であるQSR（Quality Systems Regulation）とcGTPに従い製造した製品について，研究用機器特例（Investigational Device Exemption, IDE）申請の後に臨床試験を行い，市販前承認（Premarket Approval, PMA）を通じて販売承認を得る（図3.6.1）．INDおよびIDE申請の初回審査期間は30日と定められている．

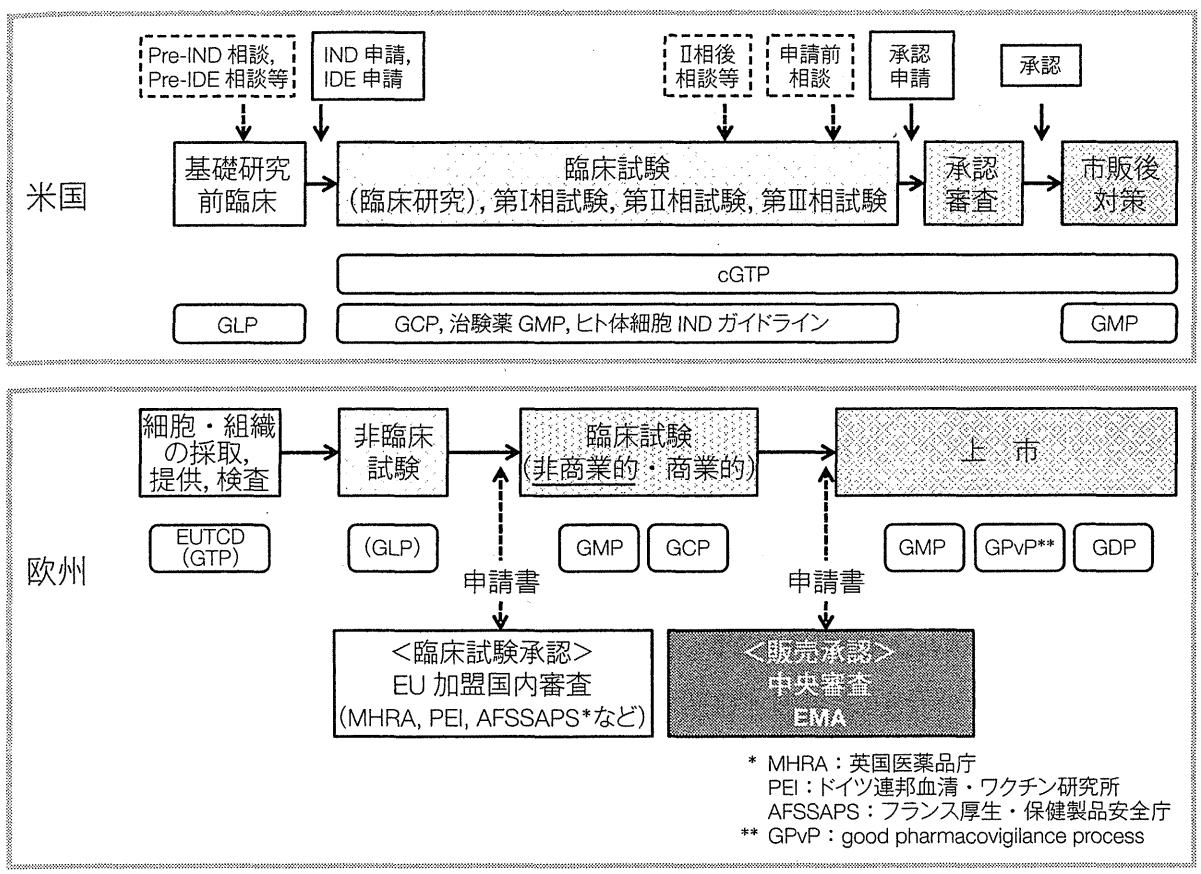

図 3.6.1　細胞・組織加工製品の開発から使用まで

(2)　欧　州

EUにおけるATMPの臨床試験は，日本における臨床研究に相当する区分は存在せず，日本における臨床研究に相当する試験であっても，すべて日本の治験に相当する規制が適用される．臨床試験（治験）に関しては，遺伝子治療医薬品および体細胞治療医薬品の場合には，既に日米EU医薬品規制調和国際会議で合意された医薬品の臨床試験に関する基準（International Conference on Harmonisation of technical requirements for registration of

pharmaceuticals for human use. Good Clinical Practice, ICH-GCP）に基づいたGCPを順守することが必要であったが，Regulation（EC）No 1394/2007施行後のATMPの臨床試験に関しては，これに加えてATMP向けの新しいGCPを順守する必要があるとされている．EU内の国境を越えた医薬品・医療機器の流通に関しては，EMAのヒト医療製品委員会（Committee for Medicinal Products for Human Use, CHMP）が欧州委員会（EC）からの委任を受けて承認審査を行っており，そこで品質・安全性・有効性に関する科学的評価が行われている．しかしながらATMPは従来の医薬品・医療機器よりも専門的かつ多分野にわたる評価を要することから，CHMPの下部諮問組織として2008年12月に設置された先端医療委員会（Committee for Advanced Therapies, CAT）での品質・有効性・安全性の評価意見書案をもとにしてCHMPが承認審査を行い，CHMPが作成した評価意見書をもとにしてECが承認の判断をする，という体制が取られている．また，ATMPは多くの患者の体の一部となることから市販後の安全対策として，トレーサビリティの確保（患者から製品・材料・ドナーまでの追跡可能性，GMP/GDP），ファーマコビジランス（有害事象の監視，GPvP），リスクマネジメントシステムの構築（リスクの最小化）と有効性のフォローアップ（事後評価）が申請者に求められる．ただしEMAはあくまでも薬事承認審査を行う機関であり，臨床試験（治験）の開始・実施に関する手続きはすべて加盟国の管轄となっている（図3.6.1）．

3.6.4　欧米の相談制度

(1)　米　国

生物製剤では開発者が臨床試験前にFDAと相談する制度（Pre-Pre-IND相談，Pre-IND相談），販売承認申請前の相談制度（Pre-BLA相談）等がある．医療機器に関しては，臨床研究前の相談（Pre-Pre-IDE相談，Pre-IDE相談），販売承認申請前の相談（Pre-PMA相談）等がある．これらの相談は，臨床試験の目的が商業的・非商業的にかかわらず利用可能で，製品に特化した

実用化までの道筋を議論することができる．また，生物製剤および医療機器の分類の判断が困難な場合は，複合製品室（Office of Combination Product OCP）に相談し，OCP が相談受付後 60 日以内に判断する．

(2)　欧　州

ATMP の開発を促進するためには，製品に関して企業が EMA と相談する場合に，その手数料をできるだけ安くする必要がある．現在 EMA では，中小企業が ATMP についての科学的助言や試験プロトコルの補助を必要とする場合，通常の手数料の 90％割引で相談に応じている．それ以外の開発者でも対象品目が ATMP ならば通常の 65％割引で相談に応じている．これらの相談は，臨床試験の目的が商業的・非商業的にかかわらず利用可能である．また，EU 加盟各国の規制当局も独自に開発者向けの無料ないし安価な相談制度を整備している．

3.6.5　未承認の細胞・組織加工製品の臨床利用

(1)　米　国

米国では未承認の生物製剤および医療機器の臨床利用は，重篤・致死的・代替療法のない疾患に対する緊急的もしくは人道的使用において認められている．生物製剤に関しては，IND 申請を行うことができない緊急時，臨床プロトコル外の患者，特定の個人の患者に使用することが可能である．医療機器に関しては，臨床試験中の緊急時での使用，臨床試験の基準外の患者への使用，臨床試験途中の患者の追加，臨床試験完了後で販売承認前使用が可能になっている．

さらに米国内で年間 4,000 人以下の患者を対象とした製品で他に有効な医療機器が存在しないものについては，薬事承認の一種である人道機器適用免除（Humanitarian Device Exemption, HDE）の承認を受けることができる．HDE は一般の市販前承認（PMA）と異なり有効性を合理的に立証する臨床試験結果は必要とされないが，想定されるベネフィットがリスクを上回るこ

などが必要とされる．また，使用される医療施設の倫理委員会（Institutional Review Board, IRB）の承認が必要など市販後に上乗せの要件が課せられる．

(2) 欧　州

販売未承認ATMPの中央審査の原則の例外として，「病院免除」（Hospital Exemption），「人道的使用」（Compassionate Use），「特別免除」（Special Exemption）の3種類がある．「病院免除」は，

① 特定の一患者向けの特注品の処方箋に従って，
② 明確な品質基準に基づき，
③ 非反復的に製造され，
④ 医療従事者の職務責任のもと，
⑤ 同一加盟国で，
⑥ 単一病院において使用される，

という条件をすべて満たすATMPはEMAの中央審査とはならないという規定である．ただし「病院免除」に該当する品目の場合も，生産国の機関からの製造・品質に関する承認，ファーマコビジランス，トレーサビリティが必要である．「人道的使用」は，代替法のない疾患に使用されるATMPに関して，販売承認申請予定や臨床試験中の品目の承認前使用が認められるというものである．「特別免除」では，患者個人からの自発的な要望に応じて供されるATMPで，医師の直接的な責任のもとに使用されるものについては，中央審査が免除されると定めてある．同等な作用を持つ承認薬がないような場合に適用される．

3.6.6 最近の話題

米国においては，コロラド州のThe Centeno-Schultz Clinicが，FDAの承認を得ることなく関節傷害に対する自己由来培養骨髄幹細胞の注入療法を行っていることが大きく取り上げられた[5]．クリニック側は，単一州内での医療行為のため，FDAの規制は受けずINDもBLAも不要と主張している．FDAの

見解としては,

① 通常の骨髄移植と異なり最低限以上の加工を施した細胞は生物製剤としてFDAの規制を受けなければならない.

② GMPに準拠していないため安全性・有効性の証明がない.

としている. FDAは2010年8月にクリニックの業務停止命令を地裁に請求したが, 未決着の状態である. このように自己由来培養細胞に関しては医療行為か医療製品かという議論は欧米でも存在する.

また, 前述したが, 欧州においてはRegulation (EC) No 1394/2007の施行後3年もしくは4年以内にEMAの中央審査による再承認を得なければ, そのATMPへの承認は取消しとなる. EMAの中央審査開始以前に承認され, EU内で流通していたATMPは15品目ほどであるが, その中でEMAから再承認を得ることができたのは, いまのところTiGenix社のChondroCelect 1品目のみである. 一方2011年5月時点におけるドイツでの「病院免除」は, 体細胞治療薬が3品目, 組織工学製品が21品目, 組織工学製品（非相同使用）が6品目にも上っている[6]. EMAの承認審査状況があまり順調ではない一方で, 多くのATMPの開発者が「病院免除」の道を積極的に活用していることがうかがえる.

3.6.7 おわりに

欧米で臨床研究が治験レベルで可能なのは, 研究者支援体制, 規制当局の人員・予算, 医療機関内・機関間の臨床試験支援体制が日本より充実していることが反映していると考えられる. 商業的・非商業的にかかわらず臨床試験においてICH-GCPを準拠することは, リスクベースアプローチの立場からは合理的である. しかしながら資金や労力面で莫大なコストがかかることも事実であり, 欧米でも大学の臨床医や中小ベンチャー企業にとってGCP準拠の条件は非常に厳しいものとなっているのも事実である[7]. ただし, 欧米の規制当局はこうした厳しい原則に則りつつも, 先端的製品の開発を促すための規制・環境整備に積極的に努めている. 日本において先端的製品の実現化を効率的に推進

するためには，彼らの努力も参考にしつつ，日本における規制・開発環境のあり方について継続的に関係者が知恵を出し合っていくことが重要であると考えられる．

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

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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 + $CaCl_2$ ($CaCl_2$ 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 $CaCl_2$ treatment. The morphology of hMSCs differed on $CaCl_2$- and $Ca(OH)_2$-treated disks. The osteopontin (OPN) expression in hMSCs cultured on $CaCl_2$-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 $CaCl_2$-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 β3 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 $CaCl_2$ 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 $CaCl_2$ treatment had a limited effect. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A: 000–000, 2013.

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

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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 hMSCs[5] to some titanium 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 biocompatibility[23] and bone formation[24] *in vivo*. Fluoride ion modification of a TiO_2 grit-blasted surface enhances osteoblastic differentiation in hMSCs in the presence of osteogenic supplements.[25] Calcium ion incorporation into a titanium surface by $CaCl_2$ 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,

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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 $CaCl_2$- 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 $CaCl_2$- 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. $CaCl_2$ or $Ca(OH)_2$ treatments were performed by soaking the NaOH-treated disks in 0.1 mol/L $CaCl_2$ 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% CO_2 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% NaN_3 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/cm^2 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

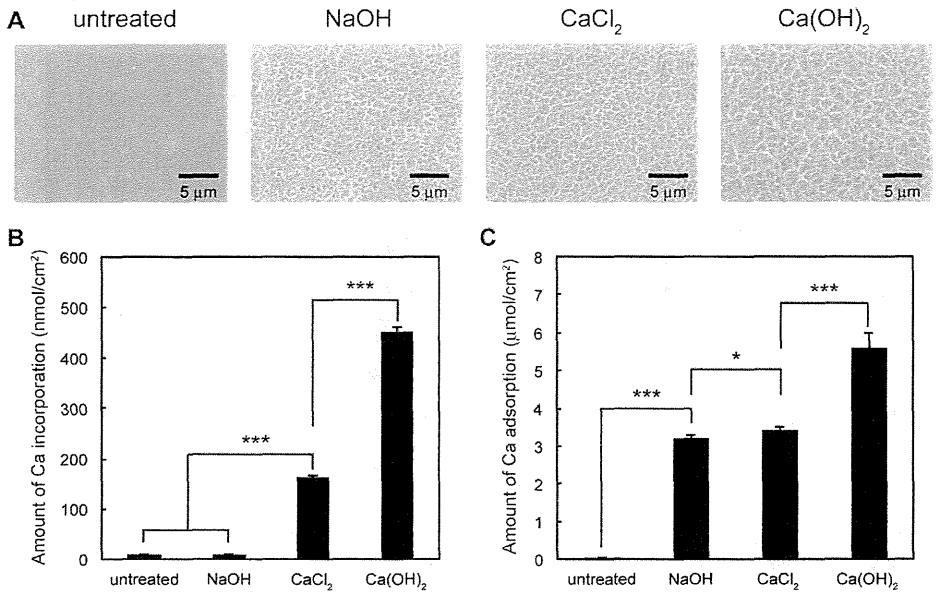

FIGURE 1. SEM images of the surface of untreated, NaOH-, NaOH + $CaCl_2$ ($CaCl_2$)-, and NaOH + $Ca(OH)_2$ ($Ca(OH)_2$)-treated titanium disks (A). Images are representative of three independent experiments. The amount of Ca^{2+} incorporation into the chemically modified titanium disks (B; left) and the amount of Ca^{2+} 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-, $CaCl_2$-, 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 + $CaCl_2$ ($CaCl_2$ 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 $CaCl_2$-treated disks.

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

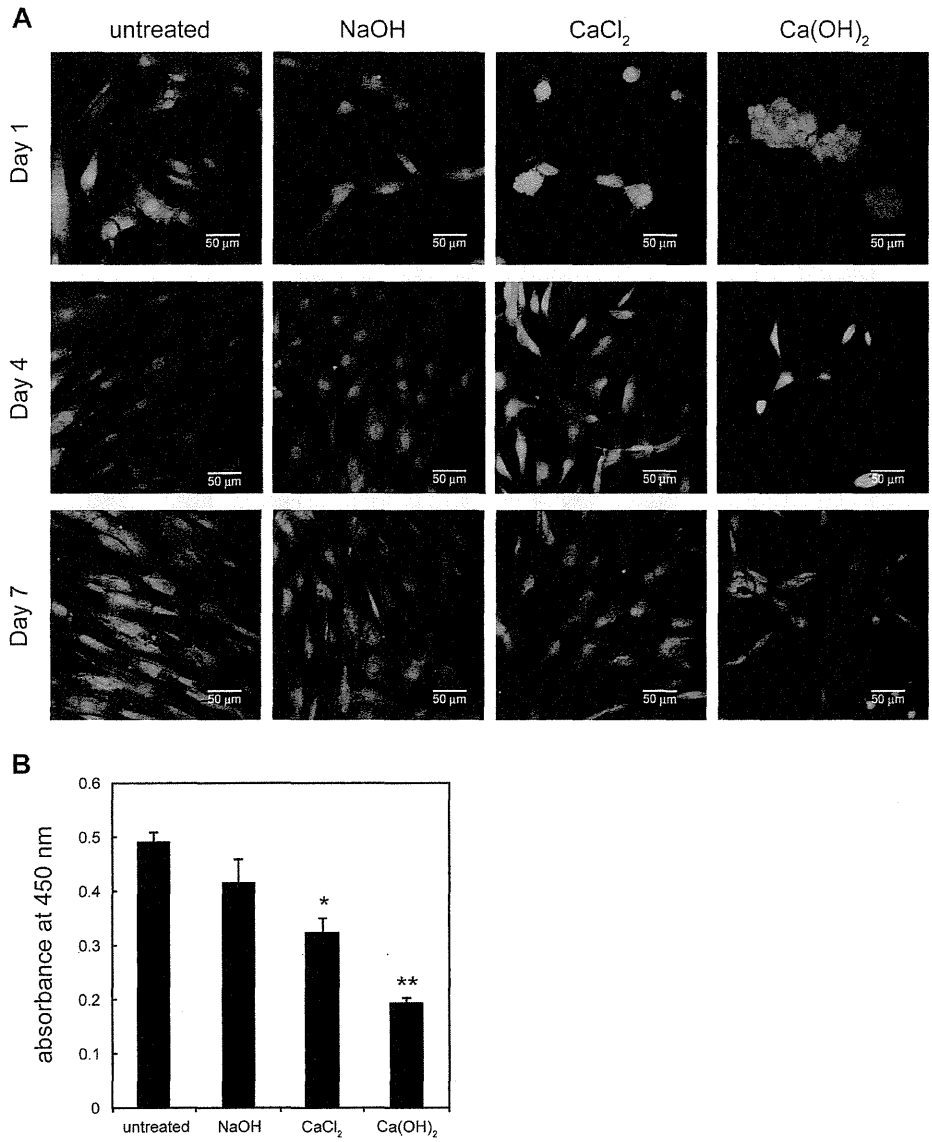

FIGURE 2. Confocal microscopy of hMSCs cultured on untreated, NaOH, $CaCl_2$, and $Ca(OH)_2$ treated titanium disks for 1, 4, and 7 days (A). Images are representative of five independent experiments. Cells were stained with CellTrackerTM Green Fluorescent Probe (Lonza). Proliferation of hMSCs for 7 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)

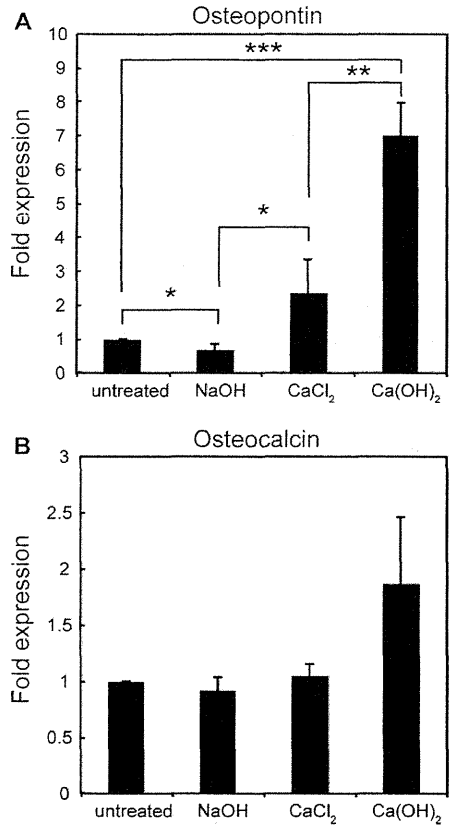

FIGURE 3. Relative expression of osteopontin (A) and osteocalcin (B) were analyzed by real-time PCR at 7 days after hMSC seeding ($n = 3$). The value was normalized to GAPDH. Significant differences between groups are shown as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

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

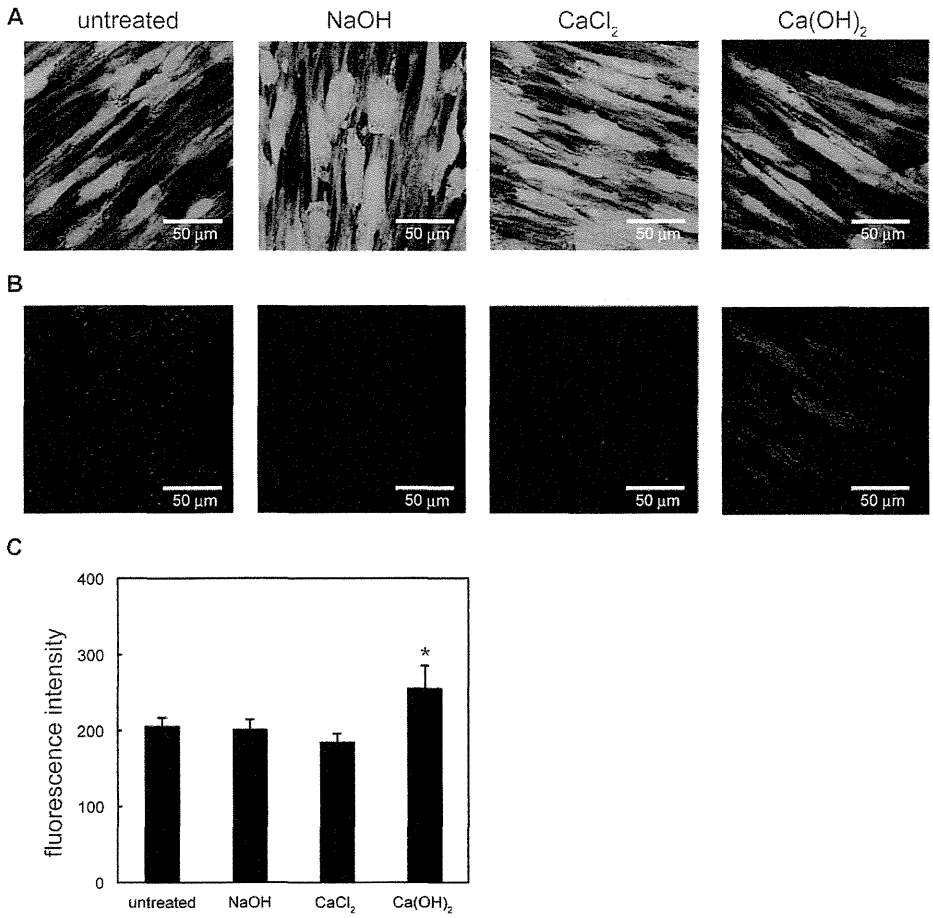

FIGURE 4. Confocal microscopy of hMSCs (A) and osteocalcin in hMSCs (B) cultured on chemically modified titanium disks for 28 days. Green: *CellTrackerTM Green Fluorescent Probe; red: Alexa Fluor 647 donkey anti-mouse. Images representative of three independent experiments are* shown. Fluorescence intensity 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,

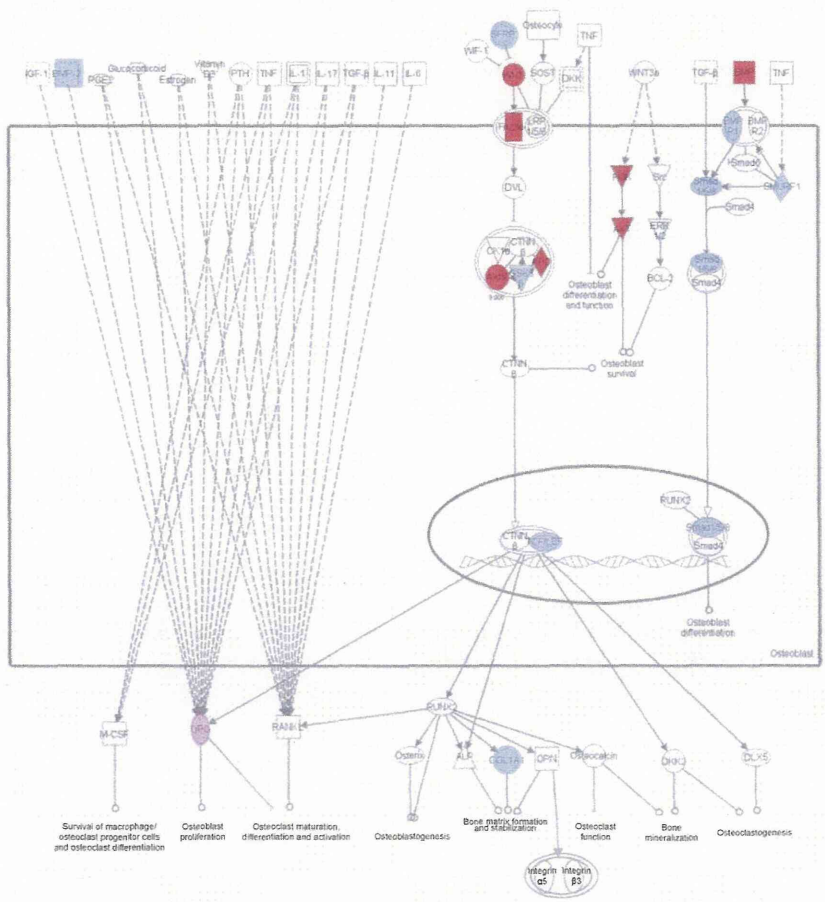

FIGURE 5. Differentially expressed genes in the canonical pathway in osteoblasts were significantly changed by NaOH treatment versus untreated conditions. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

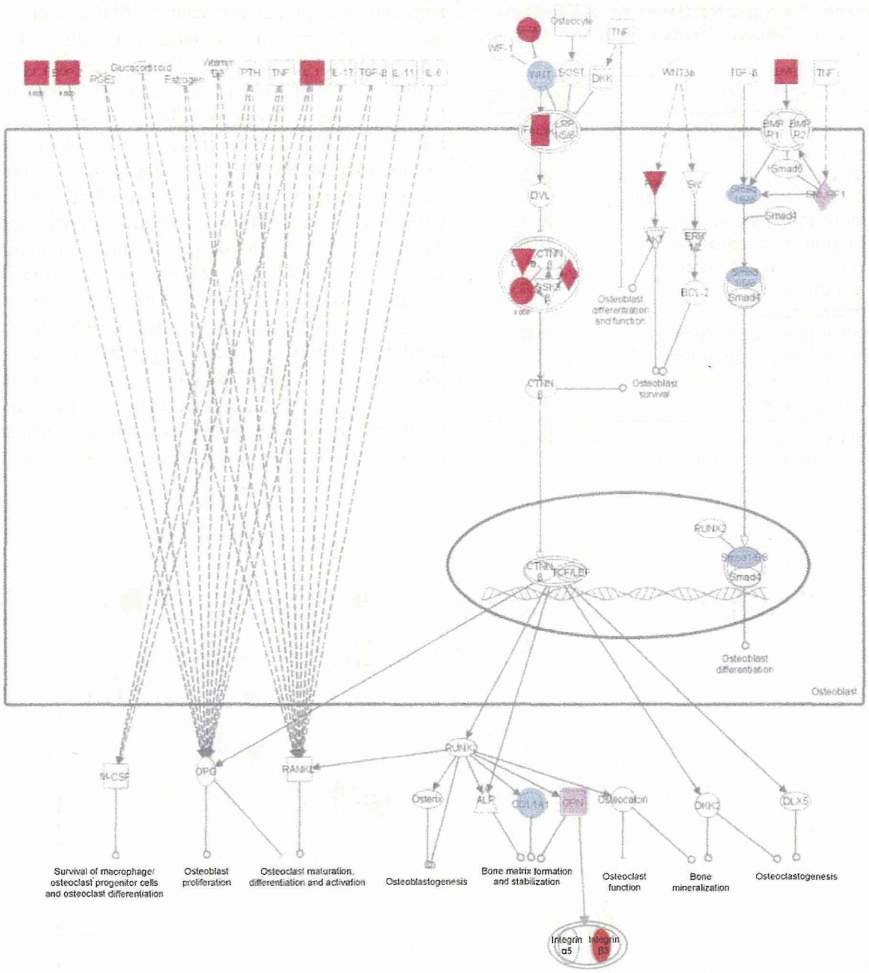

FIGURE 6. Differentially expressed genes in the canonical osteoblast pathway on $CaCl_2$-treated disks versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

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

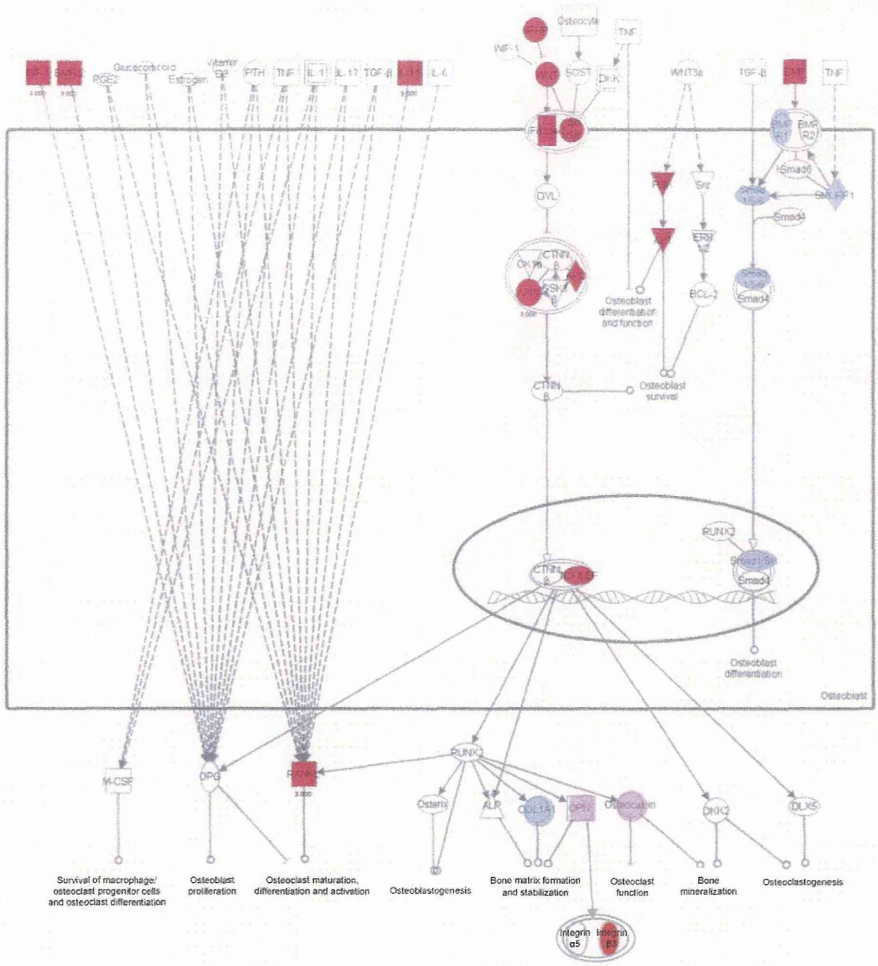

FIGURE 7. Differentially expressed genes in the canonical osteoblast pathway on $Ca(OH)_2$ versus untreated disks. Upregulated (more than twice), downregulated (less than 1/2), induced, and suppressed genes are indicated in pink, green, red, and light blue, respectively.

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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Identification of BC005512 as a DNA Damage Responsive Murine Endogenous Retrovirus of GLN Family Involved in Cell Growth Regulation

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Abstract

Genotoxicity assessment is of great significance in drug safety evaluation, and microarray is a useful tool widely used to identify genotoxic stress responsive genes. In the present work, by using oligonucleotide microarray in an *in vivo* model, we identified an unknown gene BC005512 (abbreviated as BC, official full name: cDNA sequence BC005512), whose expression in mouse liver was specifically induced by seven well-known genotoxins (GTXs), but not by non-genotoxins (NGTXs). Bioinformatics revealed that BC was a member of the GLN family of murine endogenous retrovirus (ERV). However, the relationship to genotoxicity and the cellular function of GLN are largely unknown. Using NIH/3T3 cells as an *in vitro* model system and quantitative real-time PCR, BC expression was specifically induced by another seven GTXs, covering diverse genotoxicity mechanisms. Additionally, dose-response and linear regression analysis showed that expression level of BC in NIH/3T3 cells strongly correlated with DNA damage, measured using the alkaline comet assay,. While in p53 deficient L5178Y cells, GTXs could not induce BC expression. Further functional studies using RNA interference revealed that down-regulation of BC expression induced G1/S phase arrest, inhibited cell proliferation and thus suppressed cell growth in NIH/3T3 cells. Together, our results provide the first evidence that BC005512, a member from GLN family of murine ERV, was responsive to DNA damage and involved in cell growth regulation. These findings could be of great value in genotoxicity predictions and contribute to a deeper understanding of GLN biological functions.

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Introduction

Genotoxicity assessment plays an important role in both toxicity screening during early drug discovery and regulatory drug safety evaluation in the preclinical stage [1]. Although a great number of genotoxicity assays have been developed, there is still a requirement for tests with both high specificity and sensitivity [2]. The use of microarray technology in toxicology, known as toxicogenomics, can potentially identify novel genotoxicity biomarkers and provide mechanistic insights into the mode of action of genotoxic compounds [3,4,5,6,7,8]. We identified an unknown gene BC005512 (official full name: cDNA sequence BC005512), whose expression was specifically induced by genotoxins (GTXs) but not by non-genotoxins (NGTXs) in an *in vivo* microarray study. Elevated expression of BC005512 has been reported previously in thymocytes of Parp-2 deficient mice [9], suggesting that it is relevant to DNA damage. Further analysis of this gene uncovered that it is a member of the GLN family of murine endogenous retrovirus (ERV).

ERV sequences, most probably originating from infections of germ-line cells by ancient exogenous retroviruses during evolution [10], account for approximately 8% of the human genome [11] and 10% of the mouse genome [12]. ERVs were once thought to be junk DNA, but a number of studies have shown that some have important physiological roles [13,14,15] or are implicated in certain diseases [16,17]. Several studies have reported elevated expression of ERV-related sequences in hepatocarcinogen treated rodents [18,19].

The GLN family, designated due to an unusual primer-binding site sequence corresponding to $tRNA^{Gln}$, is one of a number of murine ERV families. It was first identified over two decades ago [20], but remains little-studied [21,22]. The relationship between GLN and genotoxic stress and the biological function of GLN family members are largely unknown. Here we report that BC005512, a member of the GLN family of murine ERV, was responsive to DNA damage and involved in regulation of cell growth.

Results

1. Selection of specific and sensitive genotoxic stress responsive genes using microarray

Microarray is a powerful way of examining genomic scale gene expression changes. To identify specific and sensitive genotoxic