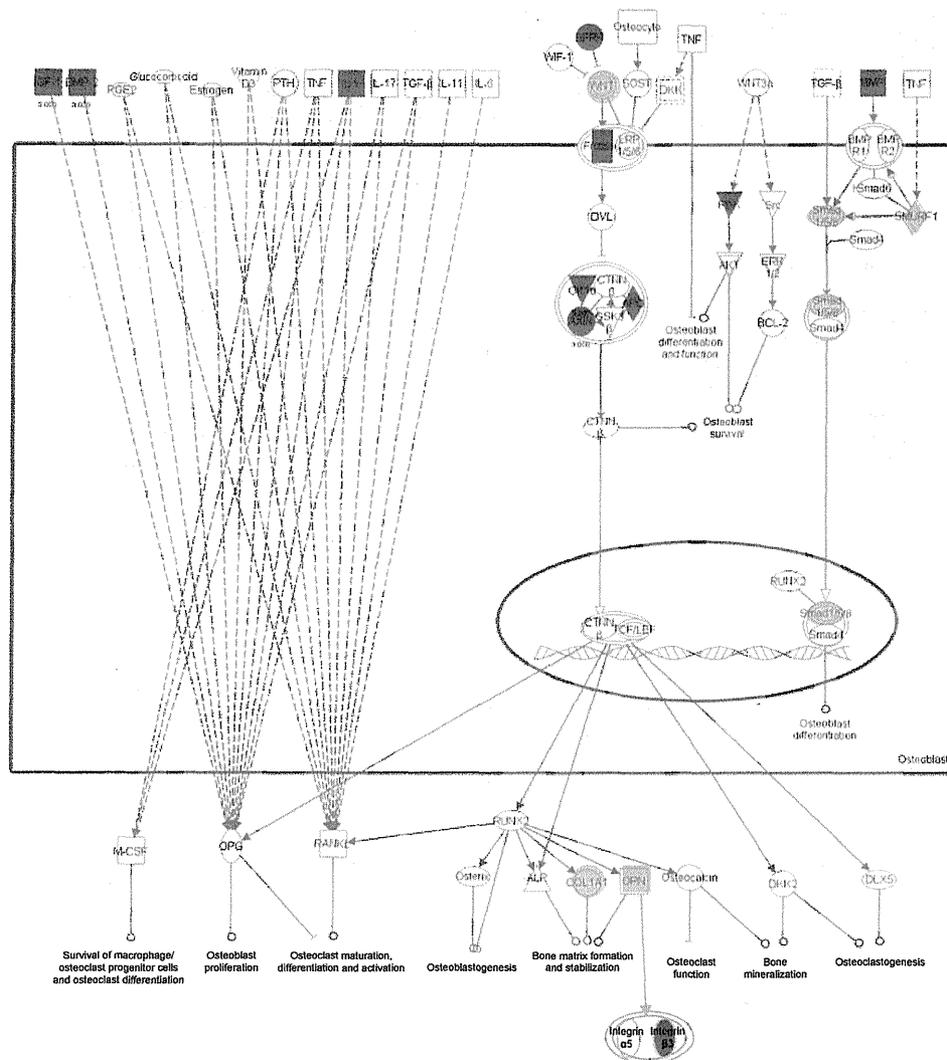


FIGURE 6. Differentially expressed genes in the canonical osteoblast pathway on CaCl_2 -treated disks versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

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 $\beta 3$ 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 $\beta 3$ 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 differentiation⁵⁰; 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 $\beta 3$ 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

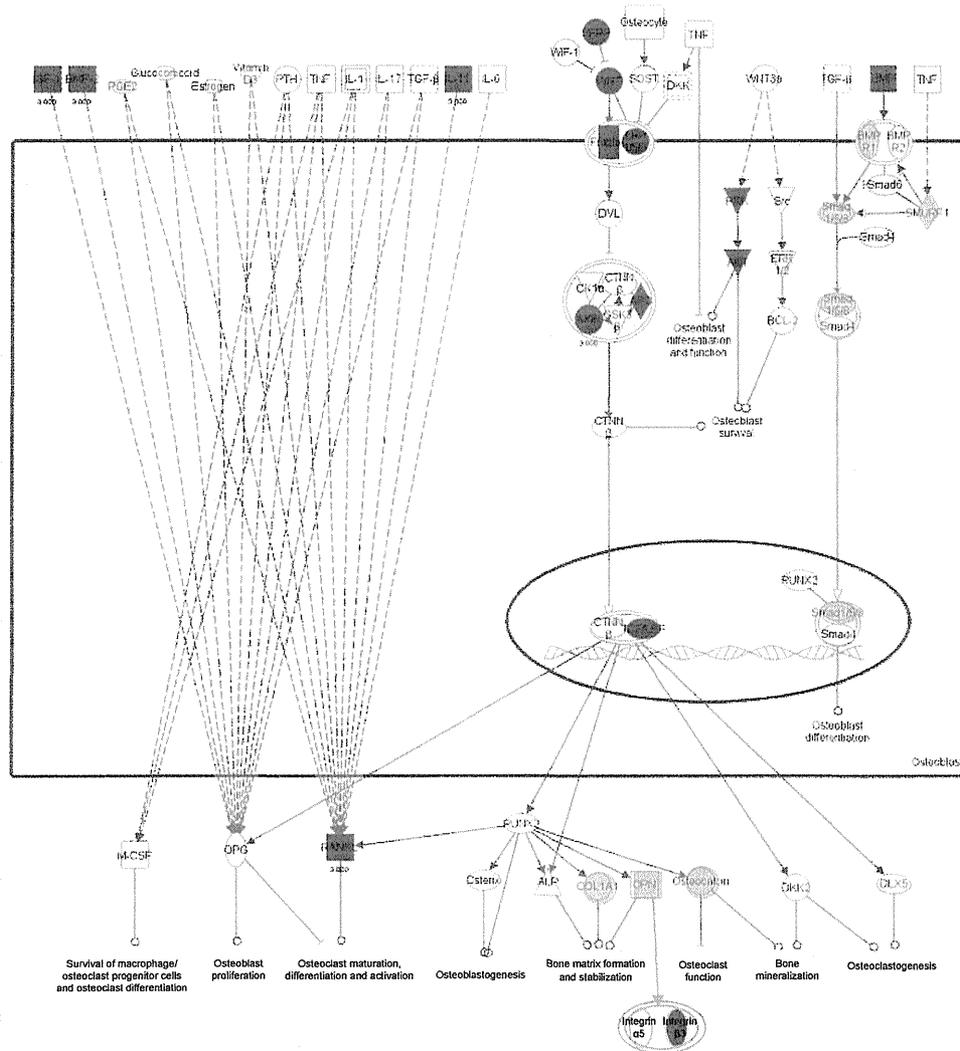


FIGURE 7. Differentially expressed genes in the canonical osteoblast pathway on Ca(OH)₂ versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

suggested that Ca(OH)₂ treatment of titanium disks induced osteogenic differentiation in hMSCs by the upregulation of BMP2, Cox2, and PTHLH compared with CaCl₂ treatment, and the activation of Wnt/β-catenin signaling.

CONCLUSIONS

In this study, we chemically modified titanium surfaces with CaCl₂ 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 CaCl₂ 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 CaCl₂ 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

This work was supported by the Health and Labour Sciences Research Grants for Research on Regulatory Science of Pharmaceuticals and Medical Devices (H22-IYAKU-IPPAN-009, H24- IYAKU-SHITEI-018) from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

- Walivaara B, Aronsson BO, Rodahl M, Lausmaa J, Tengvall P. Titanium with different oxides: In vitro studies of protein adsorption and contact activation. *Biomaterials* 1994;15:827–834.